Biological Effects of Novel Poly(Adenosine Diphosphate Ribose) Polymerase Inhibitors.

by

Sallyanne Boulton

Thesis submitted to the University of Newcastle-upon-Tyne

for the degree of Doctor of philosopy, August 1995.

With love

to my Mum and Dad

PUBLICATIONS

Parts of this thesis have been published as:

Boulton S., Pemberton L.C., Porteous J.K., Curtin N.J., Griffin R.J., Golding B.T. and Durkacz B.W. (1995) Potentiation of Temozolomide-induced Cytotoxicity: A Comparative Study of the Biological Effects of Poly(ADP-ribose) Polymerase Inhibitors. **Br.J.Cancer** 72:849-856.

Published abstracts:

Boulton S.,Porteous J., Curtin N.J., Durkacz B.W. (1994) A Comparative Study of the Effects of Novel Poly(ADP-ribose) Polymerase Inhibitors in Temozolomide Treated L1210 Cells. **Br.J.Cancer** 69:supplement XXI.

Boulton S., Jones S., Durkacz B.W. (1995) Functional Dissociation between the Effects of Poly(ADP-ribose) Polymerase Inhibitors on Enhancement of Temozolomide Cytotoxicity and DNA Strand Break Repair. **Br.J.Cancer** 71:Supplement XXIV.

Jones S., Boulton S., Porteous J., Durkacz B.W. (1995) An Adenyltransferase Deficient Cell Line is Hypersensitive to Temozolomide and to the Chemopotentiating Effects of Poly(ADP-ribose) Polymerase Inhibitors. **Br.J.Cancer** 71:Supplement XXIV.

INDEX

FIGURES AND TABLES

CHAPTER 1

FIGURES

TABLES

CHAPTER 4

FIGURES

TM, and either 3AB or NU1025.

TABLES

CHAPTER 5

FIGURES

TABLES

CHAPTER 6

FIGURE

ACKNOWLEDGEMENTS

I would like to thank my supervisor Barbara for all her advice, support and encouragement, especially during the last 8 months whilst I have been writing this thesis.

I would also like to thank Julia Porteous and Karen Bowman who kindly gave permission for me to include their *in vitro* permeabilised cell data for the PADPRP inhibitors.

Special thanks to Suzanne Jones, who has offered constant support throughout the last four years and allowed me to use her NAD^+ depletion data and tiazofurin resistance data.

I would like to acknowledge the financial support of both the NECRC and the Cancer Research Unit. I would also like to thank the Cancer Research Unit for the use of facilities.

Thank you to all my friends, especially Claire, for being extremely patient whilst I completed this thesis.

A big thank you goes to my Mum and Dad, who have always been supportive, encouraging and interested in everything I have done.

Finally, I should like to say a special thank you to Phil who has given endless support and encouragement, and without whom this thesis would certainly have taken a great while longer to write.

DECLARATION

This thesis records the work carried out at the University of Newcastle-upon-Tyne between October 1991 and December 1994, and is my own original work except where otherwise stated.

ABSTRACT

Poly(ADP-ribose) polymerase (PADPRP) is a nuclear enzyme with a well documented role in DNA repair. Inhibitors of PADPRP, (e.g. 3′ substituted benzamides) potentiate the cytotoxicity of a wide range of antitumour drugs. The results presented in this thesis represent, to the best of my knowledge, the first comprehensive and quantitative assessment of the ability of a range of PADPRP inhibitors to modulate the cellular responses to damaging agents. Two novel PADPRP inhibitors, 8-hydroxy-2-methyl quinazolin-4(3*H*)-one (NU1025) and 3,4 dihydro-5-methoxyisoquinolin-1-(2*H*)-one (PD 128763) were compared with two "classical" PADPRP inhibitors, 3-aminobenzamide (3AB) and benzamide (BZ). The relative potencies for 3AB, BZ, NU1025 and PD 128763 as PADPRP inhibitors *in vitro* were 1.0, ~1.0, ~43 and ~53 respectively. All compounds potentiated the growth inhibition and cytotoxicity of the monofunctional alkylating agent temozolomide (TM) in L1210 cells. For example, 10μM NU1025 and PD 128763 gave dose enhancement factors (DEF) of ~2 at 10% survival, whereas 1mM 3AB and 0.5mM BZ where required to give similar DEF values. Cellular NAD^+ levels were depleted up to 50% by 1-2mM TM and this depletion was completely prevented by coincubation with 50-100μM PD 128763 and 1-3mM 3AB. TM induced DNA single strand break levels were increased in a concentration dependent manner by the PADPRP inhibitors. Overall, the relative potencies for ability of the compounds to potentiate TM induced growth inhibition, cytotoxicity and DNA single strand breaks showed good correlation with those determined in an *in vitro* inhibition study, with both NU1025 and PD 128763 exhibiting ~60 fold increased inhibitory activity as compared to 3AB. The PADPRP inhibitors *per se* did not effect the growth or survival of the L1210 cells, nor increase DNA strand breakage.

NAD^+ is the substrate for PADPRP. A L1210 cell line made resistant to tiazofurin (TZ) utilising a step wise selection protocol was shown to be deficient in nicotinamide

mononucleotide adenyl transferase (NMNAT), the final enzyme required for NAD^+ biosynthesis. The consequences of a reduced NMNAT activity (<3% of the parental line) and an ~40% reduction in intracellular NAD^+ levels were determined. The resistant cells showed an ~3 fold increased sensitivity to TM as compared to the parental cells. Upon coincubation with increasing concentrations of NU1025 in the presence of a fixed concentration of TM, growth inhibition was potentiated ~70 fold in the resistant cells but only ~10 fold in the parental cell line, demonstrating the reduced level of competition between NAD^+ and NU1025 for PADPRP. However, DNA single strand breaks were increased in the resistant compared to the parental cell line only when NU1025 was coincubated with TM. In contrast, in the presence of the PADPRP inhibitors alone, equivalent growth inhibitory effects were observed in each of the cell lines, suggesting inhibition of PADPRP was not the cytotoxic effector. The ~40% NAD^+ depletion observed could therefore suggest, that NAD^+ levels in the resistant cells were reduced to, or near to the K_{mNAD+} for PADPRP.

ABBREVIATIONS

AADH	amino acid dehydrogenase
AAF	N-2-acetyl 2-aminofluorene
3AAB	3-acetylaminobenzamide
3AB	3-aminobenzamide
ADH	alcohol dehydrogenase
AMP	adenosine 5′monophosphate
6 AN	6-aminonicotinamide
AP	apurinic/apyrimidinic
ATP	adenosine 5′triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	base excision repair
BZ	benzamide
BrdUrd	bromodeoxyuridine
CDK	cyclin dependent kinase
cADPR	cyclic (ADP-ribose)
cAMP	cyclic adenosine 5′monophosphate
cDNA	complementary deoxyribonucleic acid
dCTP	2′deoxyribosylcytosine 5′triphosphate
DBD	DNA binding domain
DHFR	dihydrofolate reductase
DMS	dimethyl sulphate
DNA	deoxyribonucleic acid
dNTP	2′deoxyribosylnucleoside 5′triphosphate
dTTP	2′deoxyribosylthymine 5′triphosphate
DTIC	dacarbazine

FPLC	fast protein liquid chromatography
5FU	5 fluorouracil
FUrd	fluorouridine
GMP	guanosine 5′monophosphate
GDP	guanosine 5′diphosphate
GTP	guanosine 5′triphosphate
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high pressure liquid chromatography
HSSB	single stranded DNA binding protein
HU	hydroxyurea
IMP	inosine monophosphate
IMPDH	inosinate monophosphate dehydrogenase
MEP	molecular electrostatic potential
3-MBZ	3-methoxybenzamide
5MeNic	5-methylnicotinamide
MGMT	methyl guanine methyl transferase
mRNA	messenger ribonucleic acid
MMS	methyl methanesulphate
MNNG	N-methyl-N′-nitro-nitrosoguanidine
MNU	1-methyl-1-nitrosourea
MTIC	5-(3-methyl-1-triazeno)imidazole-4-carboxamide
NU1025	8-hydroxy-2-methylquinazolin-4(3*H*)-one
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NER	nucleotide excision repair
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide mononucleotide adenyl transferase

PAGE	polyacrilamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PRPP	phosphoribosyl pyrophosphate
PADPRP	poly(ADP-ribose) polymerase
PRAMP	phosphoribosyl-AMP
$(PR)_2AMP$	diphosphoribosyl-AMP
PHA	phytohaemoggluttin
PD 128763	3,4 dihydro-5-methoxyisoquinolin-1-(2*H*)-one
R.E.	relative elution
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
TAD	thiazole-4-carboxamide adenine dinucleotide
TM	temozolomide
TZ	tiazofurin
tRNA	transfer ribonucleic acid
TFIIH	transcription factor IIH
XMP	xanthine monophosphate
XP	xeroderma pigmentosum

CHAPTER 1 : INTRODUCTION

1.1 CELLULAR NAD^+ METABOLISM

1.1.1 Introduction

In mammals, the synthesis of the pyridine nucleotide, nicotinamide adenine dinucleotide (NAD^+) occurs via three pathways, a *de novo* pathway in addition to two salvage pathways (see Figure 1.1). The *de novo* synthesis occurs solely in the liver and kidney cells, and utilises the essential amino acid, tryptophan. The tryptophan is degraded to quinolinate, which then accepts a phosphoribosyl group from PRPP to form nicotinate mononucleotide. This reaction is catalysed by quinolinate phosphoribosyl transferase. The AMP moiety from ATP is then linked to nicotinate mononucleotide by means of a pyrophosphate linkage to form nicotinate adenine dinucleotide. This is catalysed by NAD^+ pyrophosphorylase [EC.2.7.7.1], which is also referred to as nicotinamide mononucleotide adenylyl transferase (NMNAT), and will be thus designated in this thesis. The final step of the pathway converts the nicotinate adenine dinucleotide to NAD^+ via a transamidation reaction with glutamine acting as the NH_2 donor.

The first salvage pathway involves the phosphoribosylation of nicotinate (niacin/vitamin B3) to nicotinate mononucleotide via nicotinate phosphoribosyl transferase, which is then converted to NAD^+ as described above. The second salvage pathway originates with nicotinamide. Following phosphoribosylation, the nicotinamide mononucleotide (NMN) formed is converted directly to NAD^+ by the transfer of an AMP moiety from ATP. This reaction, catalysed by NMNAT, was identified in 1950 by Kornberg and co-workers. Mammalian cells in both *in vitro* and *in vivo* situations utilise nicotinamide salvage as their main source for NAD^+ biosynthesis (Olsson *et al*, 1993). In mammals only liver and kidney cells are capable of utilising the *de novo* pathway (Ikeda *et al*, 1965). However, a diet lacking any of the three starting compounds, tryptophan, niacin or nicotinamide

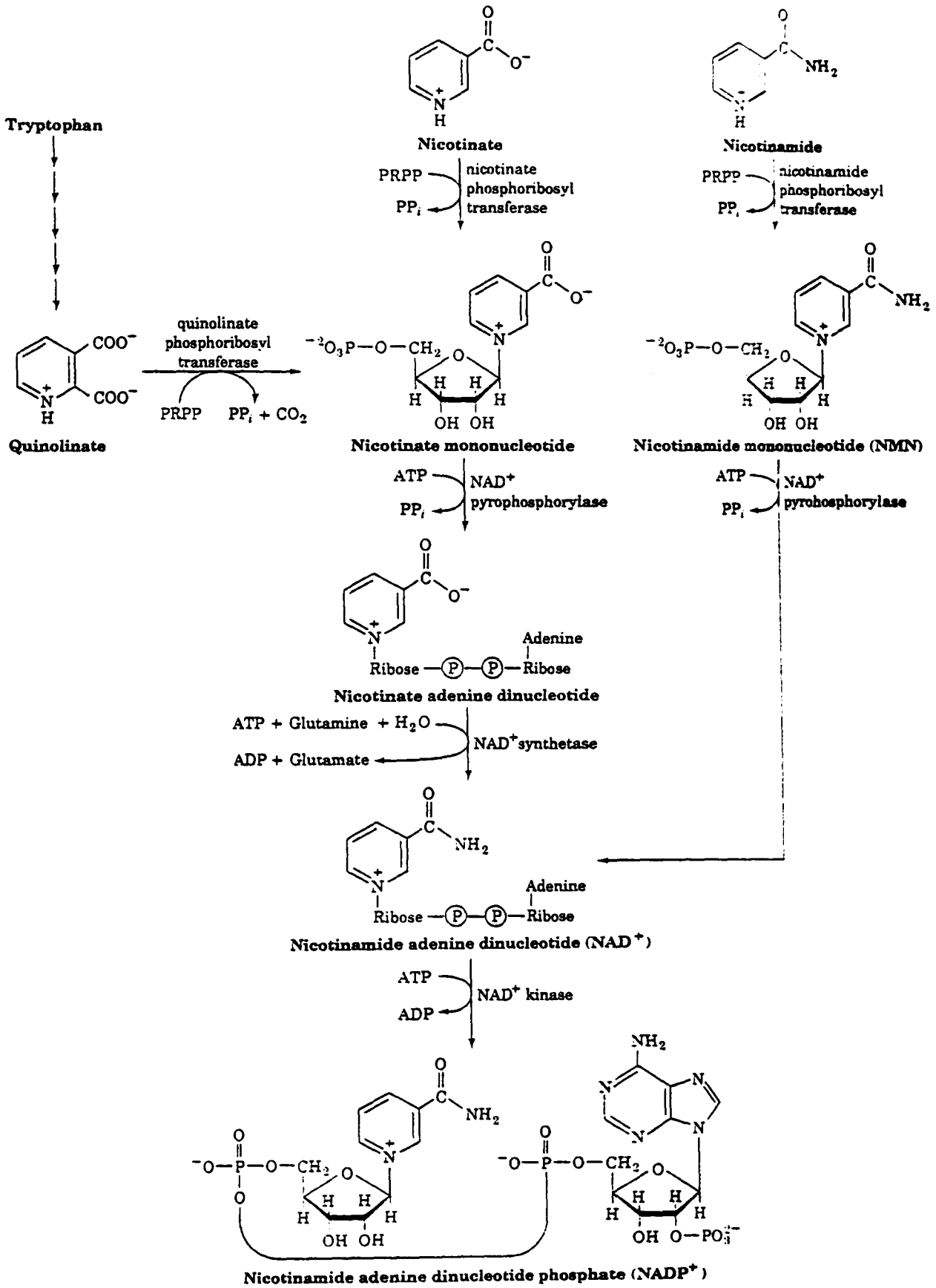


Figure 1.1: The biosynthetic pathways of NAD^+ and $NADP^+$ (Voet & Voet, 1990).

leads to the deficiency disorder, pellagra with symptoms including dermatosis, especially in areas exposed to the sun, and in cases of profound deficiency, encephalopathy resembling the Wernicke-Korsakoff syndrome (disorder of the CNS caused by abusive alcohol intake and nutritional depletion resulting in paralysis of the eye muscles, the inability to stand and an eventual derangement of mental functions) (Hendricks, 1991). Initially, the function of NAD^+ was exclusively considered to be as an electron carrier in the metabolic reactions of the cell, particularly oxidative phosphorylation. These reactions are all situated in the cytoplasm and mitochondria. Consequently, the identification of NMNAT as a nuclear enzyme denoted the possibility of additional roles for NAD^+, with over 90% of the total activity located in the nucleus (Hogeboom & Schneider, 1952). The rate of NAD^+ synthesis and degradation was calculated utilising a double-label pulse-chase technique. [^{14}C] Adenine and [^{3}H] nicotinic acid were used to label the pyridine nucleotides, but as NAD^+ breakdown occurred faster than that of the ATP pool, the overall rate of loss of [^{14}C] adenine from the NAD^+ was calculated as a balance between the rate of NAD^+ breakdown and the rate of re-entry of [^{14}C] adenine from the ATP pool. The rate of NAD^+ synthesis was found to be ~25-fold greater than that required to maintain exponential cell growth (Haines *et al*, 1969; Rechsteiner *et al*, 1975). However, a compensatory rate of NAD^+ bio-degradation was observed, and estimations have indicated that 95% of the NAD^+ synthesised replaces that catabolized, with only 5% required to maintain the pool size required for growth (Rechsteiner *et al*, 1976).

1.1.2 NAD^+ metabolism and the role of Poly(ADP-ribose) Polymerase.

Poly(ADP-ribose) polymerase (PADPRP) [EC 2.4.2.30] is a nuclear enzyme that utilises NAD^+ as its substrate to form homopolymers of ADP-ribose (see Sections 1.2 and 1.5 for detailed literature reviews on PADPRP enzymology and function). The activation of PADPRP via DNA strand breaks reduces the half-life of the (ADP-ribose) polymer from

minutes to seconds with a concomitant increase in the consumption of the cellular NAD^+ pool. In fact, PADPRP activity is considered the major cellular function responsible for the high rate of NAD^+ degradation. Therefore, the positive correlation between NMNAT activity and the cellular NAD^+ content (Haines, 1969), coupled together with the activation of PADPRP and the subsequent decrease in the NAD^+ levels, indicates the biosynthesis and degradation of cellular NAD^+ to be a unique nuclear process, with control over the cellular NAD^+ supply. The post-translational modification of nuclear proteins by (ADP-ribose) polymers has been observed to modulate their activity or function (see Section 1.2.6). A report by Ruggieri and colleagues (1988), who purified NMNAT from yeast, suggested that a possible modification of the NMNAT by poly(ADP-ribose) resulted in an inhibition of enzyme activity. Utilising fast protein liquid chromatography (FPLC) on a highly purified NMNAT preparation, three protein peaks were isolated in addition to the enzymatically active peak. Each of the three peaks contained 3-4 moles of adenine derivatives, and were proposed as inactive modified forms of the NMNAT. Although the existence of PADPRP has yet to be substantiated in yeast, Ruggieri showed an incorporation of [^{3}H] adenine into acid precipitable material that was prevented in the presence of the PADPRP inhibitor, benzamide (BZ).

1.2 POLY(ADP-RIBOSE) POLYMERASE

1.2.1 General introduction

Poly(ADP-ribose) polymerase, (PADPRP), (EC.2.4.2.30), is a unique enzyme found in the nuclei of eukaryotic cells, although several terminally differentiated cells were found to lack PADPRP activity , including mature granulocytes (Ikai *et al*, 1980a & b), mature erythrocytes (Nishizuka *et al* 1967) and intestinal epithelial cells (Porteous & Pearson, 1982). Initially in 1956, Roitt demonstrated that after the treatment of ascites tumour cells with a cytotoxic agent, glycolysis was inhibited with a concomitant increase in the

catabolism of NAD^+. This was inexplainable by NADase action alone. Several years later in the course of RNA polymerase research, Mandel and co-workers (Chambon *et al*, 1963) detected the presence of a new polymer formed from the adenylic moiety of ATP, synthesised by an enzyme dependent on DNA, and in response to added NMN. The polymer was at first thought to be poly (A), but later evidence indicated a novel polymer consisting of ADP-ribose units. The utilisation of radiolabelled NMN and ATP showed that both the ribose and phosphate groups of NMN, as well as the adenylic moiety of ATP were incorporated into the polymer. Both NMN and ATP are precursors for NAD^+. The addition of unlabelled NAD^+ reduced the incorporation of NMN and ATP indicating NAD^+ to be the substrate for PADPRP (Chambon *et al*, 1966).

PADPRP is dependent upon, and activated by, breaks in the DNA strands (Janakideveh & Koh, 1974; Miller, 1975; Halldorsson *et al*, 1978; Benjamin & Gill, 1980). The activation of PADPRP results in the formation of (ADP-ribose) homopolymers that are covalently added to itself (automodification), and to proteins involved in nuclear organisation and biosynthetic reactions, e.g. histone proteins, topoisomerase I, DNA ligase II, RNA polymerase (heteromodification) (see Section 1.2.6 for details). This post-translational modification is totally dependent on breaks in double stranded DNA. An increased activation of PADPRP, represented by an increase in poly(ADP-ribose) synthesis was observed after treatment of DNA with DNase I (Janakidevi & Koh, 1974; Miller, 1975). Gill *et al* (1974), then showed the nucleotide sequence of the DNA to have little or no importance, but that the shearing of the DNA increased the activity of PADPRP, approximately in proportion to the number of strand breaks. The effectiveness of different break ends was analysed utilising purified calf-thymus PADPRP in the presence of either covalently closed plasmid, or plasmid digested with restriction enzymes producing different end structures. It was found that double strand breaks with flush ends were most effective at initiating (ADP-ribose) polymer synthesis, followed by breaks with

3′overhangs, then breaks with 5′overhangs and single stranded breaks (Benjamin & Gill, 1980).

PADPRP function appears to be an important regulatory mechanism of the cell. Therefore this Chapter will aim to review the literature characterising the structure of both the PADPRP and its polymer, and provide an account of the proposed roles the enzyme plays within the cell.

1.2.2 Cellular distribution of PADPRP

PADPRP is a basic, non-histone protein comprised of a single polypeptide chain, and is tightly associated with the chromatin. Early evidence implicated PADPRP with transcriptionally active regions of the chromatin (Mullins *et al*, 1977) and (ADP-ribose) polymer has since been located in these regions, although not exclusively (Hough & Smulson, 1984). The basic repeat unit of chromatin is the nucleosome, consisting of ~200bp of DNA, in association with an octomeric core of histone proteins $(H2A,H2B,H3,H4)_2$. The length of DNA wrapped around the core particle is 140bp with the remainder comprising the "linker DNA", joining the nucleosomes together. 8-10 nucleosomes are organised into the subsequent level of the higher ordered chromatin structure (Stratling *et al*, 1978). Histone H1 is located at the site of DNA entry/exit into the core structure. Interaction of histone H1 with both the linker DNA (C-terminus) and the core histones (N-terminus) provides possible internucleosomal crosslinks which stabilise higher ordered chromatin structure (see Figure 1.2) (Worcel & Benyajati, 1977).

PADPRP is located in the internucleosomal linker region (Mullins *et al*, 1977; Butt *et al*, 1978; Giri *et al*, 1978). In the absence of DNA breaks, PADPRP was observed to bind to superhelical DNA. The binding of PADPRP to two distinct DNA strands results in the formation of looped structures (Gradwohl *et al*, 1987). Under such circumstances, PADPRP could compete for the same binding sites as histone H1 but upon introduction of a break, histone H1, due to preferential poly(ADP-ribosyl)ation would detach from the chromatin, so allowing PADPRP access to these binding sites. The migration rate of a

139bp fragment of plasmid DNA, containing a single strand break was found to be retarded in comparison to an intact fragment. Visualisation of the break-containing fragments by electron microscope identified "V"-like structures. The intact fragments .showed no curvature. Incubation of PADPRP with the nicked fragments visualised the PADPRP bound to the "V" shaped conformation, corresponding to the position of the break (Le Cam *et al*, 1994).

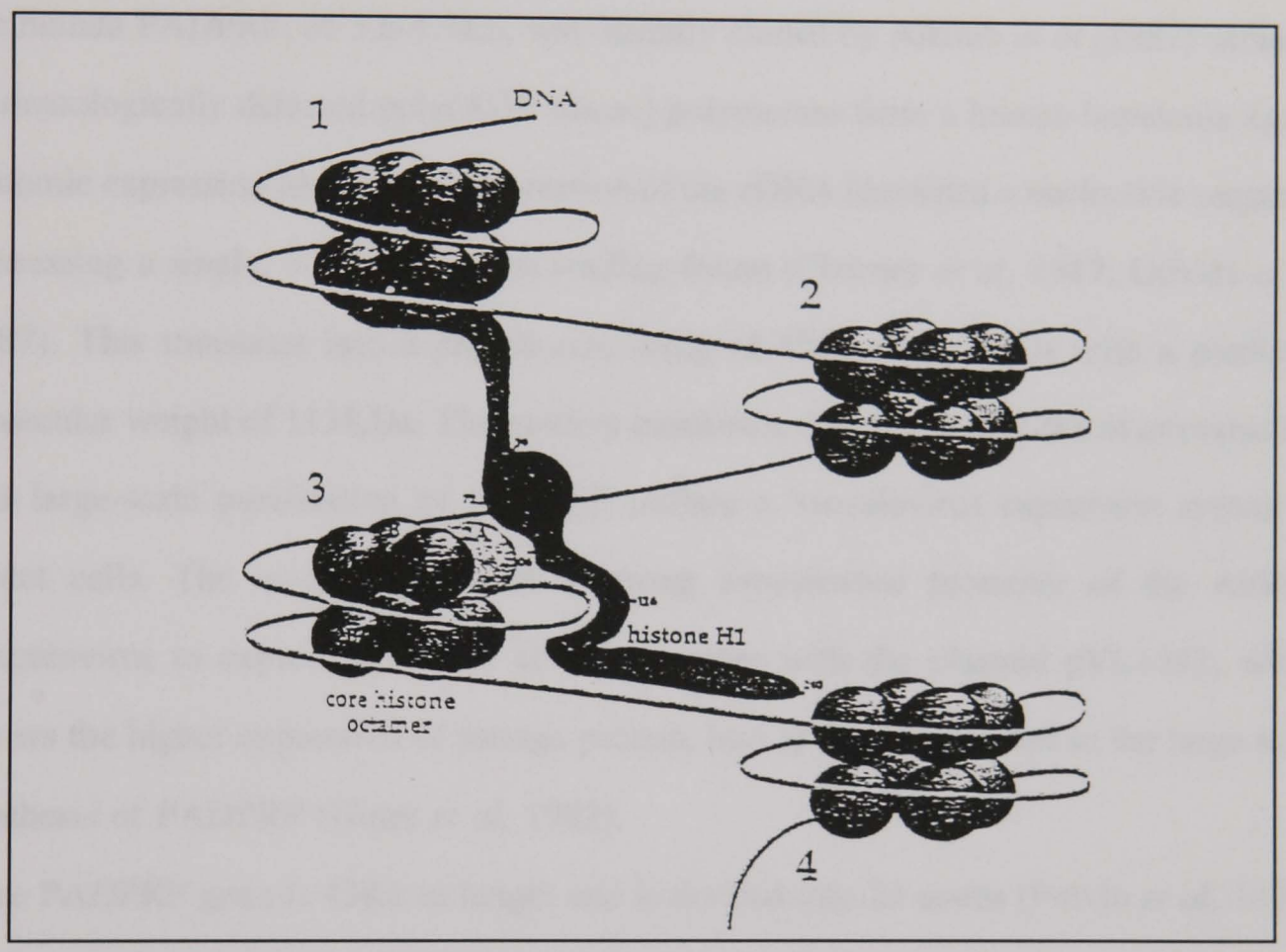


Figure 1.2 A model to show the role of the three domains of histone H1 in nucleosome and higher order chromatin structure (Boulikas, 1993)

Higher order chromatin structure also influences PADPRP activity. Enzyme activity increased until a structure containing 8 nucleosomes was reached, then, any further increase in the number of nucleosomes resulted in a decreased PADPRP activity (Butt *et al*, 1978). The increasing compactness of the chromatin structure may constrain the access of PADPRP to the acceptor proteins.

Chromatin structure is an important component of PADPRP activity and will be discussed further in Sections 1.2.5b and 1.5.

1.2.3 Structural analysis of PADPRP

1.2.3a Introduction

With the advent of molecular biology techniques, a detailed analysis of the structure and function of PADPRP at the molecular level has been achieved. A full length cDNA for the human PADPRP, of 3.6-3.7Kb, was initially cloned by Alkatib *et al* (1987) utilising immunologically detected poly(ADP-ribose) polymerase from a human hepatoma λgt11 genomic expression library. The expression of the cDNA identified a nucleotide sequence possessing a single, 3042 base, open reading frame (Cherney *et al*, 1987; Uchida *et al*, 1987). This translates into a protein consisting of 1014 amino acids with a predicted molecular weight of 113KDa. The modern biochemical technique for the overproduction and large-scale purification of PADPRP utilises a bacculovirus expression system in insect cells. The co-transfection of a strong bacculoviral promoter of the AcNPV bacculovirus to express PADPRP cDNA, together with the plasmid pVL1392, which allows the higher expression of foreign protein, into *Sf* 9 cells, resulted in the large scale synthesis of PADPRP (Giner *et al*, 1992).

The PADPRP gene is 43Kb in length and is divided into 23 exons (Potvin *et al*, 1993). Chromosomal mapping initially located the active PADPRP gene to chromosome 1 with pseudogenes (i.e. non-translated DNA), present on chromosomes 13 and 14, (Cherney *et al*, 1987). Fluorescence in situ mapping confirmed these chromosome locations with specific assignments of the PADPRP gene to 1q42 and the pseudogenes to 13q34 and 14q24 (Baumgartner *et al*, 1992). The PADPRP gene is considered a member of the "housekeeping" genes which are continuously expressed in most tissue. The characterisation of the 5′flanking region of the PADPRP gene revealed conserved sequences homologous with the 5′ regions of other housekeeping genes (Ogura *et al*,

1990; Potvin *et al*, 1993; Oei *et al*, 1994) including multiple transcriptional initiation sites, CCAAT and TAAT promoters located approximately 500bp upstream of the initiation CAP site, and two potential palindromic sequences which Oei *et al* (1994) postulated would facilitate transcriptional activation and could also be involved in transcriptional autoregulation.

Kameshita *et al* (1984), first demonstrated the presence of three functional domains within the PADPRP protein. Using specific protease digestion of the purified protein, a 46KDa NH_2-terminal domain possessing DNA binding properties was isolated, together with a 54KDa NAD^+ binding domain, located at the COOH-terminus of the protein. Separating the two domains was a 22KDa domain associated with auto(ADP-ribosyl)ation (see Figure 1.3).

Initial sequence alignment studies indicated PADPRP to be a sub-family member of the DNA/NAD binding proteins. 3D protein predictions suggested the presence of conserved structural motifs in each of the domains. These will be described in the following Sections.

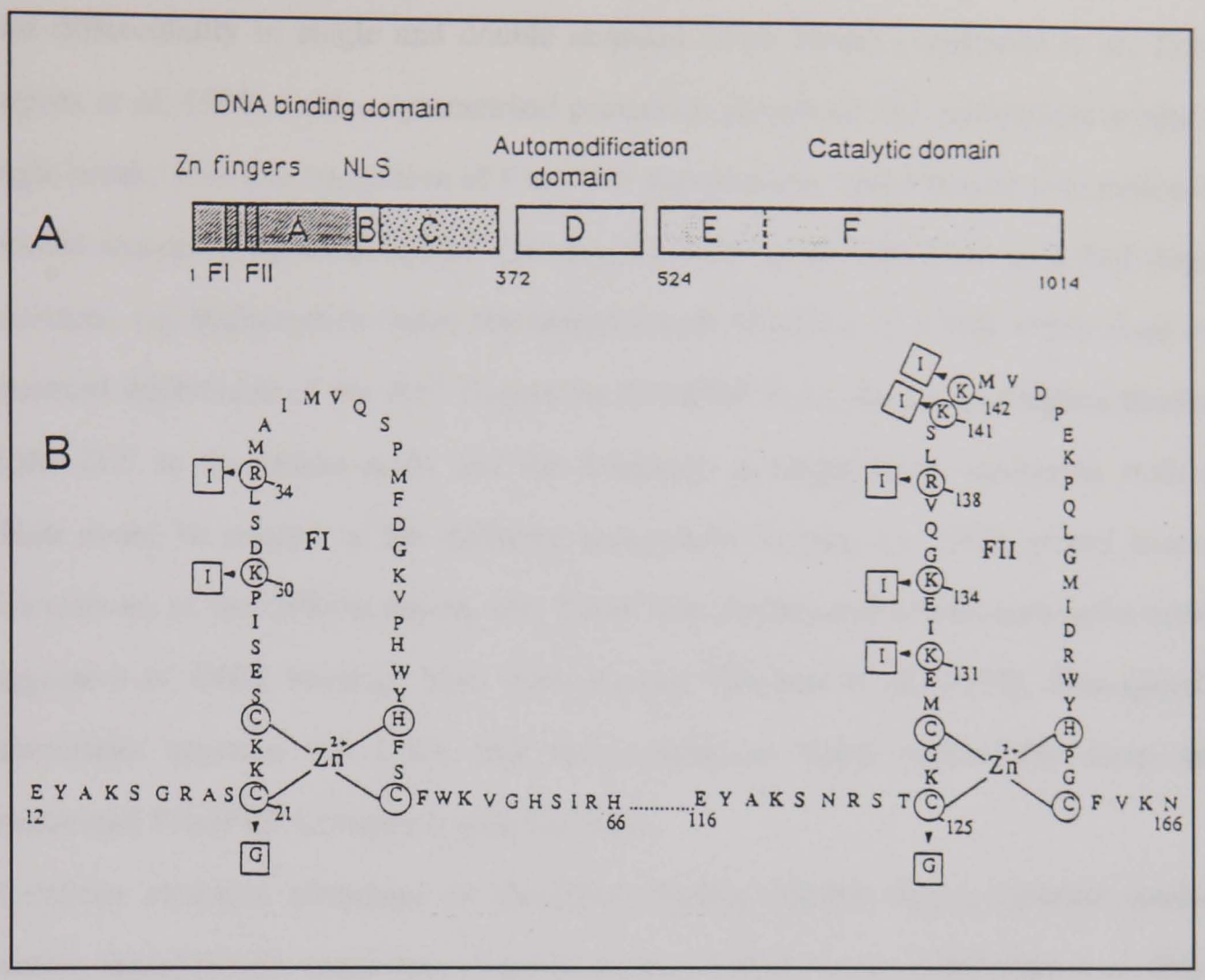


Figure 1.3 Schematic representation of:- A. the three functional domains of PADPRP; and B. the primary structure of the Zn^{+} fingers (Molinete *et al*, 1993).

1.2.3b The DNA binding domain

Energy dispersive X-ray fluorescence determined that PADPRP contained two molecules of Zn^{2+} (Mazen *et al*, 1989). Analyses utilising radiolabelled Zn^{2+} and Western blots, together with the examination of the PADPRP sequence, identified two Zn^{2+}-finger structures in the 29KDa region, closest to the amino terminal (see Figure 1.3). A molecule of Zn^{2+} bound to each finger was essential for activity (Mazen *et al*, 1989). Zn^{2+} fingers are DNA sequence motifs used for recognition and binding to the DNA. Utilising site-directed mutagenesis and footprint analysis, the finger structures were observed to

bind differentially to single and double stranded DNA breaks (Gradwohl *et al*, 1990; Ikejima *et al*, 1990), with a symmetrical protection pattern of 7±1 nucleotides around a single break. This was suggestive of PADPRP dimerisation. There was no recognition of specific sequences by the PADPRP Zn^{2+} fingers as observed with other described finger structures, e.g. transcription factor IIIa (Menissier-de Murcia *et al*, 1989). Other observed structural differences of the Zn^{2+} fingers for PADPRP were, the unusual ligand binding of the Zn^{2+} to the amino acids, and the formation of larger finger structures, both of which could be related to the different recognition system, i.e. DNA strand breaks. Downstream of this 29KDa region, two lysine rich clusters and a helix-turn-helix motif, suggestive of DNA binding, have been located (Ikejima *et al*, 1990). Non-specific interactions between the DNA and such sequences could conceivably keep any unactivated PADPRP associated with the DNA.

A further structure elucidated in the DNA binding domain was a bipartite nuclear location signal (NLS), consisting of two basic amino acid clusters (Schreiber *et al*, 1992) (see Figure 1.3). NLS directs proteins involved in the management of the nucleus from the cytoplasm, where they are translated, back to the nucleus.

1.2.3c The NAD^+ binding domain

This 54KDa domain possesses four autonomous catalytic activities, initiation and elongation of the polymer, a branching activity plus a glycohydrolase/NADase associated activity (Thibodeau *et al*, 1989; Simonin *et al*, 1993b). The domain shows a high level of interspecies sequence conservation, and initial alignment studies proposed the presence of an NTP-binding motif, GKG (Huppi *et al*, 1989; Thibodeau *et al*, 1989). Recent evidence has suggested a sequence homology between PADPRP and the NAD^+ dependent amino acid dehydrogenase enzymes (AADH) (Simonin *et al*, 1990). Site-directed mutagenesis of PADPRP demonstrated that the residue, $lysine_{893}$ and a potential β-α-β fold located at the domain terminal to be essential for activity (Simonin *et al*, 1990 & 1993a), both in accordance with the AADH enzymes. The crystal of the NAD^+-binding

PAGE
MISSING
IN
ORIGINAL

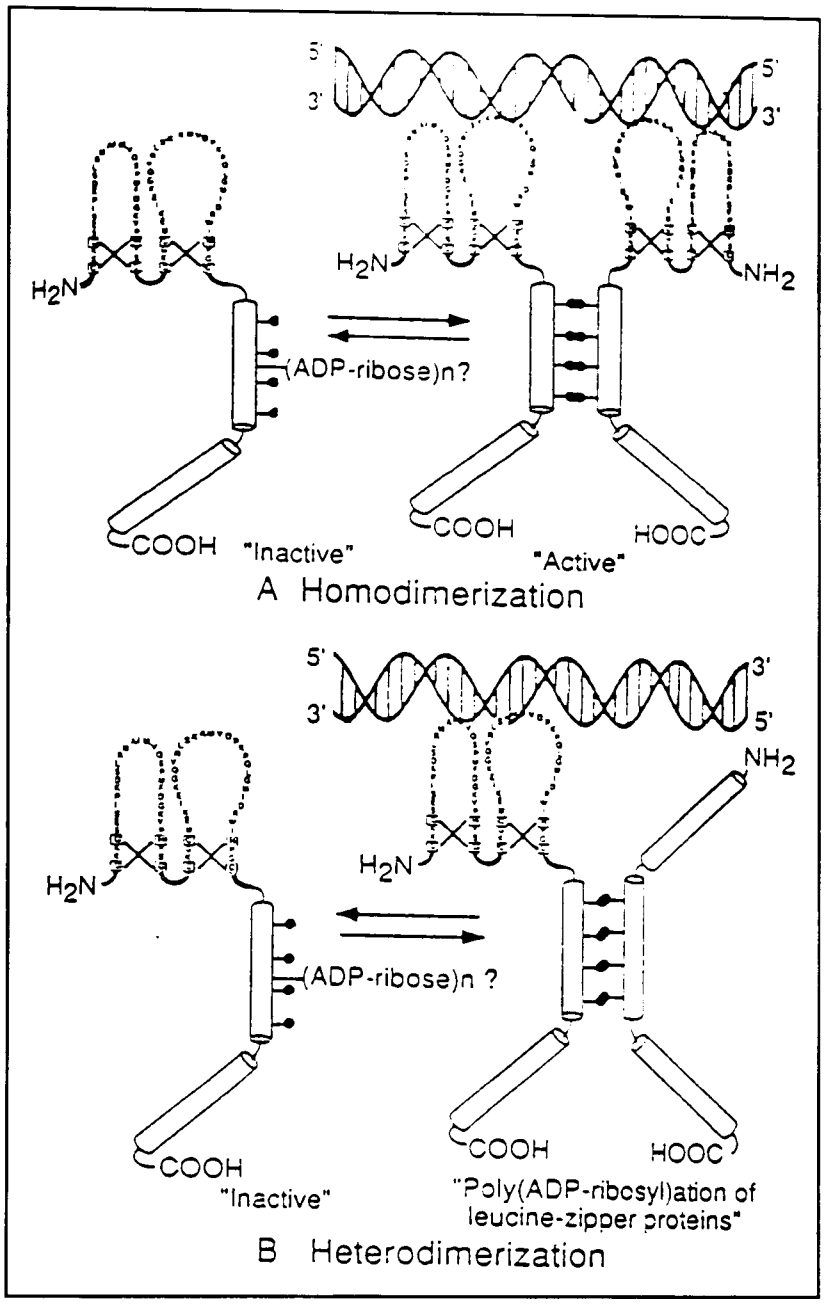


Figure 1.4 Schematic diagram to show the possible dimerisation models of PADPRP (Uchida *et al*, 1993b).

1.2.4 The poly(ADP-ribose) polymer

1.2.4a Substrate specificity

Unlike the majority of NAD^+-dependent enzymes, PADPRP utilises the pyridine dinucleotide, NAD^+ as a substrate, rather than for the transfer of electrons. The adenosine diphosphate ribose moiety of NAD^+ is used to form the homopolymer, poly(ADP-ribose). Other analogues of NAD^+, e.g. NADP(H) and desamido NAD^+ are unable to be substituted, and αNAD^+ acts as a potent inhibitor of the enzyme (βNAD^+ is the naturally

occuring form).

The physiological concentration of NAD^+ within the cell is generally between 100-1000μM (Shall, 1984). The estimated K_{mNAD} for PADPRP is ~50μM (Ueda *et al*, 1982). but as DNA and protein acceptor interactions must be considered, accurate determinations prove difficult.

1.2.4b Research techniques for the characterisation of (ADP-ribose) polymer

The characterisation of poly(ADP-ribose) in intact cells is complicated by the inability of NAD^+ to permeate the cell membrane, and although (ADP-ribose) polymer is classified as the third nucleic acid, the natural content in most tissues has been calculated to be just 3-30ng/mg DNA (Minaga *et al*, 1979). This led to the utilisation of labelled NAD^+ e.g. [^{32}P] NAD^+, [Adenine ^{14}C]NAD^+, to characterise (ADP-ribose) polymer formed in an *in vitro* situation (Miwa *et al*, 1979; Ikejima *et al*, 1987; Naegelli *et al*, 1989; Keith *et al*, 1990; Naegelli & Althaus, 1991), or the use of chloroacetaldehyde, which when incubated with the nucleosides of hydrolysed polymer (see later in this Section) created the unique fluorescent compound 1,N^6-ethenoribosyl-adenosine (Sims *et al*, 1982; Sims *et al*, 1983; Jacobson *et al*, 1983; Alvarez-Gonzalez & Althaus, 1989). Wielckens *et al* (1982) also utilised [2,8,5′-^{3}H] ATP as an *in vitro* label of (ADP-ribose) polymer. However, [^{3}H] adenosine and [^{3}H] adenine were utilised in *in vivo* labelling procedures by Tanuma & Johnson (1983) and Alvarez-Gonzalez & Jacobson (1987) respectively. A problem encountered with *in vivo* labelling of the NAD^+ pool, was the achievement of a sufficiently high specific activity, and the selectivity of labelling, as the adenine derivative used could also be incorporated into RNA and DNA, which then contaminated the $(ADP\text{-}ribose)_n$. The separation of the polymer from such contaminants was performed using dihydroxyboryl affinity chromatography (Wielckens *et al*, 1981). Utilising dihydroxyboronate biorex 70 resin, a mixed resin of boronate and carboxylate groups, the (ADP-ribose) polymers are selectively bound due to the presence of vicinal 1,2 cis-diol groups on the ribose moiety. DNA and RNA possess just a single hydroxyl group, and

hence are unable to bind. Polymers are removed by labilisation of the cyclic boronate ester bonds, which allows charge repulsion between the carboxylic groups in the resin and the phosphate groups on the polymer.

Hydrolysis of the purified (ADP-ribose) polymer with snake venom phosphodiesterase yields three products; AMP, which is the terminal residue of the polymer chain, PR-AMP, which forms the internal polymeric residues, and $(PR)_2$ AMP is the residue located at the branch points of the polymer (Miwa *et al*, 1979) (see Figure 1.5). A further assessment of the PR-AMP fraction can be performed using alkaline phosphatase, creating ribosyl adenosine (reviewed in Shall, 1984).

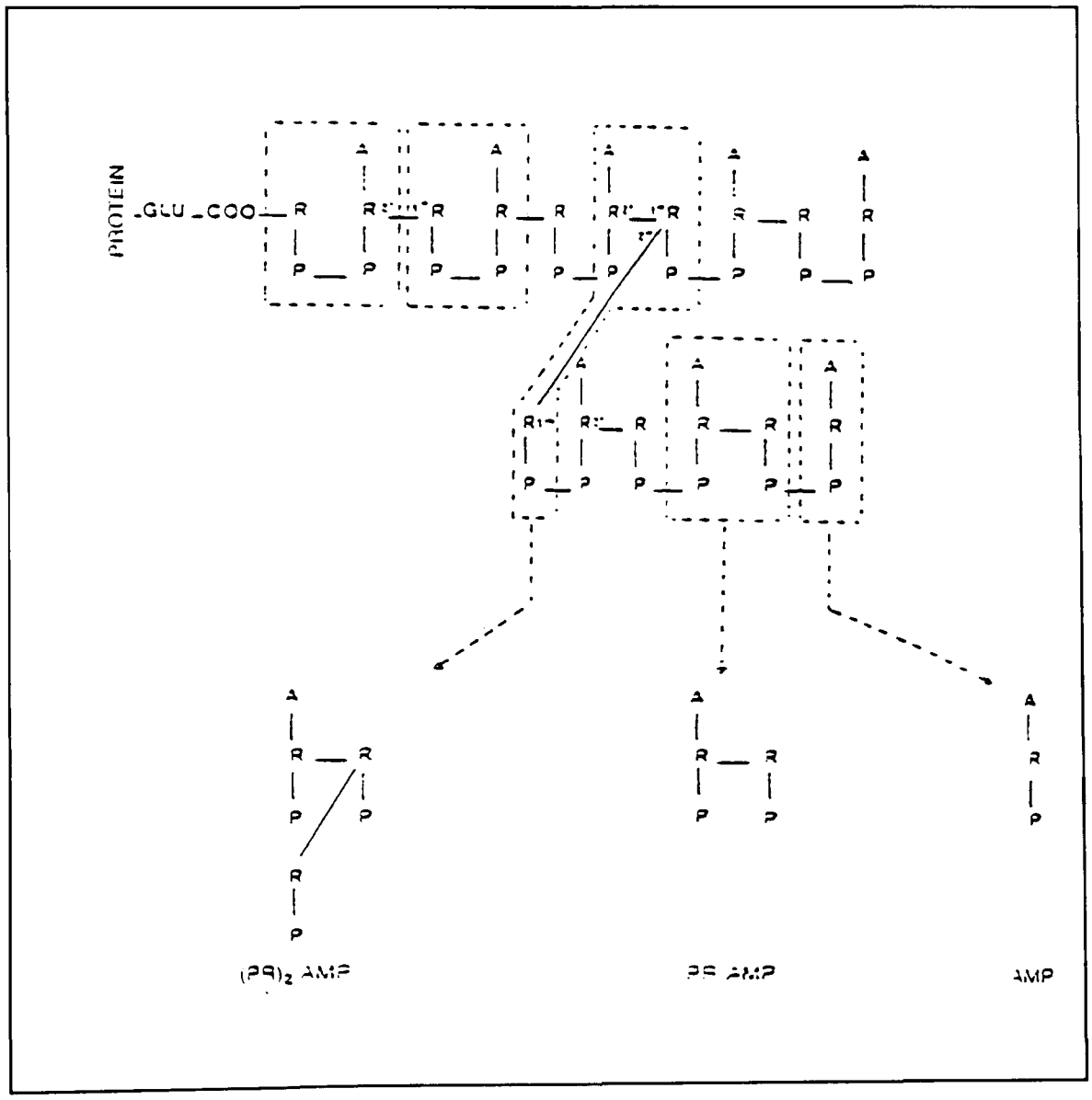


Figure 1.5 The hydrolysis products of poly(ADP-ribose) resulting from treatment with phosphodiesterase (de Murcia *et al*, 1991).

Several techniques have been used to assess the quantity of each hydrolate fraction, thereby determining the initial polymer level. High pressure liquid chromatography (HPLC) is used for the quantification of both radiolabelled and fluorescent residues (Jacobson *et al*, 1983; Sims *et al*, 1982; Sims *et al*, 1983; Alvarez-Gonzalez & Jacobson, 1987; Naegelli *et al*, 1989; Naegelli & Althaus, 1991; Kiehlbauch *et al*, 1993). Poly-acrylamide gel electrophoresis provides an excellent, high resolution, analytical tool for determining the sizes of the fractionated polymer (Panzeter & Althaus, 1990). The small fragments are observed well resolved, but multibranched polymers fail to enter the gel. However, their extraction from the gel allows further characterisation (Alvarez-Gonzalez & Jacobson, 1987; Naegelli *et al*, 1989; Naegelli & Althaus, 1991; Kiehlbauch *et al*, 1993).

1.2.4c Formation and structure of the ADP-ribose polymer

The first ADP-ribose residue of the polymer is bound covalently to an acceptor protein e.g. PADPRP, histone H1, via an alkali labile ester bond, formed between the terminal ribose residue and a carboxyl group of the protein, usually a glutamate or lysine residue. The generation of a linear polymer of ADP-ribose units ensues, linked via $\alpha(1'\text{-}2'')$ glycosidic bonds (see Figure 1.6). The structure formed resembles the secondary helical structure of single stranded DNA (Minaga & Kun, 1983). Branch points within the polymer, linked by $1'''\text{-}2''$ glycoside bonds were isolated by Miwa *et al* (1979), with an estimated frequency of one branch point every 40-50 ADP-ribose residues (Alvarez-Gonzalez & Jacobson, 1987; Keith *et al*, 1990). Overall, polymers containing up to 200 residues with multiple branch points have been isolated *in vitro* and in cultured cell systems (Alvarez-Gonzalez & Jacobson, 1987; Kiehlbauch *et al*, 1993). The size of the polymer formed is dependent upon the experimental conditions, as is the polymer half-life. Alvarez-Gonzalez & Althaus (1989) monitored the degradation pattern of poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase [EC.3.2.1.-] (see Section 1.2.5e) in intact cells, utilising the PADPRP inhibitor, 3-aminobenzamide to prevent additional polymer

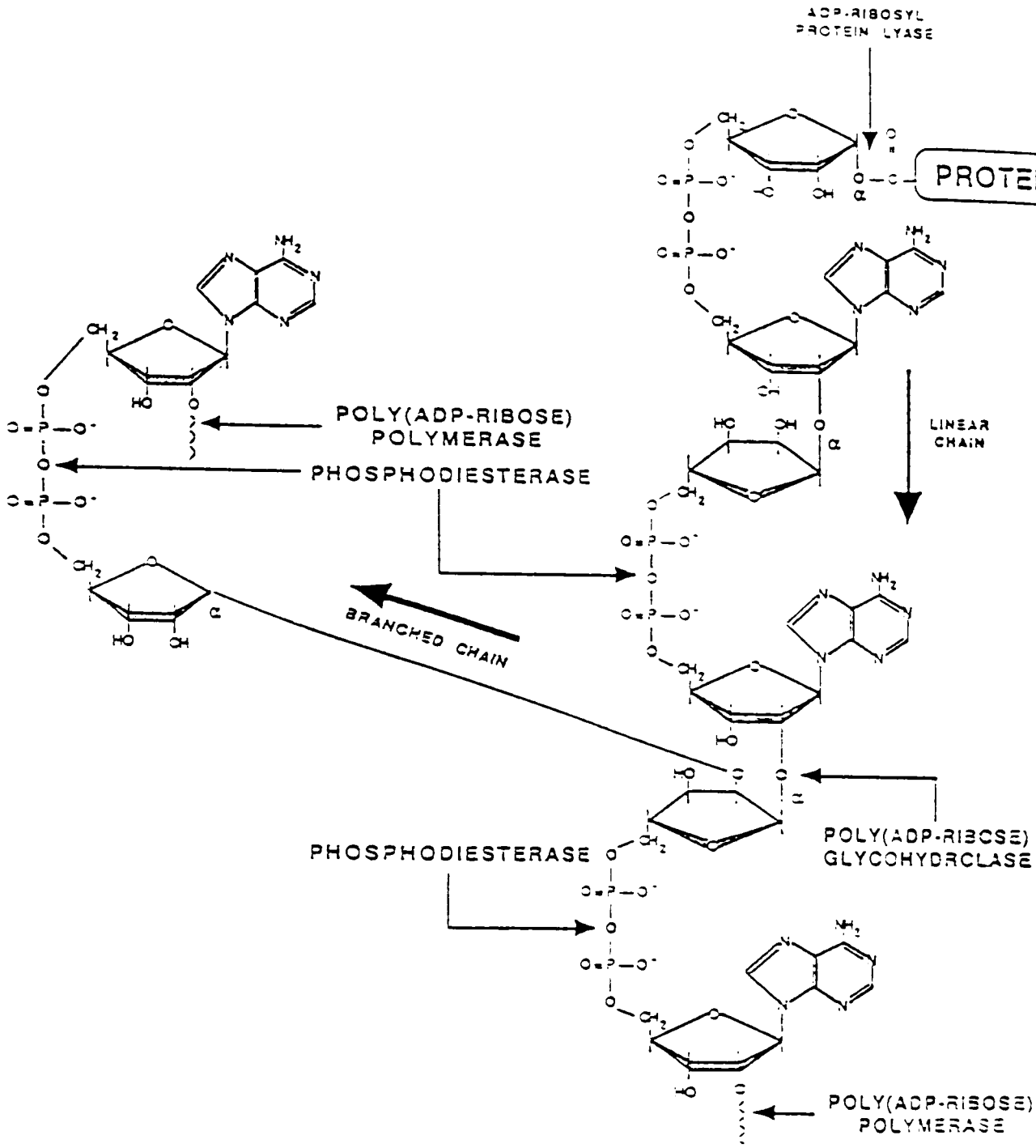


Figure 1.6: The chemistry of poly(ADP-ribose), and the enzymes involved in its synthesis and degradation (Boulikas, 1993).

synthesis. Polymer levels were determined utilising the fluorescent derivative protocol (see Section 1.2.5b). The degradation of (ADP-ribose) polymer was observed to be biphasic. The initial 60% of the polymer degraded with a $t_{1/2}$ of 6 minutes, and the remaining polymer had a $t_{1/2}$ of 7.7 hours. Treatment of intact cells with an alkylating agent, e.g. MNNG or UV irradiation, reduced the $t_{1/2}$ to less than 1 minute, although the dose administered was found to affect the rate of degradation. The greater the polymer synthesis, the faster the initial catabolism (see Section 1.2.5e)(Alvarez-Gonzalez & Althaus, 1989).

1.2.4d Polymer elongation

Two possible modes of polymer elongation have been proposed (see Figure 1.7):

1. Proximal addition would involve the transfer of an existing polymer, bound to a site on the PADPRP, to an ADP-ribose moiety bound at an adjacent site. Alternation between the two sites would result in polymer elongation (Ikejima *et al*, 1987). This proposal suggests an intramolecular reaction, with only completed polymer chains being transferred to the acceptor proteins. **2.** Polymerisation via the distal mechanism requires the addition of ADP-ribose residues to the 1′-OH terminal ribose residue of a polymer chain attached to the acceptor protein. An intermolecular reaction, with two enzyme molecules forming the polymer simultaneously has been proposed.

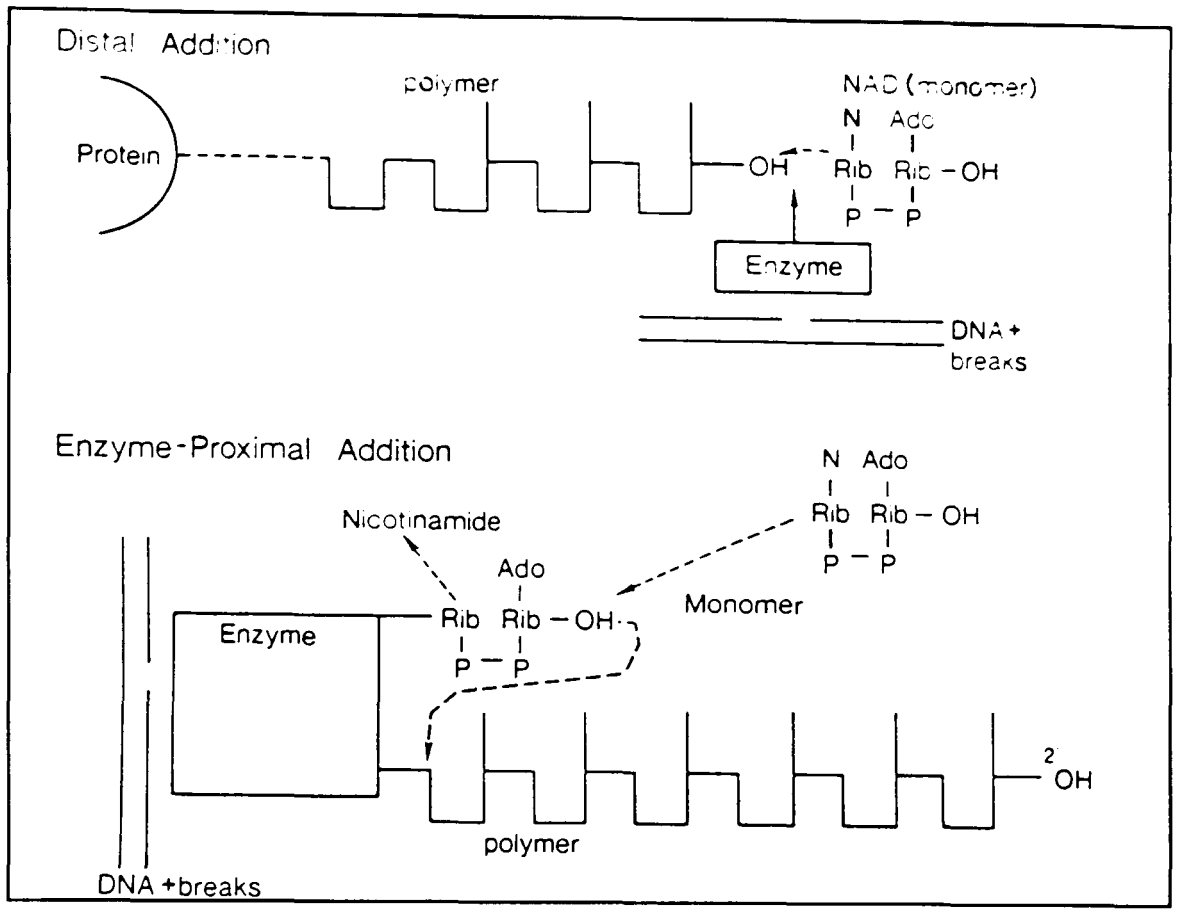


Figure 1.7 Two possible mechanisms of poly(ADP-ribose) chain elongation (Ikejima *et al*, 1987).

1.2.4e Polymer size patterns

Naegelli *et al* reported in 1989, that in a reconstituted *in vitro* system, specific polymer patterns were formed processively in both the auto- and heteromodification reactions. Identification procedures involved the purification by boronate affinity chromatography, followed by digestion with snake venom phosphodiesterase, and either HPLC or PAGE separation (see Section 1.2.5b). The histone proteins, already known to be ADP-ribosylated, were now identified as possessing individual patterns of polymerisation. Also, observations indicated that the configuration of polymer formed during the automodification of purified PADPRP to be dissimilar to that found when in the presence of histone (automodification reaction - polymer sizes ranged from 15-18 residues up to >45 residues, the histone proteins - polymer sizes ranged from 5 residues to >45 residues, with most observed between 25-45 residues). The most potent regulator was histone H1

which appeared to modulate the termination reaction (Naegelli & Althaus, 1991). These elegant experiments raise questions concerning the role of histones in the regulation and modification of PADPRP.

1.2.4f Degradation of poly(ADP-ribose)

The rapid turnover of poly(ADP-ribose) recorded within the cell, especially after DNA damage, requires the consecutive action of poly(ADP-ribose) gycohydrolase and ADP-ribosyl protein lyase.

Poly(ADP-ribose) glycohydrolase

Poly(ADP-ribose) glycohydrolase is a 71KDa, monomeric protein (Uchida *et al*, 1993a) which is the major enzyme involved in the degradation of poly(ADP-ribose) (Hatakeyama *et al*, 1986). The enzyme hydrolyses the ribosyl-ribose bonds, from the adenosine terminus of both the linear and branched portions of the polymer, in an exoglycosydic manner (see Figure 1.8) (Miwa *et al*, 1974). Monomers of ADP-ribose are released from the distal terminus. Endoglycosidic degradation occurs on large, branched polymers (Braun *et al*, 1994; Brochu *et al*, 1994). Known inhibitors include ADP-ribose, cyclic 3′5″ monophosphate, histones (Tavassoli *et al*, 1983), and the recently identified potent inhibitors, the nobotanins B, E and K (Tsai *et al*, 1992; Aoki *et al*, 1993).

A biphasic mode of action for the glycohydrolase has been suggested (Wielckens *et al*, 1982; Hatakeyama *et al*, 1986) with ADP-ribose monomers liberated in a processive manner by the sequential degradation of the polymer branches. Degradation of the remaining oligomer core was distributive, i.e. collective degradation of the polymers. However, Braun *et al* (1994) recently demonstrated three degradative mechanisms:- 1. endoglycosidic cleavage, 2. endoglycosidic cleavage with exoglycosidic, processive degradation, and 3. exoglycosidic distributive degradation. The secondary structure of the polymer, which occurs after the linkage of ~9 ADP-ribose units could influence the glycohydrolase activity. *In vitro* studies demonstrated that large polymers, generally associated with automodified PADPRP, were preferentially degraded, with a K_m 100-fold

lower than the K_m calculated for the oligomers (Hatakeyama *et al*, 1986).

ADP-ribosyl protein lyase

ADP-ribosyl protein lyase specifically cleaves the proximal ADP-ribose unit attached to the acceptor protein. This forms the rate determining step of ADP-ribosylation turnover reactions (Ueda *et al*, 1985) (see Figure 1.8).

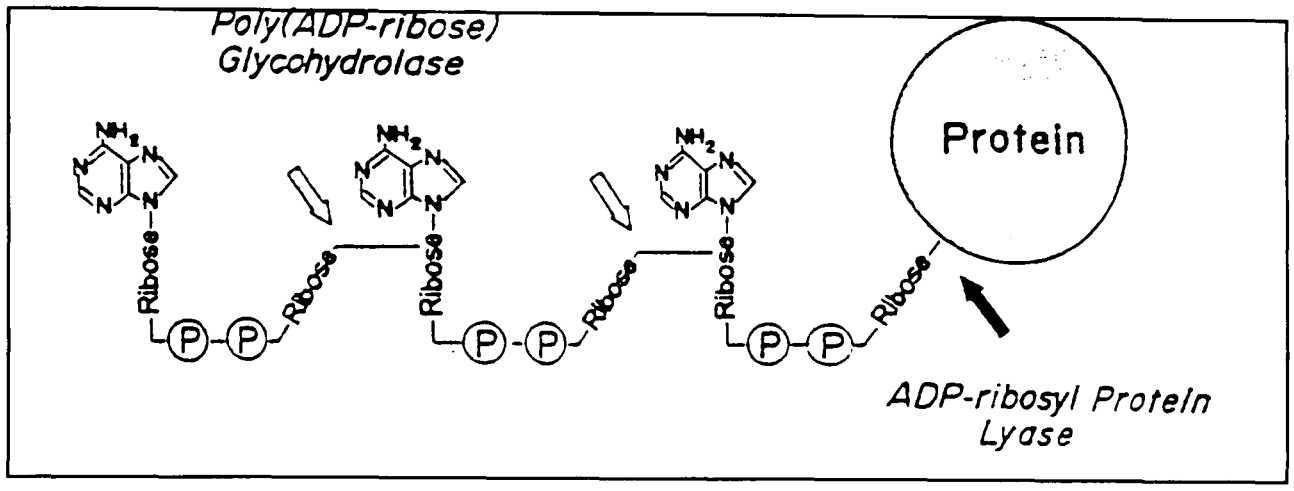


Figure 1.8 Mode of poly(ADP-ribose) degradation (Ueda *et al*, 1985).

1.2.5 Acceptors of Poly(ADP-ribose)

1.2.5a Introduction

The major acceptor for the (ADP-ribose) polymer is PADPRP itself, as was described in Section 1.2.4d. However, a large number of nuclear proteins have been identified *in vitro* as acceptors of the ADP-ribose polymer, with several also characterised in intact cells. Caution is required in extrapolating from *in vitro* to intact cell data, otherwise a distinct impression could occur that any nuclear protein possessed the potential to be modified.

The modified proteins are either structurally or enzymatically connected with the biochemical processes suggested for PADPRP, and are capable of interacting with the DNA. ADP-ribosylation increases the molecular weight and the charge of the protein which could enormously influence both the conformation of the protein and its cellular activity. As has been described, the main acceptor for poly(ADP-ribose) is PADPRP itself

1.2.5b Histone

Poly(ADP-ribosyl)ation of all histone proteins has been observed *in vitro* (Thi Man & Shall, 1982; Huletsky *et al*, 1989; Thibeault *et al*, 1992) but only histone H1 and H2B are known to be modified *in vivo* (Adamietz & Rudolph, 1984). Histone H1 is the second major acceptor of ADP-ribose polymers, and upon modification, a decondensation of the nucleosomal structure was observed utilising electron microscopy (Poirier *et al*, 1982b; Neidergang *et al*, 1985; De Murcia *et al*, 1986). Subsequent treatment with poly(ADP-ribose) glycohydrolase resulted in the rapid renaturation of nucleosomal structure, which suggested that histone H1 did not dissociate entirely from the nucleosomes (Poirier *et al*, 1982; Aubin *et al*, 1983; De Murcia *et al*, 1986). The dimerisation of histone H1, linked by an ADP-ribose polymer was initially thought to be formed during hypermodification. However, as the action of glycohydrolase is exoglycosidic, this concept becomes highly doubtful, and a more conceivable proposal would involve the repulsion of modified histone H1 due to an increased negative charge (De Murcia *et al*, 1986). *In vitro* evidence has suggested that after H1 modification, H2A and H2B become the main acceptors (Adamietz & Rudolph, 1984). Antibody studies have shown a destabilisation of core histones after modification and this may also sterically hinder recondensation (Mathis & Althaus, 1987; Huletsky *et al*, 1989; Thibeault *et al*, 1992).

The effect of poly(ADP-ribosyl)ated histones has been predicted as an early event of the DNA metabolic processes to allow enzymes access to damaged sites on DNA (Neidergang *et al*, 1985). The implications of histone modification within the DNA repair context will be discussed in Section 1.5.

1.2.5c Nuclear matrix

The nuclear matrix consists of a network of proteinaceous fibres, and is thought to provide the structural framework for interphase nuclei. Experimental evidence has indicated the dynamic involvement of the matrix in chromatin replication, repair and transcription (Pardoll *et al*, 1980).

A number of groups, utilising both *in vitro* and intact cell techniques, have identified a large range of nuclear proteins modified by poly(ADP-ribose) (Weisierka-Gadek & Sauermann, 1985; Singh *et al*, 1985; Adolph & Song, 1985a & b; Adolph, 1987; Cardenas-Corona *et al*, 1987; Pedraza-Reyes & Alvarez-Gonzalez, 1990). The modification was preventable in the presence of PADPRP inhibitors. In all cases, modified proteins between 60-70KDa were observed, and Pedraza-Reyes & Alvarez-Gonzalez, 1990) suggested these to be the lamins. The lamins are the most abundant proteins found in the nuclear matrix. The use of 3′dNAD$^+$, an analogue of NAD$^+$ recognised by PADPRP, but from which oligomers of only four residues can be formed, demonstrated covalent interactions between the lamins and poly(ADP-ribose) (Pedraza-Reyes & Alvarez-Gonzalez, 1990).

1.2.5d High mobility group proteins (HMG proteins)

HMG proteins are basic, low molecular weight proteins that are loosely associated with the chromatin. Several *in vitro* reports of HMG-proteins modified by poly(ADP-ribosyl)ation have been published (Poirier *et al*, 1982a; Tanuma *et al*, 1983). An intact cell study, showed a small fraction of HMG proteins to be ADP-ribosylated, with different levels of modification for each protein type. PADPRP inhibitors prevented this modification (Tanuma & Johnson, 1983a). The association of HMG proteins with actively transcribed genes has been suggested, especially HMG 14 & 17, thought to be responsible for the DNase I sensitivity of active chromatin (Einck & Bustin 1985).

1.2.5e Topoisomerase I

The topoisomerases are ubiquitous nuclear enzymes of eukaryotes that catalyse the concerted breakage and rejoining of phosphodiester bonds within the DNA, so altering topological conformation (reviewed in Wang, 1987).

In 1983, Jongstra-Bilen *et al*, and Ferro *et al*, individually published evidence to show the co-purification of a topoisomerase I with calf-thymus PADPRP. The activity of the topoisomerase was inhibited in the presence of NAD$^+$, and in this inhibited state was

found to be modified by ADP-ribose polymers (Jongstra-Bilen *et al*. 1983; Ferro *et al*. 1983). Purified topoisomerase I also acts as an acceptor for polymer resulting in an inhibition of activity (Ferro & Olivera, 1984). Observations suggested complex formation is reversibly inhibited with a repulsion reaction similar to the automodification of PADPRP. *In vivo* data supportive of such a theory indicates topoisomerase bound to chromatin containing PADPRP to be inhibited (Kasid *et al*. 1989). The co-purification and modification of the topoisomerase I could indicate a dual existence and function with PADPRP. Interestingly, the physiological roles suggested for topoisomerase I include DNA replication, DNA repair and recombination (reviewed in Wang, 1985). Sister chromatid exchanges were observed to increase when inhibitors of PADPRP were present (see Section 1.2.7c). The frequency of topoisomerase I within the chromatin has been estimated as 1 enzyme molecule/10-20 nucleosomes, a similar value to that predicted for PADPRP. Topoisomerase I has also been located bound to the polymer acceptors, HMG 17 and histone H1, which results in a stimulation of topoisomerase activity (Kasid *et al*, 1989).

An inhibition of topoisomerase II activity by poly(ADP-ribosyl)ation was shown in vitro (Darby *et al*, 1985). Topoisomerase II is implicated in the organisation of higher ordered chromatin structure. Inhibition of this topoisomerase may be required during periods of chromatin relaxation, e.g. after DNA damage.

1.2.5f Ca^{2+}/Mg^{2+} dependent endonuclease

Endonucleases are nuclear enzymes which hydrolyse phosphodiester bonds within the DNA. Evidence has suggested a role for eukaryotic endonucleases in DNA synthesis (Burzio & Koide, 1973).

In 1975, Yoshihara isolated a Ca^{2+}/Mg^{2+}-dependent endonuclease activity using a purified enzyme system. Activity of the endonuclease appeared to be inhibited by poly(ADP-ribosyl)ation with endonuclease activity partially restored upon incubation with snake venom phosphodiesterase (see Section 1.2.5b) (Yoshihara *et al*, 1975). DNA binding

proteins were found in crude preparations of the enzyme, that were capable of stimulating endonuclease activity but which also acted as acceptors for poly(ADP-ribose) (Tanaka *et al*, 1984). Inhibition of the DNA binding protein activity via ADP-ribosylation would be observed as an inhibition of endonuclease activity. Conceivably, by inhibiting Ca^{2+}/Mg^{2+}-dependent endonuclease activity, especially during periods of DNA damage, extensive degradation of the DNA could be prevented, e.g. as occurs during apoptosis (see Section 1.4.7c).

1.2.5g RNA polymerase II

RNA polymerase II is located in the nucleoplasm and synthesises precursor mRNA and small nuclear RNA. Transcription is initiated more readily on a nicked than an intact DNA template *in vitro*, but in the presence of PADPRP, transcription was inhibited (Slattery *et al*, 1983). A possible competition between the two enzymes for the DNA binding sites was proposed (Slattery *et al*, 1983). Taniguchi, in an *in vitro* study, observed RNA polymerase II from wheatgerm to be modified by ADP-ribose polymers (Taniguchi *et al*, 1985). The presence of a random nick suppressor especially during cellular stress, e.g. DNA damage, may prevent the production of unwanted transcripts.

1.2.5h DNA Ligases

In mammalian cells, three distinct DNA ligases have been isolated (Tomkinson *et al*, 1991). All three enzymes catalyse the formation of phosphodiester bonds, in an ATP dependent reaction sequence, at breaks in the DNA backbone. DNA ligase I has been implicated in the replication process, but physiological roles for DNA ligases II & III have yet to be fully elucidated. Evidence suggests an involvement in repair replication and/or recombination events (Higashitani *et al*, 1990).

Yoshihara *et al* (1985), showed DNA ligase II to be modified *in vitro* by (ADP-ribose) polymer, which resulted in the inhibition of enzyme activity. Intact cell data suggested DNA ligase II activity to be modulated by poly(ADP-ribosyl)ation (Creissen & Shall, 1982). DNA ligase I was not ADP-ribosylated *in vitro* (Yoshihara *et al* 1985). The

modification of DNA ligase II activity within the DNA repair mechanism will be considered in Section 1.5. The effect of PADPRP activity on DNA ligase III is at present unknown. Preliminary suggestions have associated the enzyme with recombination, a mechanism PADPRP is identified with. Therefore DNA ligase III could possibly be controlled via ADP-ribosylation.

1.2.5i SV40 Large T antigen

The large T antigen (T Ag) is the multifunctional early protein of the SV40 tumour virus, involved in the initiation of DNA replication, regulation of gene transcription and control of transformation. Post-translational modification reactions are thought to permit the many functions of this single protein. Both phosphorylation and acetylation have previously been identified. In 1981, Goldman first showed T Ag to be poly(ADP-ribosyl)ated both *in vitro* and in intact cells (Goldman *et al*, 1981). Utilising immunofractionation with anti-poly(ADP-ribose)-sepharose, minichromosomal DNA from SV40 cells containing replicative intermediate DNA was observed to bind with greater affinity than mature DNA (Baksi *et al*, 1987). Modification of T Ag by poly(ADP-ribose) polymers could possibly cause repulsion from the DNA thereby slowing the number of initiated replication cycles.

The Herpes simplex virus early protein, ICP4 has recently been shown to be modified by ADP-ribose polymers (Blaho *et al*, 1992). This protein is also phosphorylated, so perhaps post-translational modifications as a means of regulation have been adopted by many viruses.

1.2.5j Terminal dNTP transferase, DNA polymerase α and DNA polymerase β.

Yoshihara and colleagues (1985), also showed, using an *in vitro* poly(ADP-ribosyl)ation system the suppression of activity of terminal dNTP transferase activity, the enzyme responsible for addition of nucleotidyl units to the ends of polynucleotide chains, and both DNA polymerases α and β, enzymes involved in DNA replication and repair. Mild alkaline treatment, which hydrolyses the ester-linkage between the (ADP-ribose) polymer

and the protein acceptor restored enzyme activity.

1.2.6 Role of PADPRP within the cell

1.2.6a Introduction

The physiological role for the polymerisation/degradation reactions performed by PADPRP and poly(ADP-ribose) glycohydrolase has yet to be clearly defined. A vast body of evidence unquestionably implicates PADPRP in the DNA repair process and this will be reviewed in Sections 1.4.6 and 1.5, but other associations including DNA replication, recombination and differentiation have been postulated. In the following paragraphs the possible role of PADPRP in these cellular mechanisms will be discussed. The use of chemical inhibitors of PADPRP played an important role towards identifying the functions of PADPRP. However, the inhibitors used, e.g. nicotinamide, benzamide, 3-aminobenzamide (see Section 1.3) are of low potency towards PADPRP, and lack specificity. A partial inhibition of PADPRP activity, or the modification of other metabolic processes within the cell could therefore limit the interpretation of the results.

1.2.6b DNA replication & cellular proliferation

Both the synthesis and activity of PADPRP appear to be regulated throughout the cell cycle. The activity of PADPRP rose steadily through G1, peaking in mid-S phase (Kidwell & Mage, 1976; Leduc *et al*, 1988), which correlated with an increased synthesis of the enzyme (Leduc *et al*, 1988). Upon co-incubation with a PADPRP inhibitor, a transient inhibition of G1/S was observed followed, by an S phase delay. A requirement for PADPRP activity prior to entry into S phase may be necessary. PADPRP enzyme levels were also elevated during the G2/M phase of the cycle, although the increase was insufficient to account for the increased level of activity observed (Leduc *et al*, 1988). Alterations in the levels of poly(ADP-ribosyl)ated proteins upon the transition from interphase to metaphase has also been recorded (Adolph, 1987). Contradictory evidence characterising the involvement of PADPRP in cellular proliferation exists within the

literature. Similar PADPRP activities were observed in both proliferating and non-proliferating rat liver tissue (Hilz & Kittler, 1971; Hilz *et al*, 1972), but in lymphocytes stimulated with the mitogen, phytohaemoglutanin (PHA), PADPRP activity was increased (Rochette-Egly *et al*, 1980). Co-incubation with the PADPRP inhibitors, 3-aminobenzamide (3AB) or benzamide (BZ), prior to the addition of PHA inhibited the PHA stimulated DNA synthesis, cellular proliferation, and PADPRP activity, suggesting that PADPRP is involved in the initiation procedure for DNA synthesis (Ittel *et al*, 1983). Also, in Novikoff hepatoma cells (Burzio *et al*, 1972) and leukaemic cells (Burzio *et al*, 1975) PADPRP activity was higher than in the corresponding normal cells. Some of these apparent differences could be accounted for by different experimental conditions.

Overall, PADPRP function is proposed as being indirectly involved in an early event of DNA replication and proliferation. A comparison of the PADPRP and the DNA replication activities, utilising specific inhibitors, e.g. 3AB and aphidicolin which specifically inhibits DNA polymerase α, showed them to be individual processes with no dependence on each other (Berger *et al*, 1978). This was further verified using cells which exhibited temperature sensitive DNA synthesis. PADPRP activity was increased at the non-permissive temperature and expression of the mutant cells induced secondary effects of histone modification plus chromatin decondensation (Savard *et al*, 1981). The relaxation of the chromatin structure due to histone, nonhistone and HMG protein modification is a well documented event (see Sections 1.2.5b-d). and could increase the access of replication enzymes to their substrate. Electron microscopy showed the DNA that co-purifies with PADPRP contained a high proportion of replication forks (De Murcia *et al*, 1983) with PADPRP located in their vicinity. FUrd, which causes an accumulation of short replicative intermediates (Yingnian *et al*, 1989) and DNase I, which fragments the DNA (Smulson *et al*, 1975) resulted in the stimulation of PADPRP activity. During replication, gaps exist at the replication fork, and at 10kb intervals due to newly synthesised DNA intermediates. In 1985, Lonn & Lonn observed that in the

presence of 3AB, ligation of replicon intermediates was inhibited (Lonn & Lonn, 1985). This could be due to a condensed chromatin structure, so access of ligase to DNA is prevented, or a direct modification of the enzyme, thereby altering its activity.

1.2.6c Recombination

Recombination is the exchange of genetic material between two separate segments of chromosomal DNA, extrachromosomal DNA, or between chromosomal and extrachromosomal DNA. In recent years, recombination has received mounting attention, as the techniques for studying the integration of transfected DNA sequences with chromosomal DNA have advanced. This has made possible the study of gene structure and function by construction of "knock-out genes" and the use of gene therapy to correct human genetic disorders. A problem experienced is that in eukaryotic systems, the foreign DNA becomes incorporated at random sites, by processes involving illegitimate recombination (Thomas *et al*, 1986). An involvement of PADPRP in this random integration process has been observed (Farzaneh *et al*, 1988; Waldman & Waldman, 1990). The uptake and integration of transfected DNA into a host genome is a multi-stage process, but the inhibition of PADPRP activity decreased the integration of the donor DNA. At this point of the procedure, strand breaks are induced and subsequently religated. Such findings could implicate PADPRP in eukaryotic recombinational events. Perhaps chromatin decondensation, resulting in an exposure of the DNA strands could act as sites for the intergration reactions.

A well documented involvement of PADPRP activity in eukaryotic recombination is that of sister chromatid exchange (SCE) (Utakoji *et al*, 1979; Oikawa *et al*, 1980; Hori, 1981; Morgan & Cleaver, 1982; Schwartz *et al*, 1983). SCE involves the transfer of homologous DNA sequences between paired sister chromatids. Recombinational repair (see Section 1.3) could provide a significance purpose for SCEs. Initially, SCE experiments were used to assess proposed mutagenic and carcinogenic agents, as they increase SCE frequencies. However, the non-mutagenic, non-carcinogenic PADPRP

inhibitor, nicotinamide was observed to induce SCEs (Utakoji *et al*, 1979). A number of theories were suggested for its involvement. Nicotinamide is an acceptor of the $-CH_3$ group from s-adenosyl methionine, therefore if nicotinamide concentrations increase, the S-adenosyl-methionine pool would be reduced, which could in turn lead to the hypomethylation of intracellular macromolecules. The hypomethylation of DNA would lead to an increased gene expression, and an increased frequency of recombination (Utakoji *et al*, 1979). Also, a decrease in the poly(ADP-ribosyl)ation of a Ca^{2+}/Mg^{2+}-dependent endonuclease was proposed. This would increase DNA strand break levels, thereby increasing recombination sites. SCEs occur spontaneously but are also induced by agents damaging the DNA. 5-bromodeoxyuridine (BrdUrd), used to label the DNA in experiments to assess SCE levels could be recognised as repairable damage. Inhibition of PADPRP, a component of the repair process (see Section 1.5) could increase the half-life of the strand breaks formed, thereby increasing the opportunity for SCEs. Further studies revealed BZ, 3AB and nicotinamide deprivation also increased the level of SCEs following DNA damage, and this was correlated with PADPRP inhibitory ability (Oikawa *et al*, 1980; Hori, 1981; Morgan & Cleaver, 1982; Schwartz *et al*, 1983). Previous suggestions have associated the role of PADPRP with chromatin stability rather than a direct involvement in DNA replication or repair. The inhibition of PADPRP, especially in the repair process, could lead to replication on a damaged template with a persistence of strand breaks. This would extend the availability of the template for recombination.

1.2.6d Differentiation

Cellular differentiation involves the induction of specialised functions, e.g. haemoglobin production in erythrocytes. Differential gene expression has been proposed as the mechanism for the transformation.

Induction of differentiation in 1° chick myoblasts and human peripheral blood lymphocytes by mitogenic stimuli was accompanied by an increased frequency of DNA strand breaks (Farzaneh *et al*, 1982; Johnstone & Williams, 1982). The repair processes

within the cells were judged efficient, as breaks induced by the alkylating agent, dimethyl sulphate (DMS) or gamma-irradiation were proficiently repaired (Farzaneh *et al*, 1987b). Differentiation could therefore require the presence of DNA breaks for the selective expression of genes. Protein factors are known to regulate differentiation. The differentiation inducing protein MGI-2 (myeloid differentiating factor(DF)) was observed to bind to double stranded DNA, but not single stranded DNA (Weisinger & Sachs, 1983), and the purified form induced single strand breaks into a supercoiled SV40 (Weisinger *et al*, 1986). This *in vivo* nicking of the DNA could initiate the required gene expression.

Due to the presence of breaks in the DNA structure, a role, albeit contentious one, was proposed for PADPRP in differentiation processes (Farzaneh *et al*, 1982; Johnstone & Williams, 1982). Nevertheless, one point of agreement does appear to be the involvement of the enzyme in an initial event of the process. PADPRP inhibitors have been utilised to elucidate the role of PADPRP, although again, the results are somewhat contradictory. This could be partially due to the different cell lines and experimental conditions used. When mitogen stimulated human lymphocytes (Johnstone & Williams, 1982), cultured hepatocytes (Althaus *et al*, 1982) or the mitogen stimulated human promyelocytic lymphocyte, HL60 cell line (Farzaneh *et al*, 1987a) were prior incubated with 3AB, BZ or nicotinamide, all of which are inhibitors of PADPRP, induction of differentiation was prevented. Conversely, Grosso & Pitot (1984) saw an increased level of differentiation in an HL60 cell line after treatment with 10mM 3AB, but at this concentration of 3AB, cellular metabolic effects unrelated to PADPRP inhibition become apparent (see Section 1.3.2). Durkacz *et al* (1992), showed utilising a range of PADPRP inhibitors, the induction of melanogenesis in murine melanoma cell line. In a nicotinamide deprived system, the induction of differentiation was also prevented (Farzaneh *et al*, 1982) conceivably indicating a causal relationship between the induction of differentiation and PADPRP activity as nicotinamide is a precursor for NAD^+, the substrate for PADPRP.

Differentiation is a complex process that requires alterations in gene expression and transcription. In addition to increased differentiation, Grosso & Pitot (1984) found that the expression of *c-myc* in HL60 cells was reduced to ~half of control levels following either 3AB or theophylline treatment. *c-myc* expression is inversely correlated to the differentiation state of the cell. The ability of PADPRP inhibitors to induce loss of *c-myc* expression as promyelocytes were differentiated to granulocytes was also observed by Shima *et al* (1989). The up-regulation of metallothionein gene expression was observed in the presence of the PADPRP inhibitor 3AB, and this correlated with reduced levels of poly(ADP-ribosyl)ated chromatin proteins, HMG 14 and 17 and histone H1. The reduced polymer levels were proposed to allow increased access of the RNA polymerase II to the metallotionein promotor (Tanuma *et al*, 1987).

1.2.7 Mono- and cyclic- (ADP-ribosyl)ation reactions

1.2.7a Mono(ADP-ribosyl)ation

Mono(ADP-ribosyl)ation is a post-translational modification involving the transfer of a single ADP-ribose moiety of NAD^+ to an acceptor amino acid, catalysed by specific ADP-ribosyl transferases (ADPRT). Arginine, cysteine, dipthamide, which is a modified histidine residue, and asparagine have all been identified as acceptors for the ADP-ribose group. The mono(ADP-ribosyl)ation reaction does not serve as the precursor for poly(ADP-ribosyl)ation, as the former reaction involves the modification of side-chain N atoms to produce N-glycosides, whereas in contrast, the latter initiates from a carboxyl group to form an O-glycoside (Ueda & Hayaishi, 1985). In fact, mono(ADP-ribosyl)ation reactions predominate over poly(ADP-ribosyl)ation approximately 10 fold in mammalian cells (Hilz *et al*, 1982).

The bacterial toxins were initially identified as ADPRTs, and consequently are the best characterised. The diptheria toxin targets a dipthamide residue present in elongation factor-2 protein (EF-2) (Honjo *et al*, 1968), required for polypeptide chain elongation on

ribosomes in eukaryotic cells. ADP-ribosylation therefore inhibits protein synthesis and ultimately leads to cell death. The arginine-specific ADPRTs, for example, cholera toxin, were found to modify the α subunit of the GTP binding protein G_s (Cassel & Pfeuffer, 1978; Gill & Meren, 1978). A signal from a hormone/receptor complex causes GTP to displace GDP on the G_s protein. This results in the dissociation of G_s-α-GTP, which subsequently binds to adenylate cyclase, the enzyme responsible for the production of the second messenger, cAMP. The hydrolysis of GTP stops further signal transduction. When the G_s-α is ADP-ribosylated, GTPase activity is inhibited resulting in a permanent state of activation (Moss & Vaughan, 1979).

The important identification of an endogenous arginine:ADPRT in turkey erythrocytes (Moss & Vaughan, 1979), with the capability to activate adenylate cyclase in the presence of NAD^+ suggested a possible physiological role for ADPRTs in signal transduction. This suggested the bacterial toxins were exploiting physiological regulatory mechanisms. However, in the endogenous situation a reversible post-translational modification was postulated to prevent cell destruction (Tsuchiya & Shimoyama, 1994). As well as the identification of numerous endogenous ADPRTs, many of which involve GTP binding proteins, ADPRT hydrolases have been identified which catalyse the removal of the ADP-ribose group (Tsuchiya & Shimoyama, 1994; Takada *et al*, 1994).

1.2.7b Cyclic(ADP-ribosyl)ation

In 1987, Lee & colleagues identified a novel form of ADPR, cyclic ADPR (cADPR) (Clapper *et al*, 1987). cADPR functions as a messenger in the mobilisation of intracellular Ca^{2+} stores, in a mechanism distinct from the highly characterised IP_3 route (Berridge & Irvine, 1989). Identified as effecting Ca^{2+} release in sea urchin egg microsomes (Clapper *et al*, 1987; Lee *et al*, 1989), this function has been characterised in mammalian cells (Koshiyama *et al*, 1991; Takasawa *et al*, 1993a).

cADPR is formed from NAD^+ by the linking of the N at position 1 of the adenine ring to the terminal ribose of NAD^+, so displacing nicotinamide (Kim *et al*, 1993).

Surprisingly, the NAD^+ glycohydrolases (NADases), the initial enzymes to be characterised that converted NAD^+ to ADPR, for which a function has failed to be elucidated, were found to possess both the cyclase and hydrolase activities for NAD^+ (Kim *et al*, 1993).

A further exciting development was the identification that a human leukocyte antigen CD38, expressed on the plasma membrane of the cell, possessed both cyclase and hydrolytic activities for cADPR (Takasawa *et al*, 1993b).

1.3 INHIBITORS OF POLY(ADP-RIBOSE) POLYMERASE

1.3.1 Introduction

Inhibitors of PADPRP have played an important role in the elucidation of the physiological function of PADPRP. A large number of compounds have been shown to inhibit PADPRP to varying degrees (see Section 1.3.2), with the majority acting competitively with the enzyme substrate, NAD^+. Most of the inhibitors lack potency, exhibit a low solubility in aqueous solution, and are implicated in other non-specific reactions, e.g. NAD^+ metabolism. In recent years, a number of groups have developed novel PADPRP inhibitors with the aim of increasing the potency and specificity towards PADPRP. This Section will review both the early and novel PADPRP inhibitors and discuss their effects on cellular metabolism.

1.3.2 The classical PADPRP inhibitors

Nicotinamide was the first compound to be identified as an inhibitor of PADPRP (Fujimura *et al*, 1967; Clark *et al*, 1971). Preiss then observed that select, metabolically unrelated pyrimidine derivatives, e.g. thymine, thymidine, 5-bromo-2`-deoxy uridine, and the nicotinamide analogue, 5-methylnicotinamide, could also inhibit the activity of PADPRP (Preiss *et al*, 1971). Thymidine and deoxythymidine were more potent PADPRP

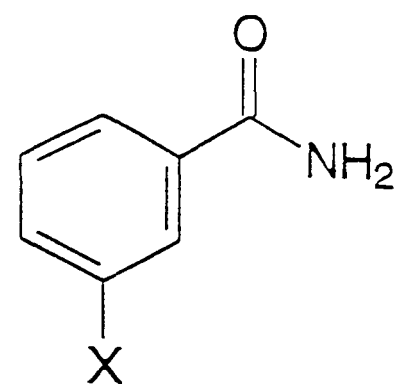
inhibitors than cytidine, deoxycytidine or uracil, suggesting the base was of greater importance than the sugar. Similarities between the pyridine and pyrimidine ring structures suggested these compounds compete for the nicotinamide binding site of PADPRP. The sugar was considered to be of some relevance, as cytosine arabinoside was completely ineffective (Berger, 1978). Inhibition was competitive for both classes of inhibitor, but the involvement of the compounds in other cellular processes questioned their observed biological effects as being attributable to the inhibition of PADPRP. Nicotinamide is a precursor for the biosynthesis of NAD^+ (see Figure 1.1), but also inhibits tRNA methylase especially in malignant cells (Buch *et al*, 1972), forms 1-methyl nicotinamide, so decreasing the S-adenosyl methionine pool (Utakoji *et al*, 1979), and decreases PRPP pools (Leiber *et al*, 1973) due to increased rates of NAD^+ synthesis, which then can result in a depletion of nucleotide biosynthesis. Thymidine has been used to prevent cell cycle progression (Tanuma *et al*, 1979) and is known to inhibit DNA synthesis due to the depletion of dCTP (Meuth *et al*, 1976). The administration of high concentrations of thymidine results in the formation of a large intracellular dTTP pool which produces a feedback inhibition of CDP reduction, thereby causing a dCTP deficiency.

A number of purine based compounds, including the methylated xanthines, and to a certain extent the cytokinins, also exhibit inhibition of PADPRP, by competition for the adenine element of the NAD^+ binding site (Berger *et al*, 1978). However, these compounds are also potent inhibitors of cAMP phosphodiesterase, the enzyme responsible for the degradation of cAMP. cAMP acts as the second messenger in the action of some hormones, e.g. glucagon, and therefore affects the regulation of a wide range of metabolic processes. Therefore, attributing PADPRP related effects derived from the use of these inhibitors in intact cells must be regarded with extreme caution.

The principal PADPRP inhibitors that have been used experimentally are the 3-substituted benzamides developed by Purnell & Whish (1980) (see Figure 1.9).

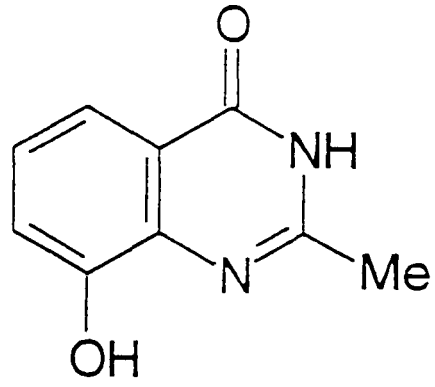
Benzamide is an analogue of nicotinamide, and was initially demonstrated to be a potent PADPRP inhibitor by Shall in 1975. The lack of nitrogen in the ring structure of benzamide was not essential for activity but this increased the specificity of the *in vivo* PADPRP inhibition, as NAD^+ biosynthetic enzymes fail to recognise it as a substrate. The 3-substituted benzamides all act competitively, and show an increased potency relative to nicotinamide. However, the cell contains other NAD^+ consuming enzymes, e.g. mono(ADP-ribosyl) transferases (see Section 1.2.8a), and NAD^+ glycohydrolases, which could also be inhibited by such competitive inhibitors. Rankin *et al* (1989) evaluated the selectivity of the 3-substituted benzamides for PADPRP inhibition. Benzamide was the most potent inhibitor tested with an IC_{50} of ~3μM against PADPRP, but when the concentration was raised to ~4mM, a panel of mono(ADP-ribosyl) transferases were also inhibited.

The elucidation of a physiological role for PADPRP, especially in DNA repair has been investigated primarily using 3-aminobenzamide. However, the specificity of PADPRP inhibition by the benzamides came into question when data was published showing benzamide and its analogues inhibited the enzyme S-adenosyl methionine:nicotinamide methyltransferase, both in liver homogenates and intact rat kidney cells (Johnson, 1981). Milam and colleagues (1984, 1986), then indicated that incubation of lymphoid cells with the benzamides caused a disruption of glucose metabolism, and DNA synthesis, via effects on *de novo* purine and pyrimidine metabolism. Therefore caution was advised in the interpretation of PADPRP dysfunction studies using these compounds. However, these results were concluded from a series of indirect metabolic assays, i.e. the level of DNA synthesis from [^{3}H]thymidine, [^{3}H]methionine and [^{14}C]glucose incorporation was determined in the presence of a benzamide analogue, rather than the determination of the cellular dNTP levels which would have offered a more definitive answer to the effects of the benzamides on nucleotide metabolism. Hunting *et al* (1985), utilising the PADPRP inhibitors 3AB, 3AAB and 3meBZ, showed that PADPRP inhibition had no significant

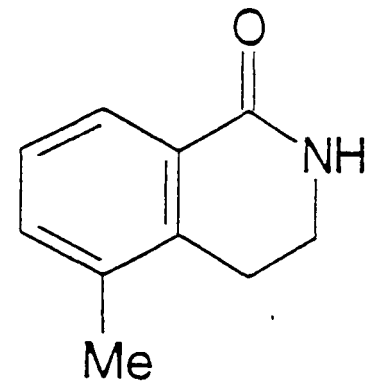


Benzamide; X = H

3-Aminobenzamide; X = NH_2



NU1025



PD128763

Figure 1.9: Chemical structures of PADPRP inhibitors.

effects on dNTP pool sizes, either in the absence or presence of DNA damage. However, utilising [^{14}C]formate and [^{14}C]glycine, the label incorporated into ATP and GTP was depleted following an incubation of the L1210 cells with 5mM 3AB, whereas when [^{14}C]hypoxanthine was used, control levels were maintained. This indicated the PADPRP inhibitor had a possible effect on either *de novo* purine synthesis or folate metabolism. The cytotoxicity of the anti-metabolite, 6-mercaptopurine was enhanced when co-incubated with 3AB and this correlated with an increased PRPP level, indicative of an effect on *de novo* purine synthesis (Moses *et al*, 1990). When a co-treatment with 5FU and 3AB was considered, the cytotoxicity of 5FU was enhanced although there was a lack of effect on both the cellular NAD^+ and the strand break frequency, effects commonly associated with PADPRP inhibition (Willmore & Durkacz, 1993). However, it was observed that 3AB increased the incorporation of FU into DNA by an increased anabolism of the base analogue. Nevertheless, Snyder (1984) found a total lack of effect on the purine dNTP pools following an incubation of human fibroblasts with 3AB.

A comparison of the potential of the benzamides to inhibit (ADP-ribose) polymer formation following MNNG treatment in an *in vitro* and an intact cell situation were in excellent agreement (BZ: *in vitro* IC_{50} = 3.3μM, *in vivo* IC_{50} = 3.5μM; 3AB: both *in vitro* and *in vivo* IC_{50} = 5.4μM) (Rankin *et al*, 1989). The intact cell data for nicotinamide and thymidine (Rankin *et al*, 1989) exhibited a 3-5 fold higher IC_{50} concentration requirement than observed for the inhibition *in vitro*. This could possibly indicate a discrepancy between the intracellular NAD^+ concentration and that used in the *in vitro* cell assay, as the inhibitors compete with the NAD^+ for the active site of the PADPRP. Other possible influences include membrane transport effects, compartmentalisation within the cell or metabolism of the compound.

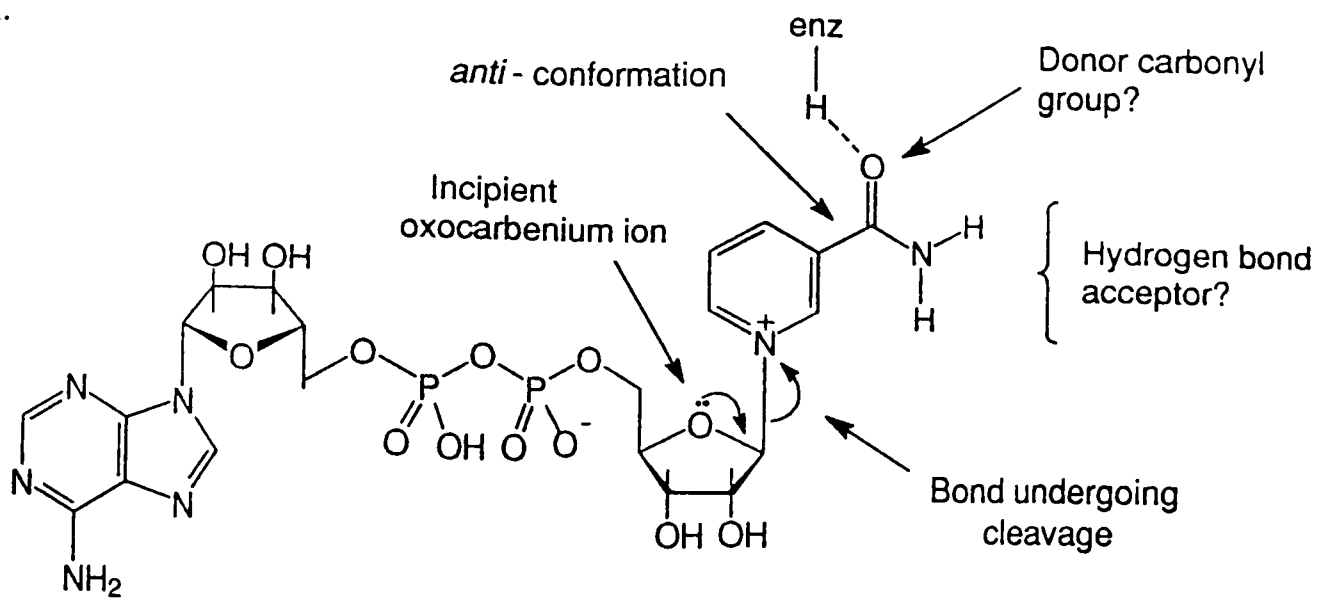
1.3.3 Development of novel PADPRP inhibitors

Although the 3-substituted benzamides displayed increased potency as inhibitors of

PADPRP compared to nicotinamide and thymidine, problems with hydrophobicity, a lack of specificity and low potency towards the enzyme still remained. In 1990, Sestili *et al* published a detailed analysis of the optimal substituted benzamide structure required for PADPRP inhibition. An unsubstituted carboxamide moiety proved essential for activity. A simple model for the binding of the nicotinamide moiety of NAD^+ to the active site of PADPRP is shown in Figure 1.10a. The benzamides are also thought to bind to this site with the 3′-substituted group positioned within the nucleoside-binding domain. Banasik also observed that all PADPRP inhibitors that possessed a carbonyl group showed inhibitory activity and that potency was increased when the carbonyl was built into a second ring (Banasik *et al*, 1992). The orientation of the amide with respect to the 3′substituted position was optimal for activity (see Figure 1.10), and by restricting the rotation of the amide by an ethane/ethene bridge, inhibitory activity was increased (Suto *et al*, 1991). From such an analysis, a series of 5′substituted dihydroisoquinolinones were developed. They retain structural features analogous to nicotinamide and are extremely potent inhibitors of PADPRP. The compound, 3,4 dihydro-5-methoxyisoquinolin-1-(2*H*)-one (PD 128763) (see Figure 1.9), was observed to potentiate radiation induced lethality (Arundel-Suto *et al*, 1991) and the cytotoxicity of monofunctional alkylating agents (Sebolt-Leopold & Scavone, 1992), with an ~10-fold decrease in the concentration of PD 128763 (0.5mM PD 128763) as compared to 3AB (5mM), and exhibiting an approximate 60-fold decrease in the inhibition of PADPRP activity compared to 3AB in an *in vitro* PADPRP assay.

The molecular electrostatic potential (MEP) records the electrostacity of a compound. A correlation was observed between the MEP and the ability to inhibit PADPRP. This was utilised to assess the inhibitory ability of novel benzamide structures (Sestili *et al*, 1990). Compounds with an amide group present in the 3′substituted position, but rotationally restricted in an aromatic ring were predicted to have PADPRP inhibitory activity.

A.



B.

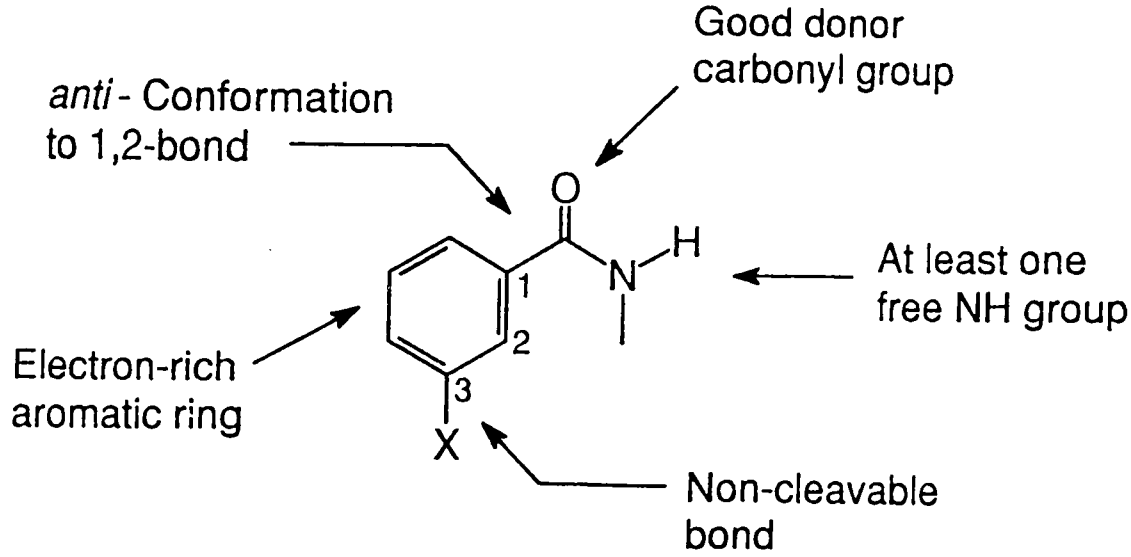


Figure 1.10: Representative structures A. The cleavage point of NAD^+ by PADPRP; B. The requirements for PADPRP inhibitory activity.

A development programme between the Cancer Research Unit and Chemistry Department, both of the University of Newcastle, is currently investigating a number of novel structures involving restriction of the 3'substituted position. The compound, 8-hydroxy-2-methylquinazolin-4(3*H*)-one, NU1025 (see Figure 1.9) has proved to be ~60-fold more potent than 3AB as a PADPRP inhibitor, and is equipotent with PD 128763 (Data shown in Chapter 3). The requirement for at least one free -NH group was demonstrated (see Diagram 1.10b), as when the H (marked X) was replaced with a methyl group the inhibitory activity of the compound was reduced from 92% to 11% at a concentration of 10μM (Griffin *et al*, 1995 in press). The biological consequences of the PADPRP inhibition by NU1025 will be discussed in detail in this thesis.

As the potency of the PADPRP inhibitors increases, their potential to be introduced into the clinics as biomodulators becomes more valid. Intact cell work (as described herein) together with *in vivo* studies will have to be performed to fully assess the specificity of these novel inhibitors and any possible toxic effects. Overall these compounds may prove valuable implements in the fight against resistance to anticancer drugs.

1.4 DNA REPAIR MECHANISMS

1.4.1 Introduction

The best understood role for PADPRP is the involvement in DNA repair, therefore an overview of DNA damage and repair mechanisms was considered essential for this thesis. Mutations in somatic cells, estimated at a frequency of 10^{-9}-10^{-12} mutations/nucleotide/cell generation, have been implicated with the ageing process, cell injury and carcinogenesis. This Section will initially aim to review the types of lesions formed from both spontaneous and environmental damage, their involvement in the mutagenic and carcinogenic processes and the mechanisms employed by the cell to repair them. Finally,

the repair mechanisms with which PADPRP has been associated will be discussed in detail.

1.4.2 Spontaneous DNA damage.

1.4.2a Replication errors

Semi-conservative replication is probably the main source of base substitutions, deletions, and mispaired bases. An estimated 1 error/100 nucleotides has been predicted, but the association of DNA polymerase with a proof-reading ability, i.e. 3′-5′exonuclease action, and the affiliation with accessory proteins, e.g. single strand binding protein, increases the fidelity of replication.

1.4.2b Depurination of bases

In vivo, DNA exists in the fully hydrated, structural B-form (Lindahl, 1993). Consequently, hydrolytic depurination by the protonation of the base, followed by the direct cleavage of the glycosyl bond is likely to be a frequent alteration. A turnover of 2000-10000 purine residues/day has been predicted (Lindahl & Nyberg, 1972), a figure 100X greater than that for depyrimidination. The rapid initiation of base excision repair (see Section 1.4.7) ensures the DNA integrity is maintained prior to the ensuing cycle of replication, thereby preventing possible mutations.

1.4.2c Deamination of bases

Cytosine, adenine and guanine can be deaminated to uracil, hypoxanthine and xanthine, respectively, due to the presence of an exocyclic amide group. Deamination results when the amide group spontaneously dissociates during pH and temperature dependent reactions of the DNA (Lindahl, 1993). The main deamination reaction found is cytosine → uracil. Uracil DNA glycosylase acts rapidly to excise the uracil residues, therefore few mutations arise (see Section 1.4.7).

Methylation of cytosine forms the analogue, 5 methyl cytosine (5mC) which is also a target for deamination reactions. Approximately 3% of all the cytosine in the cell exists

as 5mC, with ~70% of all CpG sites methylated. Methylation at CpG sites is important in the regulation of gene expression. with the patterns of methylation under stringent control (Holliday & Grigg, 1993). The deamination of 5mC results in the formation of thymine which can lead to C:G →T:A transitions during replication. Mismatch repair must be employed to correct this error (i.e. the T:G mismatch). This is a slower form of repair (see Section 1.4.5c), and so coupled with the slightly increased frequency of 5mC deamination as compared to cytosine deamination (Lindahl & Nyberg, 1974; Wang *et al*. 1982; Lindahl, 1993), 5mC becomes a preferred target for spontaneous mutation.

1.4.2d Base oxidation

A large number of metabolic reactions lead to the generation of active oxygen species. A wide spectrum of lesions form during oxidative damage to the DNA, including 8-hydroxyguanosine, planar lesions, e.g. cytosine glycols. and lesions causing major helical distortion, e.g. 8′5 cyclopurine deoxyribonucleosides. The majority are excised from the DNA by DNA glycosylase enzymes. The major mutagenic lesion is 8-hydroxy guanosine which forms a mispair with adenosine (Shibutani *et al*, 1991).

1.4.2e Base methylation

The one-carbon donor of intermediatory metabolism, S-adenosyl methionine, is capable of a weak alkylating action towards the bases of the DNA (Rydberg & Lindahl, 1982; Barrows & Magee, 1982). The main lesions formed are 7-methyl guanine (7mG) and 3-methyl adenine (3mA). Due to a lack of base mispairing during replication 7mG has no effect on the genetic sequence. and is chemically degraded over a period of days. However, 3mA is a cytotoxic lesion which can block DNA replication. An estimation of 600 3mA lesions/day has been recorded (Rydberg & Lindahl, 1982). but the presence of an efficient 3-methyl adenine glycosylase ensures their efficient excision.

1.4.3 Environmental damage

Ionizing radiation and ultraviolet radiation (UV) are the two components of the

electromagnetic spectrum known to have carcinogenic potential.

1.4.3a Ionising radiation

Ionising radiation interacts with molecular species to disrupt chemical bonds, with the three major reactive radicals formed being the hydroxyl radical OH·, a solvated electron, e^{-aq} and the hydrogen radical, H^{+} (Ward, 1975). Although these are short lived, interaction with the DNA leads to base alteration and the formation of strand breaks, by phosphodiester bond cleavage. All four bases are subject to damage, with the major form being the saturation of the C_5=C_6 bond of thymine to form the corresponding glycols and hydrates which in *E.coli* are excised by the specific thymine hydrate (TH) DNA glycosylase (Friedberg, 1984). The secondary structure of the DNA assists in minimising the level of damage, especially the bases, which gain protection due to their central location within the hydrophobic helix. Abundant free radical scavenger molecules, e.g. cysteine and glutathione, compete for the radicals formed, decreasing further the possible detrimental effects of IR on the DNA (Ward, 1975).

1.4.3b UV radiation

UV radiation incites molecules into a short lived "excited state" that renders them reactive. Interaction with aquated DNA promotes the formation of pyrimidine dimers between adjacent thymine bases, that become covalently linked to form a stable cyclobutyl ring, or cytosine residues which form the alkali labile pyrimidine-pyrimidine lesion, from C_6 of one base to C_4 of the second (6,4 photoproduct) (Lippke *et al*, 1981). UV radiation can also induce, but to a lesser extent, the stable thymine glycol (Demple & Linn, 1980), the major lesion associated with ionizing radiation. A number of minor lesions have been identified, including pyrimidine hydrates, but their short half-life indicates rapid repair and the lack of a significant role *in vivo* (Kittler & Loeber, 1977). Intercalating compounds, e.g. psoralen can absorb UV radiation, so initiating the formation of thymine-thymine dimers (Pathak *et al*, 1974). The presence of this lesion can result in the arrest of DNA replication.

1.4.4 Chemical damage

1.4.4a Aromatic amines & polycyclic hydrocarbons.

Many substances encountered during every day life can increase the risk of carcinogenesis. Two highly studied chemical groups are the Aromatic amines e.g. N-2 acetyl 2 aminofluorene (AAF) and the Polycyclic hydrocarbons, e.g. benzo(a)pyrene. Both are present in a number of industrial substances, with benzo(a)pyrene also found in coal tar, cigarette smoke and exhaust fumes. They are metabolically activated, principally in the liver, by the cytochrome P-450-dependent mono-oxygenases, located in the endoplasmic reticulum of the cells. The activated metabolites alkylate the C_8 position or the 2-amino group of guanine, to form "bulky" lesions repairable via nucleotide excision repair (Kreik, 1972; Weinstein *et al*, 1976) (see Section 1.4.5c). The latter adduct is a minor product of the interaction of AAF metabolites with the DNA, but the principle lesion formed in the dihydrodiol-epoxide intermediate of benzo(a)pyrene. The persistence of this lesion is implicated in the initiation of carcinogenesis.

1.4.4b Chemotherapeutic agents

Ironically, compounds which damage DNA and are potentially mutagenic are exploited for cancer therapy. A vast number of chemotherapeutic agents have been identified and these can be broadly divided into two classes:- 1) Direct acting agents interact directly, or following metabolic activation via the P-450 enzyme complex, with the DNA resulting in the formation of a large variety of DNA lesions e.g. mono- and bi-functional alkylating agents (e.g. MNNG and BCNU respectively), cisplatin and several of the antibiotics, e.g. actinomycin D.

2) Anti-metabolites interfere with the biosynthesis of DNA and result in the in the incorporation of incorrect bases during DNA synthesis, e.g. methotrexate, 5-flurouracil, 6-mercaptopurine.

The number of chemotherapeutic agents and all the respective modes of action are too extensive to be discussed within the scope of this thesis. Because of the focus of this

thesis, only the alkylating agents will be reviewed.

1.4.4c Alkylating agents

Alkylating agents were the first class of cytotoxic drug to be utilised in the clinic. They interact with all biological molecules, especially the DNA bases, due to an electrophilic reactive species that forms covalent links with nucleophilic groups, e.g. $-NH_2$, P, -OH. Alkylating agents can be either mono-functional (one reactive site) or bi-functional (two reactive sites), and interact with numerous potential sites on the DNA bases. Generally, the most modified sites are O^6alkyl guanine, N^7alkyl guanine and N^3alkyl adenine, but steric effects of the DNA are a major determining factor. The physiological configuration of DNA is the B form, so due to positions in the major groove, the N^7 and O^6 residues of guanine, are easily accessed by the alkylators (Friedberg, 1984). The principle mutagenic lesion formed is O^6methyl guanine which mispairs with thymine during replication, so leading to a GC:TA transitions.

A clinically relevant group of alkylating agents with a particular pertinence to this thesis are the imidazotetrazines. These are a novel group of synthetic compounds that possess a NNN cyclic linkage, which under alkaline conditions is hydrolysed to form the active, corresponding linear alkyl triazenes (Stevens *et al*, 1987; Tisdale, 1988). Mitozolomide, is activated to a chloroethylating species that forms crosslinks with the DNA. Promising results from Phase I clinical trials were obtained (Newlands *et al*, 1985) and mitozolomide was entered into Phase II trials. However, the minor antitumour activity towards small cell lung cancer and malignant melanoma was accompanied by severe myelosuppression, which halted the further clinical useage. Temozolomide (TM) is a 3-methyl substituted analogue of mitozolomide that is unable to form DNA crosslinks, but retains antitumour activity. Under alkaline conditions TM is chemically degraded to the alkylating agent, MTIC (Stevens *et al*, 1987; Tsang *et al*, 1991) (see Figure 1.11).

The metabolically activated linear triazene, dacarbazine (DTIC) gave disappointing results in the clinic, possibly due to interpatient variations in the level of metabolising

enzymes. A similar mechanism of cytotoxicity for DTIC and TM has been suggested, but as TM is chemically degraded, improved clinical results were anticipated. Due to low toxicities in the preclinical screens, TM was selected for entrance into a Phase I clinical trial. Clinical activity was observed in malignant melanoma, mycosis fungoides and high grade gliomas, with only a limited myelosuppression (Newlands *et al*, 1992). Phase II trials are at present ongoing (Woll *et al*, 1995).

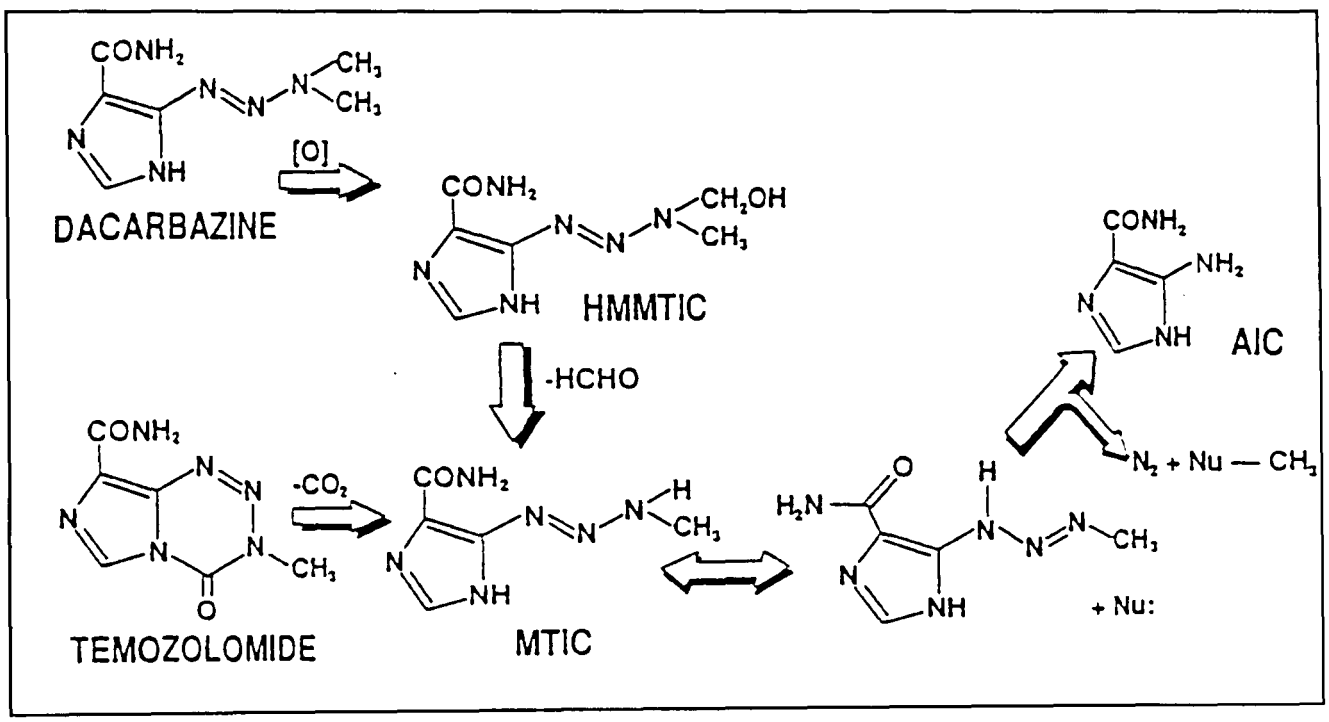


Figure 1.11 Pathway of decomposition of temozolomide (Tsang *et al* 1991).

Cells treated with TM show increased levels of alkali labile sites in the DNA that correlate with the removal of 7meG (70% of total lesions) and 3meA (9% of total lesions) (Hepburn & Tisdale 1991). The lesion O^6meG, was also observed and correlated with cytotoxicity, but the level of sensitivity was decreased in those cells with high O^6 methyl guanine methyl DNA transferase (MGMT) levels (see Section 1.4.5b). However, upon co-incubation of such cells with an inhibitor of MGMT, the sensitivity to TM was increased (Tisdale, 1988; Hepburn & Tisdale, 1991; Baer *et al*, 1993).

Terminal differentiation in TM treated cells has been observed (Tisdale, 1988; Zuccetti *et al*, 1989; Tsang *et al*, 1991) but the mechanism of action has still to be elucidated. An increased expression of the haemoglobin gene was correlated with its hypomethylation,

in TM treated K562 cells (Tisdale, 1988) but the methylation patterns of the globin genes or the oncogenes, *c-myc* or *ras* treated with TM remained unaltered (Zucchetti *et al.* 1989).

1.4.5 Carcinogenesis

The term "cancer" is the general term used for a malignant tumour. A tumour is initiated due to the ability of a cell to prevail over the normal growth regulatory mechanisms, resulting in the formation of a clone of cells. This then competes for the space and nutrients of the adjacent tissues. The potential to invade other areas of the body, i.e. metastasis, is the extremely pernicious feature that signifies malignancy.

Carcinogenesis is defined as "a multistage process driven by carcinogen induced genetic and epigenetic damage in susceptible cells that gain a selective growth advantage and undergo clonal expansion as a result of the activation of proto-oncogenes and/or deactivation of tumour-suppressor genes" (Harris, 1991) (see Figure 1.12).

The initial postulate for the induction of carcinogenesis consisted of two phases:-

1. Initiation - is the exposure of a cell to a chemical, physical or microbial carcinogen, or spontaneous endogenous damage that results in an irreversible genetic change, leading to the suppression of regulated growth.
2. Promotion - is the increased proliferative state which promotes the possibility of further genetic damage, e.g. mutation or amplification of proto-oncogenes or deactivation of tumour-suppressor genes, thereby increasing the risk of malignant development.

The majority of cancers originate from initial mutagenic events. However, most genotoxic agents exhibit a threshold dose below which tumours do not develop, due to the activation of the cellular repair mechanisms that correct the defects imposed on the genome. If these repair processes become saturated or are defective in any way, the errors in the DNA persist and are replicated leading to the fixation of the mutations and induction of carcinogenesis (Loeb, 1989). Mutagenesis is only part of the carcinogenesis

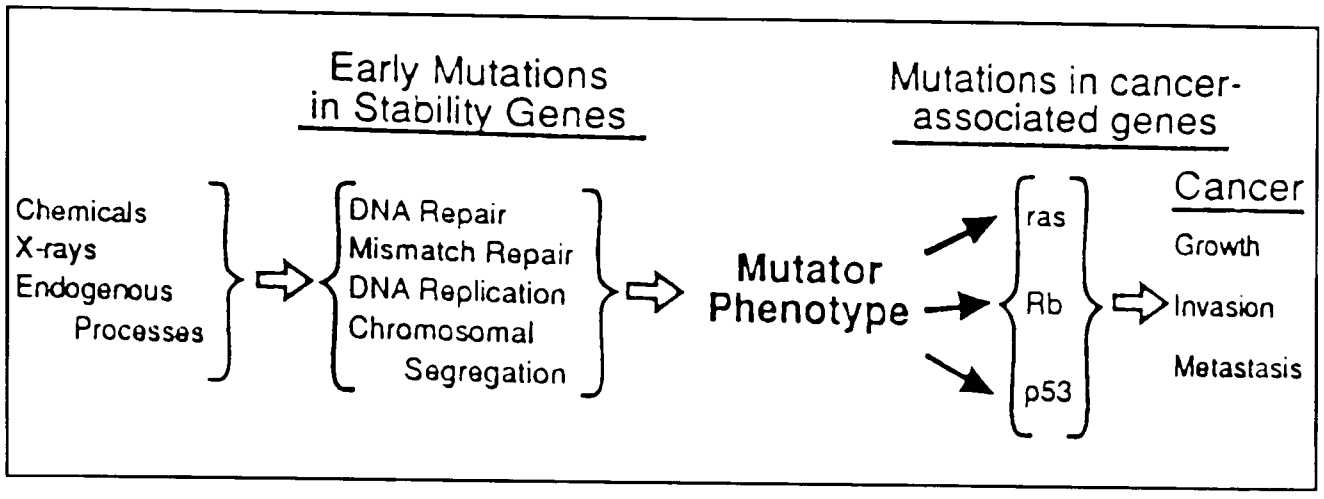


Figure 1.12 Schematic representation of the sources for multiple mutations in cancer (Loeb, 1994).

process, and this is highlighted by the fact that some compounds are carcinogenic, but not mutagenic (Clayson, 1989; Grasso *el al*, 1991). These compounds, e.g. the antibiotic, sulphonamide, activate endogenous mitogens, e.g. growth factors, thereby increasing the proliferative capacity of the cell and so the chance of replicative errors (Shaw & Jones, 1994).

Many genes in normal mammalian cells are homologous to viral genes whose products are involved in the transformation of the normal cell to a malignant state. Such genes are termed "protooncogenes", and many encode for proteins involved in the control of cell proliferation and differentiation, e.g. the *ras* family proteins possess GTPase activity and are considered the intermediatary signal between the cell surface receptor and the adenylate cyclase second messenger; *c-src* is a protein tyrosine kinase involved in phosphorylation cascades. Protooncogenes are endogenously found in low levels, but once a cell has been transformed, e.g. by amplification of the protooncogene, chromosome translocations, or when under viral transcriptional control, the oncogene subsequently fuctions at high levels. Over activation may then lead to inappropriate activity, e.g. amplified or continuous signals, increased kinase activity, which would thereby alter cellular activities. In contrast, tumour suppressor genes, e.g. p53 (see Section 1.4.7b), when inappropriately inactivated cause dysregulation of growth and differentiation

(reviewed in Harris, 1991).

Following the mutation of genes whose products sustain genome stability, e.g. proteins involved in DNA replication, repair, recombination, the cell has a reduced ability to overcome any further genomic damage. Thus, the probability of preventing the induction of carcinogenesis, following subsequent mutational events would be significantly decreased. Definitive evidence for such a mutator phenotype has been observed in colonic cancer cells (Parsons *et al*, 1993; Ionov *et al*, 1993). In families suffering from hereditary nonpolyposis colorectal cancer (HNPCC), an increased hypervariability in the CA microsatellite repeat regions, known mutation hotspots (Schlotterer & Tautz, 1992), has been observed. A mutation in the gene homologous to *mut S*, the bacterial gene responsible for methyl directed mismatch repair was also identified in HNPCC patients (Modrich, 1991), and a lack of functional mismatch repair has been observed in extracts from colon cancer cells (Leach *et al*, 1993). Together, these pieces of evidence provide a logical reasoning for the microsatellite instability. Mismatch repair corrects single-base substitutions, deletions, additions, frameshifts and small sequence rearrangements (see Section 1.3.5c), so increasing the accuracy of replication 10-400 fold. The CA repeats are usually considered "junk" DNA, but a possible function could be in the prevention of non homologous recombination (Radman & Wagner, 1993). Many cancer cells show an increased rate of non homologous recombination, so perhaps instability of microsatellite DNA will be identified in other forms of cancer.

1.4.6 Mechanisms of DNA repair

1.4.6a Introduction

The persistence of DNA damage, especially during DNA replication results in an increased risk of carcinogenesis. Therefore, in response to the damage, the cell initiates repair mechanisms that restore the genetic integrity prior to replication, thus protecting the cell from mutagenesis and eventual carcinogenesis.

The repair processes are broadly divided into two main classes 1) Direct repair and 2) Excision repair.

1.4.6b Direct repair

Direct repair involves the removal of the DNA lesion without the loss of material and leaves the DNA intact. Two examples of this form of repair are enzymatic photoreactivation and the repair of O^6 alkyl guanine lesions. The former involves the activation of the light absorbing DNA photolyase to remove cyclobutane pyrimidine dimers that have the potential to inhibit DNA replication (Sancar & Sancar, 1988). O^6alkyl guanine lesions are mutagenic, causing GC→AT transitions. A repair mechanism has evolved that utilises the protein, O^6methyl guanine methyl DNA transferase (MGMT) [EC 2.1.1.63] which stoichiometrically transfers the methyl group from the guanine residue to a S-methyl cysteine residue within itself (Demple & Karran, 1983; Pegg, 1990). This results in the restoration of the guanine residue in the DNA, but the MGMT protein is not regenerated, and for this reason has been termed a "suicide enzyme".

The mammalian MGMT gene has recently been cloned (Hayakawa *et al*, 1990; Rydberg *et al*, 1990). The preferred substrate is O^6me G, although longer alkyl groups, e.g. propyl, butyl, and branched structures also act as substrates, but with a decreased affinity (Pegg, 1984). The human MGMT level ranges from a low expression in the brain and mammary gland to high levels in the liver and spleen (Pegg, 1990). These differences in the rate of O^6me G repair confer a tissue dependent susceptibility to tumour promotion.

1.4.6c Excision repair

During excision repair, the damaged bases/nucleotides are excised from the DNA by specialised enzymes. The DNA integrity is then restored by the action of DNA polymerases and DNA ligase. The concepts of excision repair were initially conceived in 1964 (Boyce & Howard-Flanders, 1964; Setlow & Carrier, 1964), and two major excision mechanisms, base and nucleotide excision have been identified in all living organisms. However, mismatch repair, which corrects base mispairs, and recombinational

repair that is essential for the repair of crosslinked DNA. have also been distinguished and will be briefly discussed later in this Section.

Base excision repair (BER)

The multi-enzyme process of BER (see Figure 1.13) has been characterised most fully in *E.coli*, but significant advances towards elucidating the mammalian process are being made.

The initial event of BER is the hydrolysis of the N-glycosidic bond that links the modified or damaged base to the sugar/phosphate backbone. This reaction is catalysed by DNA glycosylase enzymes, each enzyme having specificity for a single type of base. The DNA glycosylases are ubiquitous globular enzymes, ranging from 20-50KDa with no requirement for cofactors (Friedberg, 1984; Lindahl, 1990). In *E.coli*, six DNA glycosylase enzymes have been isolated to date. The sequence analysis of four of the enzymes showed no homology and lacked characteristic DNA binding motifs, e.g. Zn^{2+}-fingers, helix-turn-helix (Sakumi *et al*, 1986; Varshney *et al*, 1988). The characterised enzymes are:-

Uracil DNA glycosylase removes the misincorporated and deaminated base, uracil. Encoded by the *ung* gene in *E.coli* the survival of *ung*- mutants indicate this to be non-essential (Duncan, 1985). Hypoxanthine DNA glycosylase removes the product of adenine deamination. 3methyl adenine (3meA) DNA glycosylase is important for the removal of alkylation damage. In *E.coli* two forms of the enzyme exist, TagI, encoded by the *tag* gene shows specificity for 3meA (Riazuddin & Lindahl, 1978), and TagII encoded by the *AlkI* gene. TagII, recognises a broader range of lesions mainly located in the minor groove, including 3meG, 7meG, 7meA, O^2meT, O^2meC plus the ethyl derivatives. As the minor groove is usually unmethylated (Friedberg, 1984), TagII has been proposed to "patrol" the domain. Formamidopyrimidine DNA glycohydrolase encoded by the *fpg* gene (Boiteux *et al*, 1987), removes the C_5=C_6 pyrimidine lesions resulting from ionizing and oxidative interactions. Pyrimidine dimer DNA Glycosylase removes pyrimidine

dimers and associated lesions with the concerted action of an AP endonuclease. All these enzymes are also found in mammalian cells with the exception of the pyrimidine dimer DNA glycosylase. Also, only a single 3meA DNA glycosylase has been identified with specificity for both 3meA and 7meG. In murine plasmocytoma cells a further DNA glycosylase has been identified that removes hydroxymethyluracil, a product of ionizing radiation and oxidative damage (Hollstein *et al*, 1984).

After removal of the base, the lability of the apurinic/apyrimidinic (AP) phosphodiester bonds is increased. Spontaneous hydrolysis via a β-elimination reaction can result, but the $t_{1/2}$ of 40-100 hours is limiting (Lindahl & Andersson, 1972). Located in all organisms are special nucleases that specifically hydrolyse these bonds. Again, the nucleases of *E.coli* are the most intensively studied with the identification of several functional enzymes although, as many are minor enzymes, they are unlikely to be of biological significance. The main nuclease is exonuclease III encoded by the *xth* gene. The initial identification of a 3′→5′exonuclease action gave the enzyme its name, although the endonuclease activity is the main enzymic function (Weiss & Grossman, 1987). Catalysis of the phosphodiester bond occurs 5′ to the AP site producing 3′OH and 5′P termini (Gossard & Verly, 1978). The second most prevalent endonuclease is the inducible, endonuclease IV which shares characteristics with the endonuclease enzymes found in yeast (Johnson & Demple, 1988), drosophila (Kelley *et al*, 1989), and human cells (Kane & Linn, 1981). It also cleaves 5′ to the damaged base, usually as a result of oxidative damage. The mammalian AP endonucleases have been characterised from many sources, including HeLa cells, human fibroblasts and placenta (Linn, 1982). The placental AP endonuclease is capable of incising at either the 3′ or the 5′ sites but not both.

Until recently the BER mechanism had a "missing link", as following 5′ incision there remained a base-less deoxyribosephosphate residue attached to the 5′ termini. Due to obstruction by the residue, the DNA polymerase I of *E.coli* was shown to promote strand displacement synthesis rather than nick-translation, therefore increasing the possibility of

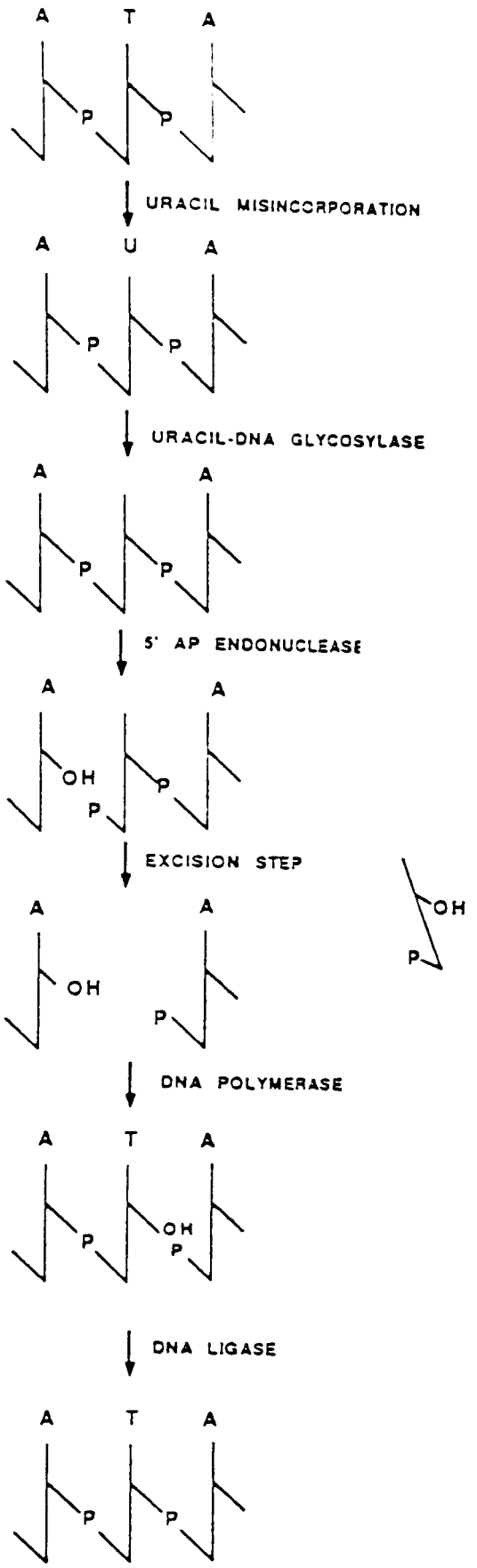


Figure 1.13: DNA base excision repair pathway for the removal of damaged bases and AP sites (Franklin & Lindahl, 1988).

inaccurate replication (Mosbaugh & Linn, 1982). Initially, an enzyme activity was identified in *E.coli* with the ability to remove this incomplete nucleotide (Franklin & Lindahl, 1988). A similar enzyme activity has been isolated in mammalian cell extracts (Franklin & Lindahl, 1988). Recently this action has been identified as a function of the DNA exonuclease, encoded by the *E.coli* recJ protein involved in the recombinational repair pathway and mismatch repair (Dianov *et al*, 1994).

Nucleotide excision repair (NER)

NER is the most versatile of all the cellular repair mechanisms with a vast array of DNA lesions corrected, e.g. UV dimers, photoproducts, chemical lesions,(e.g. metabolites of aromatic amines and benzo(a)pyrene), cisplatin adducts and even oxidative damage (Huang *et al*, 1994). Such adaptability within a single procedure is partially due to the creation of distortions in the DNA helix by the "bulky" adducts, that are then recognised by the repair enzymes. However, the multitude of specialised proteins that work collectively as an excinuclease complex to remove the damaged sites must also offer versatility.

The initial process of NER was elucidated in *E.coli*, and this has been used as a simplified basis for the determination of the eukaryotic system. Elucidation of the eukaryotic NER pathway (see Figure 1.14) has advanced significantly only in the last three years. Cleaver initially identified patients suffering from the genetic disorder, Xeroderma pigmentosum (XP) which confers sensitivity to UV irradiation, to be deficient in the incision step of the NER mechanism. Complementation analysis identified seven different groups, indicating the involvement of at least seven proteins in the recognition and incision of damage (Cleaver, 1983). Recently, the cloning of the yeast (Prakash *et al*, 1993) and human repair genes (Van Duain *et al*, 1986; Tanaka *et al*, 1990; Weeda *et al*, 1990; Legerski & Peterson, 1992; Weber *et al*, 1990; Thompson *et al*, 1994; Scherly *et al*, 1993; MacInnes *et al*, 1993) has been the factor responsible for establishing the role of each protein, and the inter-protein relationships within the NER process. A high degree

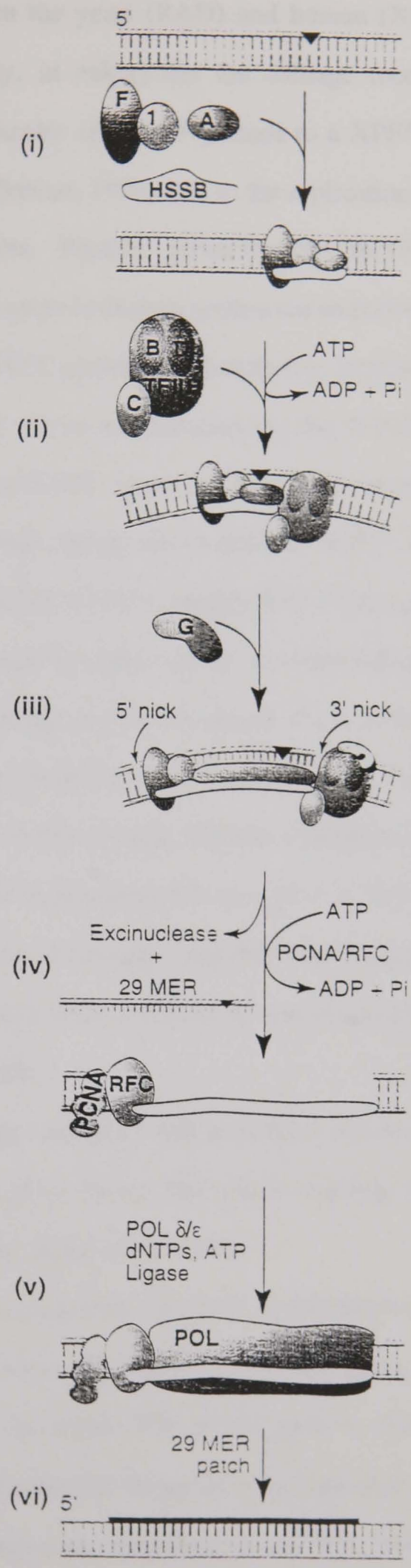


Figure 1.14: Molecular mechanisms of mammalian nucleotide excision repair (Sancar, 1994).

of evolutionary conservation between the yeast (RAD) and human (XP/ERCC) repair genes has been established. Initially, in eukaryotes the damage recognition protein RAD14/XPA (Robins *et al*, 1991; Guzder *et al*, 1994) binds to a XPF/ERCC4:ERCC1 heterodimer (Li *et al*, 1994; Park & Sancar, 1994) and to the replication protein, HSSB, which binds to the damaged site. Protein subunits, RAD3/XPB/ERCC2 and RAD25/SSL2/XPB/ERCC3, which possess helicase activities are recruited to the damaged site by the RAD14/XPA. The RAD4/XPC protein is subsequently installed at the damage site, either through an attraction to, or a loose association with, the TFIIH (Sancar, 1994). The presence of the helicases presumably opens up the helix structure as in the prokaryotic system. This conformational change allows the RAD2/XPG (Habraken *et al*, 1993; O'Donovan *et al*, 1994) and RAD1/RAD10 complex/XPF (Tomkinson *et al*, 1993; Sung *et al*, 1993), both of which possess nuclease ability, to make a dual incision at the 3′ side (O'Donovan *et al*, 1994) and the 5′ side (Bardwell *et al*, 1994) of the lesion, respectively. The excision of the oligonucleotide and the removal of the repair proteins is facilitated by the proliferating cell nuclear antigen (PCNA) (Shivji *et al*, 1992), and the ensuing "gap" is infilled by either DNA polymerase δ or ε prior to DNA ligation.

A scanning method for the recognition of damaged sites has been proposed whereby the protein(s) would "track" along the helix until a distortion was encountered (Gruskin & Lloyd, 1988a,b; Dowd & Lloyd, 1990).

Recently, the most significant finding was that NER is divided into two sub-pathways:

1) Transcription coupled repair: removes lesions that block ongoing transcription and therefore requiring urgent elimination (Bohr *et al*, 1985).

2) Genome overall repair: removes lesions from the bulk of the non-transcribed DNA.

Initially, transcription and repair were considered to be individual processes. The observation of preferential repair of the transcribed *DHFR* gene in CHO cells (Bohr *et al*, 1985) challenged this theory and further investigations highlighted the possibility that this phenomenon is common to all organisms (Madhani *et al*, 1986; Mellon *et al*, 1986;

Mellon & Hanawalt, 1989). The stalled RNA polymerase was initially considered as the signal that attracted the repair enzymes to the damaged site. However, an investigation of *E.coli* transcription coupled NER found that on encountering a blocking lesion, the RNA polymerase I complex, recruited a transcriptional repair coupling factor (TRCF), encoded by the *mfd* gene (Selby *et al*, 1991). The TRCF once bound to the complex, altered the conformation of the RNA polymerase, resulting in the release of both the enzyme and the truncated mRNA. The TRCF which possesses sequence homology with UvrB, remained bound to the DNA and attracted the UvrA protein so initiating repair of the damaged site (Selby & Sancar, 1993). The elucidation of the events in the eukaryotic transcription coupled NER has identified the proteins RAD1 and RAD10 associate to form a DNA endonuclease with a preference for single stranded DNA (Sung *et al*, 1993; Tomkinson *et al*, 1993) and that the RAD2/XPG/ERCC5 and RAD4/XPC interact specifically with the factor b/TFIIH (Freidberg *et al*, 1994). The known components of the factor b/TRIIH, i.e. RAD25/XPB/ERCC3 and RAD3/XPD/ERCC2 are of dual function which suggests the complex functions as a single unit in both transcription and repair.

Repair synthesis

Following DNA incision at the damaged site, exonucleases and DNA polymerase enzymes act at repair patch sites. DNA strand breaks associated with the repair of ionising radiation or small adducts formed by the action of alkylating agents, induce short patch repair of 1-4 nucleotides.

The mammalian DNA polymerases lack associated exonuclease activity, therefore independent enzymes are affiliated. Several 5′-3′ acting exonucleases have been isolated. DNase IV shows a preference for double stranded DNA substrates with 5′-phosphate or hydroxyl termini. Although DNase IV shows a resemblance to the polymerase I of *E.coli* it lacks polymerising activity (Friedberg, 1985). Two exonucleases have been found in Human placenta - Human placental correxonuclease and DNase VII. The correxonuclease

is specific for single stranded DNA and can initiate hydrolysis of 30-40 nucleotides from either the 5′ or 3′ termini (Friedberg, 1985). DNase VII is also specific for single stranded DNA but liberates short nucleotides exclusively from the 5′ terminus (Friedberg, 1985). In Novikoff hepatoma cells and HeLa cells, DNase V was isolated with specificity for double stranded substrates (Friedberg, 1985). The repair synthesis is executed by the DNA polymerases α and β, both of which show a preference for "gapped" DNA (Friedberg, 1985). Polymerase β shows greater versatility than polymerase α, which is only poorly processive; i.e. dissociation from the DNA occurs following the addition of ~11 nucleotides. The polymerase β will initiate synthesis from nicks or 1 nucleotide gaps in addition to resynthesising gaps of up to 50 nucleotides. However, reports have indicated that if one of the enzymes became rate limiting, then remaining enzyme activity increases to compensate (Friedberg, 1985).

The re-establishment of strand continuity is the final step in the excision repair process. DNA ligase catalyses the final phosphodiester bond between the 5′phosphate and the 3′hydroxyl groups in an ATP dependent reaction. Although DNA ligase I catalyses the bond formation in DNA replication, the distinct ligase II has been associated with the repair process, especially short patch repair (Friedberg, 1985).

Mismatch repair

Mismatch repair is a post-replicative repair process identified in bacteria, yeast and higher eukaryotes. Two major forms of the process have been distinguished in *E.coli*, "long patch repair" and "short patch repair". The most versatile of the two is long patch repair which corrects mispairs from all replication errors, except C:C, and recognises mispaired bases in the early intermediates of homologous recombinations (Rayssiguier *et al*, 1989).

The process most fully characterised is that found in *E.coli* (see Figure 1.15), with initiation dependent upon the presence of an unmethylated DNA daughter strand, i.e. hemi-methylated DNA at d(GATC) sites. The MutH protein, a Mg^{2+}-dependent

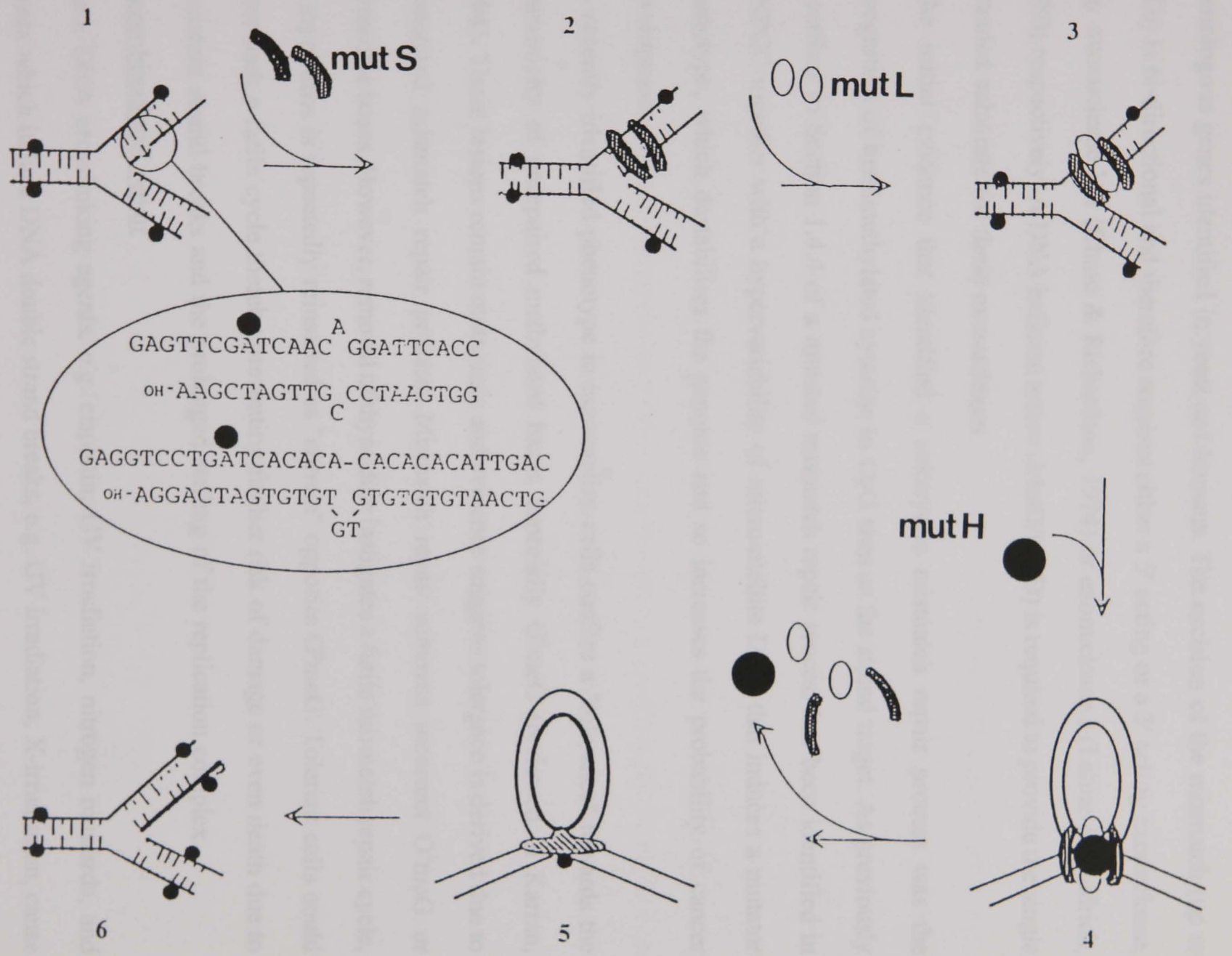


Figure 1.15: Mismatch correction in *E.coli* (Karran & Bignami, 1994).

endonuclease, incises at the hemi-methylated d(GATC) 5′ to the G residue (Welsh *et al*, 1987). This activity is dependent upon the protein, MutS binding to a mispaired base, which then complexes with the protein, MutL that aligns the mispair with a complementary sequence. The MutS, L and H proteins are highly conserved, with homologous genes identified in yeast and humans. The excision of the mismatch (up to 1Kb) is bi-directional and therefore requires either a 5′ acting or a 3′ acting exonuclease, e.g. exonuclease VII (Chase & Richardson, 1974) or exonuclease I (Lahue & Modrich, 1989) respectively. A DNA helicase action (MutD/UvrD) is required to provide the single stranded substrate for these exonucleases.

The initial evidence that identified a eukaryotic mismatch repair process was the recognition of hemimethylated cytosine in CpG sites as the strand target. As previously described in Section 1.4.4 of a mutated mismatch repair process has been identified in HNPCC together with a hypervariability of microsatellite DNA that induces a mutator phenotype, which destabilises the genome and so increases the probability of cancer development.

A recently identified phenotype in mammalian cells confers a "tolerance" towards the cytotoxicity of unrepaired methylated bases, especially O^6meG (reviewed in Karran, 1994). These lesions remain mutagenic and evidence suggests tolerance is derived due to a mutated mismatch repair process. Mismatch repair removes incorrect O^6meG or thymidine bases. However, removal of thymidine instigates a futile mismatch repair cycle, as thymidine is repeatedly reinserted as "correct" opposite O^6meG. Tolerent cells could avert such a futile cycle thereby preventing further risk of damage or even death due to persistent strand breaks and the prolonged stalling of the replication complex.

Recombinational repair

Both DNA crosslinking agents, e.g. cisplatin, UV irradiation, nitrogen mustards, and agents which induce DNA double strand breaks, e.g. UV irradiation, X-irradiation, cause an increase in recombinational events. Both types of DNA damage, due to the destruction

of the two DNA strands, lack a complementary sequence from which to initiate DNA repair.

The double strand break repair model predicts that the ends of a double strand "gap" created from the double strand break, interact, by alignment with the second unbroken, homologous duplex present in the genome and utilises its sequence as the template required to repair the "gap". This represents a "gene conversion" as information is transferred from one duplex to its homologous counterpart (Deng & Nickoloff, 1994). Jessberger & Berg (1991) developed an *in vitro* system to identify the proteins involved in the mammalian recombinational repair system. Utilising extracts derived from calf-thymus nuclei a high molecular weight complex, termed RC-1 was identified. This was found to catalyse the recombinational repair of double strand gaps and deletions in DNA by gene conversion as well as cross over events. Co-purified with the RC-1 complex was DNA polymerase ε, a DNA ligase thought to be mammalian DNA ligase III, and several nuclease activities, e.g. 3′-5′exonuclease, 5′-3′exonuclease, double stranded endonuclease.

Crosslinks formed between the two DNA strands utilise a combination of nucleotide excision repair and recombinational repair. As for most of the repair pathways, the mechanism of action is most characterised in *E.coli*. The (A)BC excinuclease incises on both sides of the crosslink, but in just one of the strands. The protein RecA, which binds to single stranded DNA invades the damaged duplex, so displacing the excised oligomer. However, the excised oligomer remains bound by the crosslink to the second DNA strand. The (A)BC excinuclease then excises this in a manner similar to that of a monoadduct so causing its release. The resulting gap is infilled by the action of polymerase I, helicase II and ligase (Friedberg, 1985; Sancar & Sancar, 1988).

1.4.7 Inducible responses in DNA damaged cells.

1.4.7a Introduction

A cell responds to DNA damage with an induction of gene transcription and protein

synthesis, a transient arrest of cell cycle progression, and at least with some DNA damaging agents, apoptosis, the controlled cell death mechanism may be activated. Such cellular responses are suggestive of a sensor mechanism for the detection of DNA damage, the level of damage incurred and the survival capability of the cell. PADPRP has been proposed as a possible candidate for such a detection system (de Murcia & Menissier de Murcia. The abundance and localisation of PADPRP, the specificity for activation by breaks in the DNA and the rapid half-life of the polymer make this an excellent candidate. This Section will briefly review the mechanisms induced by the cell in response to DNA damage and the possible involvement of PADPRP.

1.4.7b Cell cycle checkpoints and the p53 inducible response following DNA damage.

The cell cycle is a highly regulated process with each phase controlled by a series of cyclin dependent kinases (CDKs) which drive the cell through successive checkpoints. Mammalian cells contain a broad range of CDKs, with CDK2 acting at S-phase and CDK1 required for mitosis. PCNA, a cofactor of DNA polymerase δ, involved in DNA replication and NER repair synthesis is found complexed with many of the early CDKs. The inhibition of the CDKs by interphase checkpoint proteins, regulates the transcription rates, degradation and phosphorylation status of the CDKs which restricts the progression through the cycle until each phase is complete. These inhibitors could also provide the mechanism for blocking the proliferation of quiescent or senescent cells, although a loss of function could lead to uncontrolled cell growth and tumour formation (reviewed in: Hunter, 1993; Pines, 1994).

The cell responds to DNA damage with an induction of specific genes and an arrest of the cell cycle at the G_1/S and G_2/M phases (Little, 1968; Kastan *et al*. 1992). This provides a period for repair, and so presumably prevents the replication and propagation of mutagenic lesions which could lead to genomic instability and tumourigenesis. The protein product of the protooncogene p53 was identified as having a pivotal role in the G_1/S checkpoint control following damage to the DNA (see Figure 1.16) (Kastan *et al*,

1991; Kuerbitz *et al*, 1992). Mutations in the p53 gene are found in 50% of all tumours (Marx, 1994), with many exhibiting a lack of the G1 arrest following γ irradiation. The survival of a knockout$_{p53}$ mouse indicated p53 to be non-essential for cell viability and development, although a high susceptibility to tumours in the young adult knockout mouse indicated a possible requirement for p53 in the maintenance of genomic stability (Donehower *et al*, 1992).

The formation of both single and double stranded breaks in the DNA are the proposed trigger for the observed transient increase of the p53 protein following DNA damage (Nelson & Castan, 1994). However, the lack of induced gene transcription or protein synthesis indicated a post-translational stabilisation mechanism. p53 is a trans-activating transcription factor that binds to specific p53 motifs in the promoters of targeted genes or the TATA box binding protein, to influence the expression of proteins involved in the regulation of the G1 arrest (see Figure 1.16) (Kern *et al*, 1991; Kern *et al*, 1992). Transcription of the protein p21$^{waf1/sdi1/cip1}$ is stimulated by p53 and inhibits the cycling of the cell by at least two independent pathways:- 1) The activity of several of the early CDKs, i.e. cyclin A-CDK2, cyclin E-CDK2 and cyclin D-CDK2, are inhibited both *in vitro* and *in vivo* by the binding of p21 (Xiong *et al*, 1993), 2) p21 bound to PCNA inhibits the activation of DNA polymerase δ (Waga *et al*, 1994). Similarly, the transcription of Gadd45, a member of the "growth arrest and damage inducible" family of genes was found to be dependent upon p53 stabilisation. A role in DNA repair was identified when complexes of Gadd45 were found associated with PCNA in the absence of CDKs, and an inducible repair-like response was observed (Smith *et al*, 1994). Following DNA damage, immunostaining techniques identified a redistribution of PCNA, perhaps indicative of a switch from replication to repair (Hall *et al*, 1993).

The expression of mdm2 mRNA is also induced by p53 (see Figure 1.16) (Barak *et al*, 1993). Mdm2 protein binds and complexes to p53 causing the inhibition of transcriptional activities and consequently the G_1 arrest (Zauberman *et al*, 1993; Oliner *et al*, 1993). In

cycling cells, a balance was proposed between mdm2 and p53 for the entrance of the cell into S phase (Barak *et al*, 1993; Otto & Deppert, 1993). The complex, transcription factor-like structure of mdm2 would suggest a role in the regulation of gene expression, in addition to a simple inhibitory binding function (Picksley & Lane, 1993).

The G_2/M phase arrest following DNA damage or incomplete DNA replication has been characterised most fully in yeast, although homologies to human genes suggest the existence of G_2 checkpoint arrest in humans. The arrest of cell division following DNA damage is under the control of the RAD9 gene, although it is not essential for the repair process (Weinert & Hartwell, 1988). RAD9 could therefore be an equivalent to p53, scanning the DNA for signs of damage, especially double strand DNA breaks. Observations suggest just a single double strand DNA break is sufficient to activate the G_2/M checkpoint (Sandell & Zakian, 1993). A number of other proteins have been identified that also prevent mitosis including RAD17, RAD24 and MEC1, MEC2 and MEC3 (Hartwell & Kastan, 1994). Interestingly, the phosphorylation of cyclin B-CDK2 complexes results in the inhibition of a negative feedback pathway, which prevents the cells with damaged or incompletely replicated DNA from entering into mitosis (El-Deiry *et al*, 1993; Harper *et al*, 1993).

1.4.7c Apoptosis

Apoptosis is the programmed death of a cell, which performs an important role in embryogenesis and development (Wylie *et al*, 1981a). However, numerous DNA damaging agents have been found to induce apoptosis (reviewed in: Wyllie *et al*, 1981b, Kauffmann *et al*, 1993). Several distinct morphological features distinguish apoptosis, including the loss of cell-cell interactions, the condensation of the nucleus into heterochromatin masses, nuclear fragmentation, and the "budding off" of apoptotic bodies, which are subsequently phagocytosed by macrophages prior to lysis (Dive & Hickman, 1991).

A considerable number of factors involved in the apoptotic process have been identified, although their functions have still to be fully elucidated. An influx of Ca^{2+} into cells

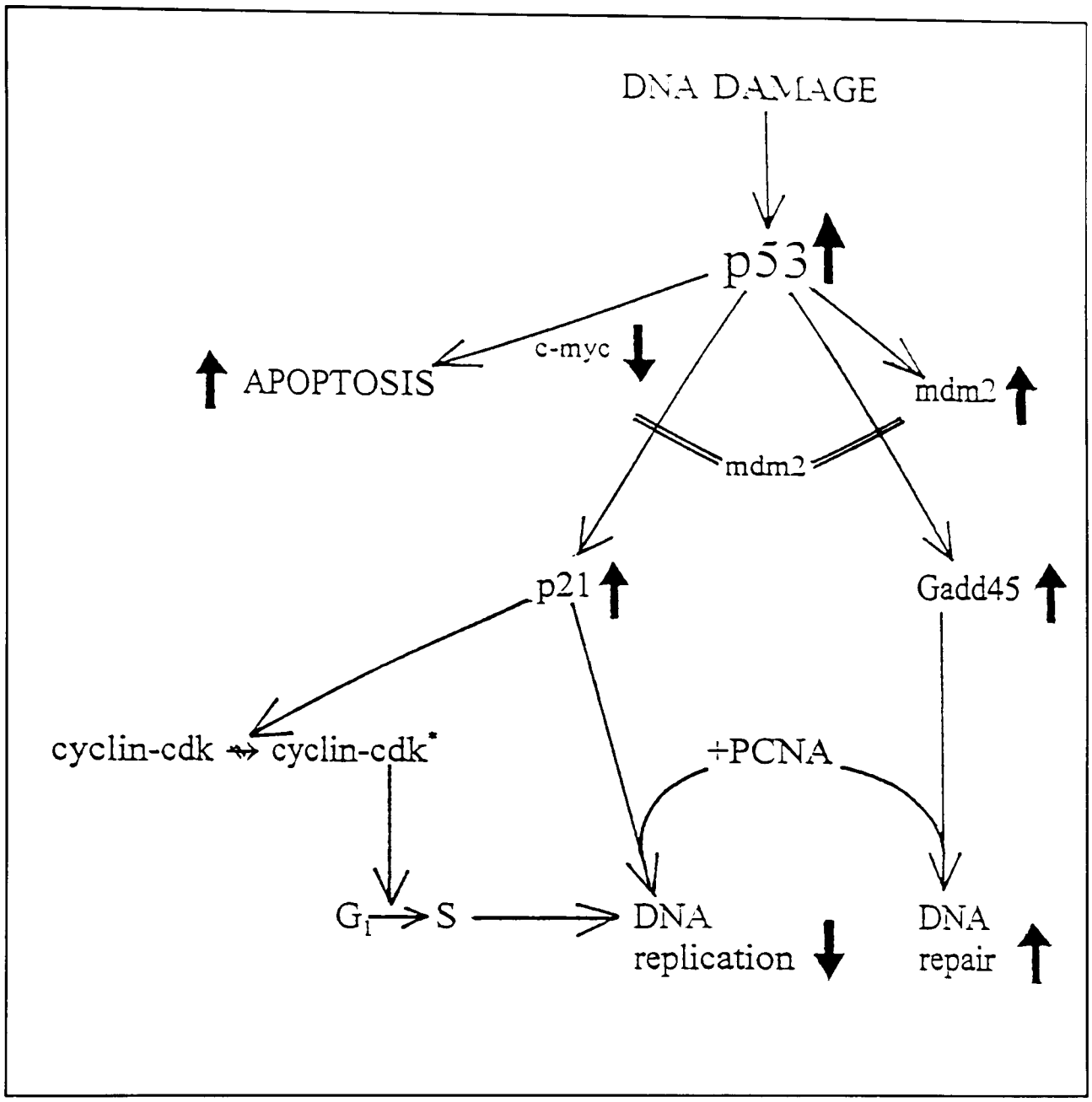


Figure 1.16 p53 damage inducible responses.

destined for apoptosis has been observed, which may act as a second messenger, signalling the action of further components of the process (McConkey *et al*, 1989; Tenniswood *et al*, 1992). The endonuclease responsible for the oligonucleosomal DNA fragmentation observed exhibits a dependency on Ca^{2+}/Mg^{2+} (Wyllie *et al*, 1981a). A possible candidate for the apoptotic endonuclease could be the Ca^{2+}/Mg^{2+} dependent endonuclease activity modulated by PADPRP (see 1.2.6f).

The accumulation of p53 was found to result not only in the arrest of the G1/S phase transition, but also in an increased frequency of apoptosis (see Figure 1.18) (Lowe *et al*,

1993). However, the effect of p53 on apoptosis appears connected with the function of the proto-oncogene c-*myc* (Hermeking & Eick, 1994). This is a protein-kinase C regulated gene, previously implicated in the positive regulation of cell proliferation (Hickman *et al*, 1992). The levels of c-*myc* decrease during apoptosis (Hickman *et al*, 1992). In normal cells, damage results in either the delayed continuance of the cell cycle or apoptosis, but in cells expressing a mutant p53, only cell proliferation is observed. One proposal indicates that c-*myc*, usually found at increased levels in comparison to p53 attempts to over-rule the p53 G_1 arrest but this results in the p53 stimulation of apoptosis (Hermeking & Eick, 1994). Uncontrolled cell growth, with a high predisposition to tumourigenesis results from a lack of apoptosis. This is observed in tumours exhibiting an increased expression of the *bcl-2* gene, known to inhibit apoptosis (Reed, 1994)

1.4.7d The role of PADPRP in apoptosis

Several possible mechanisms for the involvement of PADPRP in the apoptotic process have been proposed, including the modulation of cellular NAD^+ (see later discussion) (Sims *et al*, 1982; Sims *et al*, 1983; Nosseri *et al*, 1994), the poly(ADP-ribosyl)ation of crucial proteins involved in the apoptotic process (Darby *et al*, 1985) and the decondensation of the chromatin due to (ADP-ribose) polymer modifications which would then allow access of endonucleases to the DNA (Realini & Althaus, 1992). However, specific inhibitors of PADPRP, e.g. 3AB, nicotinamide, thymidine, have given conflicting results, with some indicating a decrease in the presence of TNF growth factor and VP16 (Agarwal *et al*, 1988; Tanizawa *et al*, 1989), and others an increase in the level of apoptosis induced by nitroso-compounds (Rice *et al*, 1993).

Associated with apoptosis is a depletion of the intracellular NAD^+ concentration. PADPRP activation in the presence of DNA strand breaks results in a rapid loss of cellular NAD^+ due to the formation of the polymer, (ADP-ribose). This could represent a mechanism for damage determination. Previously postulated by Berger & colleagues, the "suicide hypothesis" described, that in extreme instances of DNA damage, NAD^+

levels would be seriously reduced so resulting in an impaired purine nucleotide metabolism. The resulting limited DNA, RNA and protein synthesis would lead to the eventual death of the cell (Sims *et al*, 1982: Sims *et al*, 1983). Such a mechanism provides a link between excessive damage and cell death. Recently, in cells undergoing apoptosis, PADPRP was found to be proteolytically cleaved into an 85KDa fragment and a 25KDa fragment, the latter containing the Zn^{2+}-fingers (Kaufmann *et al*, 1993; Lazebnik *et al*, 1994). Utilising a specific inhibitor of PADPRP, the cleavage was shown to occur subsequent to the NAD^+ depletion (Kaufmann *et al*, 1993). PADPRP activation would ensue in response to the initial damage, but could also be activated for a second time due to the endonucleolytic fragmentation during apoptosis. Therefore, the cleavage of PADPRP may prevent attempts to repair this damage. Interestingly, the PADPRP inhibitors failed to arrest both PADPRP cleavage and endonuclease induced fragmentation suggesting that PADPRP activation does not actively participate in apoptosis, but is a passive response (Kaufmann *et al*, 1993).

1.5 THE ROLE OF PADPRP IN DNA REPAIR

1.5.1 Historical perspective

In 1956, Roitt observed a depletion in the intracellular NAD^+ concentration following treatment of tumours with alkylating agents. The mechanism of depletion was unknown, but further research indicated the decrease to be dependent on the concentration (Schein *et al*, 1973) and potential cytotoxicity of the agent used (Harrap & Furness, 1973). An initial theory proposed that non-specific damage to the plasma membrane resulted in the loss of NAD^+ through "leaky membranes" (Harrap & Furness, 1973). However, subsequent evidence showed the retainment of cell viability following the NAD^+ depletion, with the eventual replenishment of the NAD^+ level (Skidmore *et al*, 1979). Two further theories were then generated:- 1) the biosynthesis of the NAD^+ is decreased

or 2) the degradation of the NAD^+ is increased, mediated by PADPRP activity.

The involvement of PADPRP function in this depletion of NAD^+ was initially recognised by Juarez-Salinas and colleagues (1979). The induction of DNA damage by treatment with the alkylating agent, MNNG, was correlated with a vast increase in the level of poly(ADP-ribose) polymer and a concomitant decrease in the NAD^+ level. As damage to the DNA initiates the cellular repair mechanisms, activating PADPRP which utilises NAD^+ as its substrate, these results suggested an involvement of PADPRP in the repair process. Substantiation for the theory was provided when the NAD^+ depletion was prevented in a concentration dependent manner following co-incubation of the alkylating agent, MNU with 5-methylnicotinamide, theophylline, theobromine and thymidine, which had been demonstrated to be inhibitors of PADPRP (Skidmore *et al*, 1979). The degradation of NAD^+ in the cell could occur via the action of PADPRP or the enzyme, NAD^+ glycohydrolase [EC 3.2.2.6]. Following DNA damage, the level of glycohydrolase activity remained constant, and therefore was not accountable for the alterations in cellular NAD^+ concentration (Skidmore *et al*, 1979). PADPRP had been shown to be activated by breaks in the DNA (Janakideveh & Koh, 1974; Miller, 1975; Halldorsson *et al*, 1978; Benjamin & Gill, 1980), and cytotoxic drugs or γ radiation were known to lead to DNA strand scissions. Then in 1980, Durkacz and coworkers presented the first evidence to indicate the involvement of PADPRP in the cellular recovery from DNA damage (Durkacz *et al*, 1980). The utilization of alkaline sucrose density gradient centrifugation as a measure of the integrity of the DNA revealed that upon co-incubation of the alkylating agent, DMS, with the PADPRP inhibitor, 3AB, a persistence of the DNA strand breaks occurred, indicating a retardation of the excision repair process. The repair of strand breaks occurred over the same time frame as the observed increase in polymer synthesis. The role of PADPRP in repair was further corroborated by the increased cytotoxicity of the DNA damaging agent when coincubated with non-toxic concentrations of the PADPRP inhibitor, or following nutritional deprivation of

nicotinamide. The effect on cytotoxicity enhancement and DNA increased strand break levels is not simply confined to 3AB and the alkylating agent, DMS. Table 1.1 provides an overview of the effects that a wide variety of PADPRP inhibitors have on numerous DNA damaging agents. The cytotoxicity of monofunctional alkylating agents, e.g. MMS, DMS, and bifunctional alkylating agents, e.g. BCNU, melphalan, together with oxidising agents, e.g. H_2O_2, bleomycin, and antimetabolites, e.g. 5FU, hmdUrd, was enhanced when in the presence of several differing PADPRP inhibitors, e.g. 3AB, 3-MBZ, 5meNic, 3AAB, 6AN. Furthermore, where determined, a concomitant increase in DNA strand break frequency was observed. The majority of these agents result in DNA base modifications or direct DNA strand breaks. The co-incubation of 3AB with UV irradiation gave conflicting results. James & Lehmann (1982) were unable to show potentiation of UV cytotoxicity, and there was no effect on DNA strand break levels. However, Hunting & Gowans (1987), did observe increased DNA break levels when UV irradiated cells were co-incubated with 5mM 3AB. These observations will be discussed in greater detail in Section 1.5.2.

The mechanism of enhanced cytotoxicity in the presence of the PADPRP inhibitors was investigated by analysis of cell cycle perturbations. Das *et al* (1984), found that a co-treatment of MMS with 3AB resulted in a progression of cells into the G_2 phase of the cell cycle, with an ensuing block in the G_2 phase. To further characterise this effect, Boorstein & Pardee (1984), utilised a synchronised cell population, and at the entrance into each phase of the cell cycle cells were treated with MMS and 3AB, then assessed for cell survival. Cells in G_0 exhibited little sensitivity towards 3AB until advancement into S phase, when the sensitivity was increased. However, towards completion of S phase, 3AB sensitivity began to decrease. The lethal effect of 3AB in MMS treated cells was considered due to the traverse of the DNA damaged cell population through S phase. A transient inhibition of DNA repair of cells in G_0 was proposed to lack lethality, but following traverse into S phase, the lack of repair was proposed to convert non-lethal

TABLE 1.1

DAMAGING AGENT	PADPRP INHIBITOR	CYTOTOXICITY	BREAK FREQUENCY
BCNU Berger *et al*, 1982	1mM 6AN	enhanced	N/D*
DMS **γ irradiation** **UV irradiation** James & Lehmann, 1982	5mM 3AB 5mM 3AB 5mM 3AB	enhanced no effect no effect	increased no effect no effect
melphalan Brown *et al*, 1984	5mM 3AB	enhanced	N/D
streptozotocin Nduka & Shall, 1984	2mM 5MeNic	enhanced	N/D
MMS Boorstein & Pardee, 1984	4mM 3AB	enhanced	increased
MNNG Jacobson *et al*, 1984	1mM 3-MBZ	enhanced	N/D
X-rays Lunec *et al*, 1984	4mM 3AB	enhanced	increased
BrdUrd + UV Ben-Hur *et al*, 1985	20mM 3AB	enhanced	N/D
bleomycin Huet & Laval, 1985	3-MBZ	enhanced	increased
H_2O_2 Cantoni *et al*, 1986	5mM 3AB	enhanced	increased

UV irradiation Hunting & Gowans, 1987	5mM 3AB	N/D	increased
hmdUrd Boorstein *et al*, 1987	4mM 3AB	enhanced	N/D
6-thioguanine Moses *et al*, 1988	3.25mM 3AB	enhanced	no effect
MTIC Lunn & Harris, 1988	1mM 3AAB	enhanced	increased
5FU **5FdUrd** Willmore & Durkacz, 1993	3mM 3AB 3mM 3AB	enhanced reduced	no effect increased

*N/D: Not determined

lesions to lethal ones as the DNA was replicated. Structural damage to the DNA, resulting from incubation with DNA damaging agents, e.g. MMS, was suggested to result in a G_2 phase arrest, and this was amplified in the presence of 3AB. Jacobson *et al* (1985a), also observed a G_2 phase arrest with MNNG treated cells in the presence of the PADPRP inhibitor, 3-MBZ. A requirement for PADPRP activation was proposed for the successful progression of the cell cycle in DNA damaged cells.

1.5.2 In which of the repair processes is PADPRP involved ?

The unique activation of PADPRP by breaks in the DNA strands greatly determines the involvement of the enzyme in the repair process. This immediately excludes an involvement in direct repair, e.g. as carried out by MGMT, as the DNA strand integrity is maintained. The involvement for PADPRP in excision repair has only recently been clarified. Evidence indicated that the rate of the two excision repair processes was governed by different reactions:- in BER, ligation of the break determined the rate of repair, but in NER it was the incision step (James & Lehmann, 1982). This is due to their different mechanisms of action. In BER, abundant and constitutive DNA glycosylase and AP endonuclease enzymes remove damaged bases and rapidly incise the DNA respectively, so creating a surge of breaks. However, a low break frequency exists in NER as the excinuclease complex must assemble prior to excision of the damaged site. This complex then would mask the DNA strand breaks from the PADPRP. These two pathways repair different lesions, with BER removing alkylation, and at least a proportion of oxidation and gamma-radiation damage and the NER process handling the bulky chemical lesions and the dimers formed from UV-radiation. However, the involvement of PADPRP in the repair of UV damage has been a contentious subject for several years. Conflicting reports have been published. McCurry & Jacobson (1981) and Berger *et al* (1979) showed PADPRP activity and ADP-ribose polymer synthesis was increased 1.5-3-fold following UV-radiation respectively. James & Lehmann (1982) failed to observe

enhancement of cytotoxicity when UV damaged cells were co-incubated with 5mM 3AB. As a 50 fold increase in PADPRP activation was observed by alkylation damage, the break frequency appeared to determine the extent of enzyme activation. Recently Satoh *et al* (1993) utilised *E.coli* endonuclease III, an analogous enzyme to TH DNA glycosylase to remove the minor UV lesion, the pyrimidine hydrate from plasmid DNA, thereby leaving only the bulky pyrimidine dimers and 6-4 photoproducts. Following repurification of the plasmids, the treated cell extracts showed no induction of PADPRP activity. The conclusion reached therefore was that the small increase in PADPRP activity following UV-radiation was due to the BER of the minor UV-induced lesion. This theory conforms with the effect of PADPRP inhibition on other forms of chemical damage. The bifunctional alkylating agents, melphalan, BCNU, and cyclophosphamide form crosslinks with the DNA due to the presence of two chloroethylating groups. The PADPRP inhibitor, 6-aminonicotinamide potentiated the cytotoxicity of BCNU (Berger *et al*, 1982). However, although BCNU primarily crosslinks the DNA, initially, just one arm of the crosslink is formed which could possibly be a target for BER. Oxidising agents lead to the formation of a wide spectrum of lesions in the DNA, with most excised via DNA glycosylase action. However, certain lesions, e.g. 8'5'cyclopurine deoxyribonucleosides, would be removed by the NER process because their "bulky" conformations would lead to helical distortions. Overall, the general consensus is that PADPRP plays a specific role in the BER process.

By the same argument one could predict that PADPRP would not be involved in the mismatch repair process. Incision would again be rate limiting and masked by the binding of the mismatch repair complex of enzymes and proteins.

1.5.3 At which stage of excision repair is PADPRP involved ?

The excision repair process can be divided into three characteristic sections:- 1) incision at the site of damage 2) gap formation & polymerisation (repair synthesis, or unscheduled

DNA synthesis (UDS)) and 3) ligation of the final phosphodiester bond (for a more detailed account see Section 1.4.5c). PADPRP is activated in response to breaks in the DNA strands with the effect of PADPRP inhibitors delaying the rejoining of strand breaks rather than completely preventing their repair. This could indicate a lack of total inhibition of PADPRP explainable by the competitive nature of the inhibitors, or a failure to achieve sufficiently high intracellular concentrations. Alternatively the PADPRP inhibitors could alter the kinetics of strand break repair (Durkacz *et al*. 1980). At any one time the number of breaks observed represents a dynamic balance between the level of strand break formation and the rate of break ligation. The effect of the PADPRP inhibitors on repair synthesis will be considered separately in the following subsection. Generally, the function of PADPRP in the repair process is considered to be in the regulation of ligation. Following treatment of cells with an alkylating agent, the level of breaks increases. An increase in the activity of PADPRP that facillitates ligation would thereby increase the rate of strand rejoining and so augment cell survival (James and Lehmann, 1982). Creissen & Shall (1982) observed an increased activity of ligase II in the presence of DNA strand breaks which was repressed in the presence of inhibitors of PADPRP. Ligase activity was estimated by the amount of ^{32}P labelled 5′-phosphate in nicked DNA that became inaccessible to alkaline phosphatase due to religation of the break. Partially purified extracts from *in vivo* DMS treated cells exhibited a five fold enhancement of ligase II activity, but a co-treatment with 3AB resulted in a return to the basal activity. The activity of ligase I was unchanged. The modification by (ADP-ribose) polymer of the ligase II itself, or a closely associated protein was postulated. However, Yoshihara *et al* (1985) utilising a reconstituted poly(ADP-ribosyl)ating enzyme system found the activity of ligase II to be inhibited when poly(ADP-ribosyl)ated, which suggested an alternative interpretation was required. An early report showed the activity of partially purified DNA ligase to be suppressed when in the presence of histone proteins (Zimmermann & Levin, 1975). Ohashi and colleagues (1983), utilising DNA/histone and

reconstituted chromatin systems also found an inhibition of DNA ligase activity when in the presence of histone proteins. However, the addition of (ADP-ribose) polymer or activated purified PADPRP, resulted in the reactivation of the ligase activity. To explain the results Ohashi proposed two possible mechanisms. The neutralisation of the negatively charged histones by poly(ADP-ribose) could result in the decondensation of the chromatin, allowing access of ligase II to DNA, or the (ADP-ribose) polymer could facilitate the localisation of the ligase at sites of DNA damage due to the observed high affinity of the ligase for the (ADP-ribose) polymer.

1.5.4 The effect of PADPRP on repair synthesis

The effect of PADPRP inhibitors on "gap-filling" of the excised site has been intensively studied in a number of cells following alkylation or radiation damage (Berger *et al*, 1979; Berger & Sikorski, 1980; Althaus, 1980; Durkacz *et al*, 1981a; Miwa *et al*, 1981; James & Lehmann, 1982; Sims *et al*, 1982 & 1983). In each case an increase in repair synthesis was observed. Both Durkacz *et al* (1981a) and James & Lehmann (1982) showed concentration dependent increases in the level of repair synthesis following exposure to the damaging agent, DMS but that this plateaued at high concentrations. Cleaver (1985) proposed that the number of lesions repairable at any one time was limited by the cellular concentration of repair enzymes. However, the level of repair synthesis was maintained during a co-incubation of the lower range of damaging agent concentrations and the PADPRP inhibitors, but at the higher concentrations of DNA damaging agent, the levels, rather than reaching a plateau, continued to increase (Durkacz *et al*, 1981; James & Lehmann, 1982). This increase was dependent upon the potency of the compound as an inhibitor of PADPRP (Miwa *et al* 1981, Sims *et al* 1982). Therefore, until an extreme level of damage to the DNA occurred, PADPRP appeared to play no part in the polymerisation step of excision repair. Miwa *et al* (1981) proposed two possible theories to explain the effect of the inhibitors of PADPRP on repair synthesis following DNA

damage:-

1) The suppression of the ligase activity due to PADPRP inhibition (Creissen & Shall, 1982) induces a persistence of the strand breaks. The delayed religation leads to a loss of control over polymerisation, with the correction finish point not recognised, thereby resulting in a longer chain of repair synthesis.

2) A Ca^{2+}/Mg^{2+}-dependent endonuclease activated by PADPRP inhibition (see Section 1.2.6f) increases the number of breaks induced into the DNA at any one time so initiating extra sites for the polymerisation reaction.

Berger and colleagues presented a third theory (Sims *et al*, 1982 & 1983). Following low levels of DNA damage the NAD^+ and ATP reserves are maintained, with a continuance of DNA repair synthesis. However, after extreme damage to the DNA, PADPRP activation could seriously reduce the cellular NAD^+ concentration, which in turn could deplete the ATP reserves of the cell and thereby affect DNA, RNA and protein synthesis. As ATP is an essential co-factor of ligase, and the final phosphodiester bond of repair synthesis is catalysed by the action of ligase, then repair synthesis would also be limited as demonstrated by the plateau in the rate of repair synthesis at high levels of DNA damge. The use of PADPRP inhibitors would preserve the NAD^+ levels and consequently the ATP supply, which would enable the dose dependent increase in repair synthesis to continue.

The patch size formed during repair synthesis was assessed in an aim to identify which of these mechanism was involved. An increase in repair patch size would indicate the first and most supported hypothesis, but a constant sized patch would point to the second hypothesis. Cleaver (1985) incubated [^{14}C] dThd labelled cells in the presence of BrdUrd, [^{3}H] dThd and HU after treatment with the DNA damaging agent, MMS before cleaving the DNA with miccrococcal nuclease to reduce the DNA size. The incorporation of [^{3}H] Thd into the [^{14}C] labelled parental DNA would indicate repair synthesis. However, BrdUrd labelling was also utilised to distinguish further between repair and

semiconservative replication according to the density of the strands. Utilising isopynic gradients, repair synthesis was observed to increase in the presence of 3AB, but there was no alteration in the density of the DNA fragments, that would have indicated an increase in the patch size. Therefore, Cleaver (1985) reported that the increase in repair synthesis did not correlate with an increased repair patch size and declared the endonuclease theory to be the most plausible. The proffered explanation was that by inhibiting PADPRP, the activity of a Ca^{2+}/Mg^{2+} dependent endonuclease normally dormant due to (ADP-ribose) polymer modification would be increased, thereby raising the number of breaks introduced into the DNA (see Figure 1.17). At low concentrations of the PADPRP inhibitors repair synthesis would manage slight increases in DNA strand break levels. However, at higher PADPRP inhibitor concentrations, Ca^{2+}/Mg^{2+}-dependent endonuclease activity would be increased, thereby a maximal rate of repair synthesis would be observed, and consequently the number of repair patches would be increased.

However, Ruffer & Morgan (1992) were unable to validate this theory. They utilised a technique which electroporated restriction enzymes, which induce double stranded DNA breaks, into human cells containing the shuttle vector pHAZE, in the presence or absence of 3AB. A *lacZ* target gene within the vector possessed sites of action for the enzymes. Previously, a dramatic increase in the level of chromosome abberations was observed following treatment with restriction enzymes in the presence of 3AB (Chung *et al*, 1991). Utilising the restriction fragment length polymorphisms (RFLP) technique, mutations due to abberations at the restriction sites could be mapped. Mutations would result from possible recombination events at the sites of restriction enzyme incision, with the loss of the recognition sequence for the restriction enzyme. An increase in non-specific nuclease action, e.g. Ca^{2+}/Mg^{2+}-dependent endonuclease, due to the inhibition of PADPRP could be determined, as the restriction fragment pattern of recovered plasmids would be altered due to mutation outside of the known restriction sites. However, the results showed no significant differences between mutation rates of the untreated or the 3AB-treated cells

thereby indicating the absence of non-specific endonuclease activity.

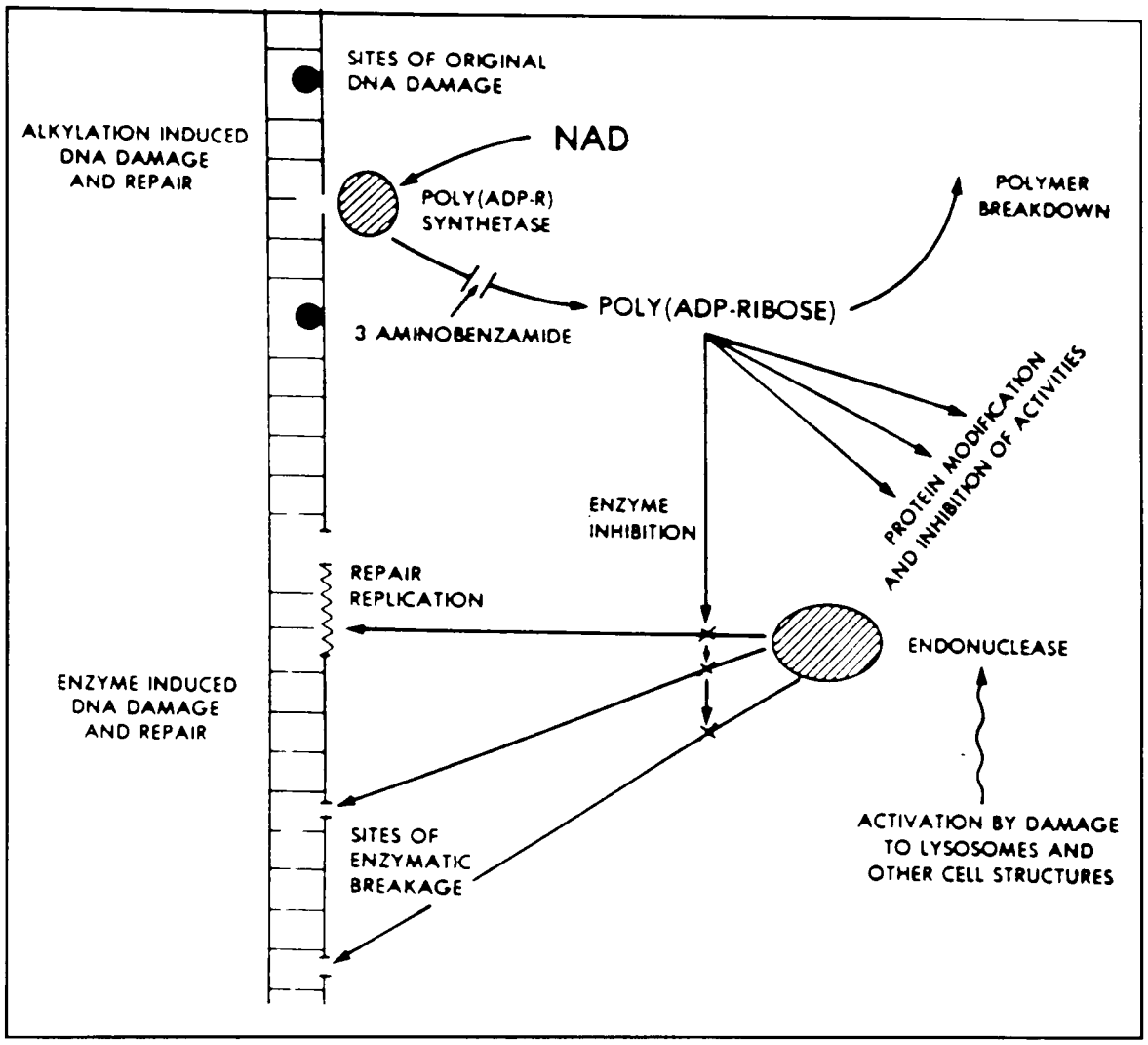


Figure 1.17 Pathway by which inhibition of poly(ADP-ribose) synthesis by 3AB may lead to a non-specific attack on DNA by endogenous nucleases after exposure to DNA-damaging agents (Cleaver & Morgan, 1985).

1.5.5 Topoisomerase I

Topoisomerase I relaxes supercoiled DNA prior to DNA replication and repair (see Section 1.2.6e). Compounds that inhibit topoisomerase I, e.g. camptothecin and topotecan, are cytotoxic towards cells due to stabilisation of the topoisomerase I:DNA complex which prevents further activity (Jaxel *et al*, 1991; Mattern *et al*, 1991). However, these inhibitors have an essential requirement for active topoisomerase I. As previously stated in Section 1.2.6e, poly(ADP-ribosyl)ation of topoisomerase I resulted in the reversible impediment of enzyme:DNA complex formation with the inhibition of enzyme activity

(Ferro & Olivera, 1984; Kasid *et al*. 1989). Under such conditions, the effect of the topoisomerase I inhibitors would be diminished, with a reduction of the cytotoxic potential. However, Mattern *et al* (1987) observed that if cells were treated with an inhibitor of PADPRP, e.g. 3AB, prior to the administration of the topoisomerase I inhibitor, the topoisomerase I would not be modified by (ADP-ribose) polymers, and a greater proportion of active topoisomerase enzymes would be present to form cleavable complexes. This was supported by the observation that 3AB was able to potentiate camptothecin cytotoxicity (Mattern *et al*, 1987).

The (ADP-ribosyl)ation of topoisomerase I in the vicinity of DNA strand breaks has been postulated as part of the mechanism to aid the temporary shutdown of DNA replication (Jongstra-Bilen *et al*, 1983).

1.5.6 Modulation of chromatin structure by poly(ADP-ribose) polymer.

Several reports in the literature have related the modulation of the chromatin structure, resulting from PADPRP activation (see Section 1.2.6b) to an early event in excision repair. PADPRP is activated in response to DNA breaks formed during BER. An (ADP-ribose) affinity column was utilised to separate oligonucleosomes modified by polymer from the bulk chromatin taken from cells treated *in vivo* with an alkylating agent. The modified regions were observed to be preferentially located adjacent to breaks in the DNA (Thraves *et al*, 1985). Both auto- & hetero-modification reactions occur following activation of PADPRP, with extensive modification of histone H1 resulting in the relaxation of the chromatin structure (Poirier *et al*, 1982b; Aubin *et al*, 1983; Niedergang *et al*, 1985; De Murcia *et al*, 1986). Modification of just 10% of the histone H1 proteins has been estimated to induce the relaxed state (Thoma & Koller, 1981). Subsequent modification of the core histones by ADP-ribose polymer would then destabilise the DNA/protein interactions at the nucleosomal level of the chromatin organisation. An antibody approach was used to assess the disruptive effect of the (ADP-ribose) polymer

on the chromatin structure. Antibodies directed against epitopes on the surface of the histone proteins showed an altered response following PADPRP activation, indicative of local rearrangement (Thibeault *et al*, 1992). Most of the *in vitro* systems designed to study PADPRP induced chromatin rearrangements fail to emulate the *in vivo* situation, with PADPRP and PADPR glycohydrolase acting concurrently. However, an *in vitro* procedure devised by Thomassin *et al* (1992), showed that in a high polymer turnover situation i.e. a high glycohydrolase activity similar to that found in intact cells, there was an overall reduction in the polymer content with the polymer distribution altered. A reduced level of automodification of PADPRP itself was observed with the percentage of polymer bound to the histone H1 and core histones increased. The increase in negative charge resulting from ADP-ribosylation would decrease the affinity of the histones for the DNA, resulting in the dissociation of the chromatin solenoid. This relaxation of the chromatin structure in the vicinity of a DNA break has been proposed to facilitate the access of repair enzymes to the damaged site. Substantiation for the theory was observed as the activity of DNA polymerase α (Niedergang *et al*, 1985) increased following the ADP-ribosylation of polynucleosomes. The chromatin rearrangement hypothesis would also support the involvement of DNA ligase activity as local chromatin disruption would facilitate a secondary effect on repair reactions. Mathis and Althaus (1986, 1990) discovered that the PADPRP associated structural alterations of the chromatin were coupled with the repair of damage to the DNA and that the repair patches were located in the free-DNA domains. Following depletion of the chromatin-bound ADP-ribose polymers though, an inhibition of the excision repair process was observed.

As previously described in Section 1.2.5d, specific polymer patterns of the histone proteins have been associated with the regulation of the PADPRP reaction during DNA repair (Naegelli & Althaus, 1991; Malanga & Althaus, 1994). The ability of the histones to modulate the polymer configuration indicated the possible presence of non-covalent interactions between the histones and the polymers. Panzeter & Althaus (1992)

acknowledged the existence of such bonding with a hierarchy of preferential interactions:- branched polymer > long linear polymers > short linear polymers. *In vivo* data, using mammalian cell extracts, found that the histones were exclusively capable of binding noncovalently to the polymer and interestingly, the histone domains involved in these interactions were identical to those involved in chromatin condensation e.g. histone H1 bound via the C-carboxy terminal, whereas histones H3 and H4 utilised the N-terminal (Panzeter *et al*, 1993). The histone shuttle mechanism, proposed by Althaus and associates describes an elegant reaction pathway for the involvement of poly(ADP-ribose) metabolism and the modulation of chromatin in response to DNA damage (Realini & Althaus, 1992) (see Figure 1.18). PADPRP, targeted by breaks in the DNA, is activated which results in the extensive automodification of the enzyme. The presence of polymer, especially large branched structures, attract the histone proteins which bind non-covalently to the polymer upon dissociation from the DNA. This renders the DNA free for the action of repair enzymes. Finally, degradation of the polymer by PADPR glycohydrolase eliminates the binding sites for the histones causing the reassembly of the nucleosomal structure with the DNA.

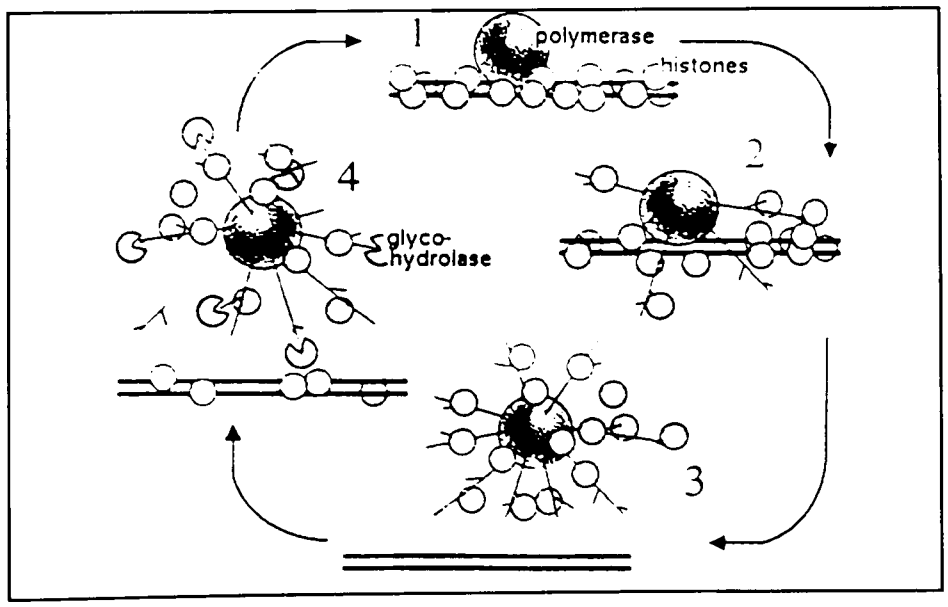


Figure 1.18 A schematic representation of the poly(ADP-ribosyl)ation-dependent histone shuttle on DNA.

1.5.7 New advances in the quest to identify the function of PADPRP in repair.

Recent advances in molecular technology have enabled PADPRP research to enter new avenues.

The development of mutant cells with a reduced PADPRP activity has provided a means to bypass the use of PADPRP inhibitors and the possible involvement of secondary effects on the cell metabolism. A replica-plating mutagen treatment was used, and the CHO colonies were subsequently screened using a [^{32}P]NAD^+ permeable cell-screening assay for reduced PADPRP activity (MacLaren *et al*, 1990). Initially, two mutant CHO lines were characterised, PADR-1 and PADR-2, both of which possessed just 50% of the parental PADPRP activity, but maintained a similar growth rate. The sensitivity to the DNA methylating agent, ethyl methanesulphonate (EMS) was found to be increased 1.3-fold in the PADR-1 line as compared to the parental CHO cells (MacLaren *et al*, 1990). PADR-1 was subsequently utilised for a second mutagen treatment. PADR-9 was isolated and found to exhibit just 17% of the parental PADPRP activity but again maintained a similar growth rate (Witmer *et al*, 1994). The sensitivity towards EMS was further increased in the PADR-9 cells to 2.8-fold. A direct correlation between the reduced PADPRP activity in the mutant cells and the increased sensitivity to cell kill by alkylating agents was observed.

The depletion of endogenous PADPRP in HeLa cells by the transfection of an antisense RNA_{PADPRP} plasmid construct showed PADPRP activity to be decreased ~80%, and the levels of PADPRP protein reduced by ~90%. Assessment with deoxyribonuclease I showed PADPRP depleted chromatin to have an altered structure. Following treatment with MMS, PADPRP depleted HeLa cells exhibited a decreased survival as the cells were unable to initiate DNA strand break rejoining. However, normal PADPRP levels were restored after 8-16 hours, and at later times repair was found to be re-established. The concentration of PADPRP present was considered in excess for DNA repair or replication, but it was postulated that the excess levels were required for maintenance of

chromatin structure (Ding *et al*, 1992 & 1994; Ding & Smulson, 1994). The effect of antisense construct expression was then investigated in the repair of either UV or nitrogen mustard damage in the essential gene, DHFR. Antisense cells were only deficient in the repair of alkylation damage, adding support to the participation of PADPRP only in gene-specific BER of alkylation damage. This was verified further as cells expressing the antisense constructs showed an increased sensitivity to the nitrogen mustard in a clonogenic survival assay, and so indicated the requirement for the repair of essential genes in cellular survival (Stevnsner *et al*, 1994).

The development of a cell free system to assay DNA repair showed PADPRP to possess an inhibitory effect on repair. The cell free system involved the incubation of a γ irradiated plasmid containing one single strand break/molecule together with soluble cell extracts of cultured cells supplemented with Mg^{2+}, dNTPs, NAD^+, ATP and a regenerating ATP system. Repair was measured by the amount of plasmid converted to covalently closed circular DNA. The effect of each component suspected of an involvement in the repair process was investigated by its selective elimination from the assay. In the absence of PADPRP, repair occurred equally efficiently in the absence or presence of NAD^+, but following the addition of purified PADPRP to the system, an NAD^+ requirement was essential to release the PADPRP molecule bound to the strand break to allow strand rejoining to occur. The presence of a PADPRP inhibitor also aggravated the situation by the delayed release of the PADPRP. However, this system would indicate PADPRP was not essential for DNA repair. A structural role in the chromatin was again proposed, or a possible signalling role due to the PADPRP binding to the DNA strand break ends thereby signalling to the cell to arrest, and so preventing lesions replicating (Satoh & Lindahl, 1992; Satoh *et al*, 1993). Similar results were obtained when the innovative technique of overexpressing recombinant DNA binding domain (DBD) polypeptides of PADPRP in CV-1 cells was performed (Molinete *et al*, 1993). Following MNNG treatment, a trans-dominant inhibition of endogenous PADPRP

was observed due to the binding of the DBD to the strand breaks. Once bound, release of the DBD could not be effected due to the lack of the domains required for the automodification reaction. This is a similar situation to the presence of PADPRP but absence of NAD^+ in the cell-free system.

Recently, the most exciting revelation was the survival of a "PADPRP knock-out mouse" indicating PADPRP to be non-essential for cell viability and organism development (Wang *et al*, 1995). Mice devoid of the PADPRP gene would remove the possible secondary reactions and side-effects of the PADPRP inhibitors and experimental techniques previously used. A genetical approach utilised mice with a disrupted PADPRP gene and homologous recombination. Embryonic stem cells were used and mice heterozygous for PADPRP i.e. chimeras were crossed to give homozygous PADPRP negative mice. After 5-6 months, ~30% of the mice developed severe skin disease, and this was increased with age. The level of DNA repair in PADPRP deficient embryonic fibroblasts (-/-) and control fibroblasts containing PADPRP (+/+) was assessed by:- (1) the measurement of the restoration of *in vitro* damaged SV40 CAT plasmid expression levels, which had been transfected into the PADPRP deficient and control fibroblasts. 12 hours after transfection, CAT expression levels from both groups of cells containing the damaged plasmids were decreased, as compared to undamaged controls. However, 28 hours later, control values were reattained, but comparable rates of repair were observed in both the -/- and +/+ fibroblasts, indicative of similar repair efficiencies. (2) The incorporation of [^{3}H] thymidine into replication arrested -/- and +/+ fibroblasts, treated with either MNNG or UV irradiation was measured to analyse the rate of UDS. Both cell lines showed comparable concentration dependent increases in [^{3}H] thymidine incorporation, indicating PADPRP not to be required for either BER (MNNG treatment) or NER (UV treatment). This would again support the results from the cell-free system. PADPRP was postulated to be important component in the response to environmental stress, and target molecules, e.g. p53, cyclins were proposed to require ADP-ribosylation

in response to such external stresses. However, the long term effect of PADPRP deficiency has yet to be established. The efficiency of the repair processes is considered to decline with the age of the organism, with an increased probability of mutation and the development of tumours.

1.6 AIMS

15 years ago, a concept for utilising PADPRP inhibitors as resistance modifiers in the treatment of leukaemia was proposed (Durkacz *et al*, 1980). This emanated from the ability of these inhibitors, e.g. 3AB, nicotinamide, to potentiate the cytotoxicity of DNA damaging agents which activated the BER mechanism. However, due to a lack of potency and specificity towards PADPRP, and problems with insolubility, this idea has failed to be realised. The recent development of novel PADPRP inhibitors with greatly enhanced potency (~60 fold greater than 3AB) has again raised the possibility for clinical development. Before entrance onto a clinical trial, extensive *in vitro* and intact cell studies must be performed. One of the aims of this thesis was to ascertain if the inhibitory ability of two novel compounds, NU1025 and PD 128763, both of which exhibit ~60 fold increased PADPRP inhibition when compared to 3AB *in vitro*, showed the same increased potency when cellular responses to DNA damage were analysed. The two "classically" used inhibitors, 3AB and BZ were used as comparisons. The clinically relevant, monofuctional alkylating agent, TM was utilised as the DNA damaging agent, and a series of growth inhibition, cytotoxicity and NAD^+ studies were the biological endpoints chosen.

Upon examination of the PADPRP inhibitor literature, it was apparent that a comprehensive study to analyse the PADPRP inhibitor concentration range required to effect an increase in the single strand break frequency or cytotoxicity, following damage to the DNA, was missing. The majority of studies concentrated only on the use of a single, high concentration of the PADPRP inhibitor together with varying doses of the

damaging agent. Due to the availability of PADPRP inhibitors with differing levels of potency, a study of changes in single strand break levels, to determine such concentration ranges was initiated, again utilising the clinically relevant TM.

A further aim attempted to discover if disturbances to the intracellular NAD^+ concentration resulted in an alteration of the PADPRP activity, following DNA damage. NAD^+ is the substrate for PADPRP, therefore alterations to the metabolism of NAD^+ could be an important regulator of PADPRP activity. A cell line, deficient in the final metabolic enzyme of NAD^+, NMNAT, was isolated and characterised (in comparison to the parental cell line <5% NMNAT activity and ~50% lower NAD^+ levels were observed) before being used to assess the effect low NAD^+ levels and low biosynthetic capacity of NAD^+ had on the activity of PADPRP in DNA damaged cells.

CHAPTER 2 : MATERIALS AND METHODS

2.1 MATERIALS

Chemicals/reagents

3-Aminobenzamide was purchased from Pfaltz & Bauer, Phase Separations, Deesside, UK; Coomassie protein assay reagent was bought from Pierce, Rockford, Illinois 61105, USA; trypan blue was purchased from NBL, U.K, and SeaKem ME agarose was acquired from FMC Bioproducts, Rockland, ME 04841, USA. Adenosine 5'-triphosphate (ATP), β-nicotinamide adenine dinucleotide (βNAD$^+$), β-nicotinamide mononucleotide (βNMN), benzamide (BZ), dithiothreitol (DTT), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT), phenazine methosulphate (PMS) and triethanolamine were purchased from Sigma Chemical Company, St.Louis, MO, USA.

All remaining chemicals, unless otherwise stated were of Anal R grade and purchased from Sigma or BDH, Dorset, UK.

Enzymes

Alcohol dehydrogenase (ADH) and Proteinase K were both purchased from Boehringer Mannheim Biochemica, Mannheim, Germany.

Radiolabel

[2-^{14}C]Thymidine, specific activity = 52mCi/mMol, [methyl-^{3}H]thymidine, specific activity = 41mCi/mMol and [^{32}P] NAD$^+$, specific activity = 1000Ci/mMol were bought from Amersham International, Amersham, UK.

Tissue culture supplies

The constituents of the tissue culture medium were obtained from Gibco BRL, Life Technologies Ltd, Paisley, UK. Foetal calf serum (FCS) was bought from Globepharm Ltd, Esher, Surrey, UK. Dulbecco's phosphate buffered salts (modified) without Ca^{2+} and Mg^{2+} (Dul A) and sterile NaOH were purchased from ICN Flow, ICN Biomedicals inc., Irvine, UK. Tissue culture plastic ware (25cm^2 & 80cm^2 flasks, 60x15 dishes, 6-well

multi-dishes) was obtained from NUNC. Denmark. Acrodiscs (2μM pore size) were obtained from Gelman Sciences, USA for filter sterilisation.

2.2 PREPARATION OF THE STOCK DRUG SOLUTIONS

Both 8-carbamoyl-3-methylimidazo-[5,1-d]-1,2,3,5,-tetrazin-4-(3H)-one (temozolomide. TM) (kindly supplied by Professor M.F.G.Stevens, Cancer Research Laboratories, University of Nottingham) and 5-(3,3-methyl-1-triazeno) imidazole-4-carboxamide (MTIC) (synthesised in the Chemistry Department, Newcastle University) were prepared to stock concentrations of 100mM in DMSO and stored aliquoted at -20^{0}C. 2-β-D-ribofuranosylthiazole-4-carboxamide)(tiazofurin, TZ) (generously donated by Dr V. Narayanan, National Cancer Institute, Bethesda, MD) was made to a stock concentration of 200mM in sterile water, filter sterilised and stored in volumes of 500μl, at -20^{0}C.

30mM stocks of 3AB and BZ were prepared fresh in medium for each experiment. Prior to use, the stocks were filter sterilized and the required concentrations of the compounds were prepared by dilution into medium. Stocks of 8-hydroxy-2-methylquinazolin-4[3*H*]-one, NU1025 (synthesised in the Chemistry Department, University of Newcastle upon Tyne) and 3,4-dihydro-5-methoxyisoquinolin-1(2*H*)-one, PD 128763 (a gift from Dr W.R.Leopold, Parke Davis Pharmaceutical Division, Warner Lambert Company, Ann Arbor, MI 48105) were made at a concentration of 200mM in DMSO and stored frozen in aliquots at -20^{0}C. Dilutions of NU1025 and PD 128763 were prepared into DMSO. The volume of DMSO-dissolved drug, added to the cell suspensions, was such that the DMSO content was kept constant and did NOT exceed 1% in the medium. Treatment of cells with DMSO alone results in a concentration-dependent inhibition of cell growth. At 1%, the growth was reduced to approximately 70% of control cell growth. In experiments where drug-treated cells were exposed to DMSO, control cells were also given an equivalent volume of DMSO.

2.3 TISSUE CULTURE

The murine leukaemic cell line, L1210, (obtained from European Collection of Animal Cell Cultures, U.K.) was maintained as a suspension culture in RPMI-1640 containing 10% FCS, 10mM glutamine, 18mM HEPES buffer, 11mM sodium bicarbonate and 100 Units/ml penicillin, 100μg/ml streptomycin. The medium was adjusted to pH 7.2 with 1M sterile NaOH. The cells, maintained in tissue culture flasks with the lids loosened to allow gas exchange, were routinely subcultured to keep the density between $5x10^3$-$8x10^5$cells/ml, otherwise cell growth slowed due to the presence of old and dead cells. The cells, which doubled each 12-13 hours, were incubated in a Heraeus incubator at 37^0C, 5% CO_2. For experimental procedures, cells in the exponential growth phase were used.

2.4 ROUTINE TEST FOR MYCOPLASMA

Mycoplasma are a genus of bacteria that are a common contaminant of cell cultures, and which can produce various abnormal changes within the cell. The growth of the cell is not necessarily affected, thereby detection is difficult.

Utilising the fluorescent stain, Hoechst 33258 which specifically binds to DNA, the cells were routinely tested for mycoplasma (Chen 1977). L1210 cells were cocultured for 3 days in RPMI medium with an adhesive cell line, e.g. CHO-K1 (Chinese hamster ovary) grown on to glass coverslips. Contaminated L1210 cells would lead to the cross-contamination of the adhesive line. When the CHO-K1 cells were approximately 50% confluent they were "fixed" using Carnoy's fixative (3:1, methanol:acetic acid) stained and then visualised using an UV microscope. Mycoplasma are identified as small morphologically uniform fluorescent bodies located in the extranuclear and intercellular space. The L1210 cells tested negative for mycoplasma at all times.

2.5 CALCULATION OF CELL DENSITY

The haemocytometer (Neubauer, UK) was used to determine the cell density. A thick glass coverslip was positioned over two mirrored counting chambers found in the centre of the glass slide, so producing Newton's ring interference patterns. Each chamber is divided into 9 x $1mm^2$ squares and with the coverslip in place, the depth of the chamber is 0.1mm. The volume of the square is calculated as shown:-

$$(1mm \times 1mm) \times 0.1mm = 0.1mm^3 (\equiv 10^{-4}ml)$$

The counting chambers were filled by capillary action using a pasteur pipette which was touched against the edge of the coverslip. Cells were counted in either the centre or 4 corner squares, with cells touching the upper and right hand perimeter lines ignored and those touching the lower and bottom lines counted. At least 100 cells were counted for each determination.

2.6 CRYOGENIC STORAGE OF THE L1210 CELLS

Stocks of L1210 cells for long term storage were preserved in liquid N_2. Upon reduction of the temperature, ice crystals form within the cell. The presence of DMSO in the freeze media helps to prevent the formation of ice, and by freezing the cells slowly, but by thawing rapidly, damage can be kept to a minimum.

Freeze-down protocol

L1210 cells of a known cell density were centrifuged for 5 min at 400g, 20^0C (MSE Mistral 3000, Fisons, U.K.). The cell pellet was resuspended into an appropriate volume (approximately 10^6 cells/ml) of the freeze medium (RPMI medium containing 10% DMSO). To ensure sterility, the DMSO was filter sterilised into the medium immediately before use. The cells were stored in cryotubes (NUNC) in 1ml aliquots, and for the initial 24 hour maintained at -80^0C. They were then transferred to the liquid N_2 stores.

Revival protocol

An aliquot of cells taken from the liquid N_2 was thawed quickly by plunging the

cryotube into a waterbath at 37^{0}C. The cells were then added to 9ml medium and centrifuged as above to remove traces of the DMSO. before being resuspended into fresh medium. After a 24 hour incubation the cells were again pelleted and fresh medium added.

2.7 ISOLATION OF DRUG-RESISTANT L1210 CELLS

L1210 cells resistant to tiazofurin (TZ) were selected using a step wise selection protocol (Jayaram *et al* 1993). In the parental L1210 cells, TZ has an IC_{50} of 2.7μM (as determined using a growth inhibition analysis, see Section 2.9). The L1210 cells were subcultured as described above but a concentration of TZ equal to the IC_{50} was added to the medium. The TZ concentration was maintained for several weeks with routine subculturing until the growth rate had resumed that of the parent cell line. The concentration of TZ was then raised at intervals, with the cells allowed time for a similar adjustment between each new concentration. A final concentration of 2mM TZ was achieved after approximately 3 months, representing a ~1000 fold increase in the IC_{50} value (see Figure 6.2).

The resistant L1210 cells were then cloned to ensure that a cell line was obtained which represented a homogeneous population derived from a single cell. The technique for colony formation was as described in Section 2.9. A known number of the cells were seeded into the soft agar in tubes that contained TZ to a final concentration of 2mM. The cells were then left for 7 days to allow for the development of colonies, at which point the contents of the tube were transferred to plastic tissue culture dishes, and single colonies extracted using the tips of sterile glass pipettes. The colonies were placed into individual wells of a 96 well plate containing 100μl medium and agitated to cause dispersal into single cells. From this point, only a single cell suspension was taken to be subcultured as described in Section 2.3. This cell line was designated TZR (for tiazofurin resistant). Resistance was stable over a 3 month period in the absence of selection, but

to guarantee against a possible reversion to sensitivity, the cells were grown in 2mM TZ for a single passage every 4 weeks. Experiments were carried out using the cloned resistant cells, after they had been grown in the absence of TZ for at least two passages. TZ is phosphorylated to tiazafurin 5'-monophosphate (TRMP) which is then converted to the active metabolite, thiazole-4-carboxamide dinucleotide (TAD), an analogue of NAD^+ (Cooney *et al* 1982). TAD is unable to efflux from the cell. Thus it was considered necessary to grow the TZR cells in the absence of TZ for several generations prior to experimentation to ensure the loss of cellular metabolites of TZ by dilution.

2.8 POLY(ADP-RIBOSE) POLYMERASE ASSAY

The permeabilised cell assay for the measurement of PADPRP activity was as described by Halldorsson *et al*, 1978. The principle of the procedure involves the introduction of "holes" into the cell membrane through which the labelled NAD^+ can pass freely. Together, the hypotonic buffer and the ice-cold conditions result in osmotic and cold shock permeabilisation of the cells.

In the presence of a blunt ended oligomer to activate the enzyme, the labelled NAD^+, in an active poly(ADP-ribosyl)ation system is rapidly incorporated into acid-insoluble (ADP-ribose) polymer. The ability of compounds to actively inhibit PADPRP can be assessed by their inclusion into the reaction mixture.

Preparation of the permeabilised cells

L1210 cells were seeded to give a final cell number of $8x10^7$cells in 100ml on the day of the experiment. After the cell number was determined by haemocytometer count, the cells were washed once in ice cold Dul A and the pellet resuspended into ice cold hypotonic buffer (9mM Hepes, 4.5% dextran, 4.5mM $MgCl_2$, adjusted to pH 7.8 with NaOH/HCl, stored at +4°C. DTT was added to a final concentration of 5mM from a 100mM stock solution prior to use) to a density of $3x10^7$cells/ml. Following a 30 minute incubation, 9 volumes of ice-cold isotonic buffer was added (40mM Hepes, 130mM KCl,

4% dextran, 2mM EGTA, 2.3mM $MgCl_2$, 225mM sucrose, adjusted to pH 7.8 with NaOH/HCl, stored at +4°C). DTT was added to a final concentration of 2.5mM from a 100mM stock solution prior to use. The extent of permeabilisation was determined using a trypan blue exclusion haemocytometer count. The permeabilised cells (ideally >95%) take up the blue dye whereas intact cells exclude the dye. Once resuspended into the isotonic buffer, and kept on ice, the cells remain useable for up to 1 hour.

Preparation of the NAD^+

A stock solution of approximately 6mM NAD^+ was prepared in sterile water. The absolute concentration of this stock was determined from the measurement of the optical density (OD) of a 100 fold dilution at 260nm, using a UV lamp and quartz cuvettes. Based on this data, a working NAD^+ stock solution of 600µM was prepared (ε=18000, see Section 2.14) to which a small volume (2-5µl), depending on the current specific activity of [^{32}P] NAD^+ (~10µCi/µl), was added.

Poly(ADP-ribose) polymerase assay

The components of the reaction mixture, 5µl palindromic (CGGAATTCCG) oligonucleotide (200µg/ml in 10mM Tris-HCl, pH 7.8), 50µl 600µM NAD^+ containing [^{32}P] NAD^+, 8µl drug dissolved in DMSO (a final concentration of 2%) (prepared at 50X required final concentration), 37µl H_2O were added to 15ml conical bottomed tubes (NUNC) and positioned in a shaking water bath at 26°C. Each drug concentration was performed in quadruplicate.

The previously prepared cell suspension was warmed for 7 minute, before being mixed thoroughly with an Eppendorf repeat dispenser. To each of the reaction tubes, 300µl cell suspension was added at 5 second intervals, mixing the tube contents after each addition. Each tube was allowed a 5 minute incubation before the addition of 2ml of ice cold TCA (10% w/v TCA, 10% w/v Na pyrophosphate). Assay blanks (T_o), which correct for non-specific binding of the [^{32}P] NAD^+ to the filter (see next Section) were prepared by addition of the TCA to the reaction mixture before the cells were added, thereby

preventing initiation of the reaction. All the tubes were left on ice for a 1 hour incubation period.

Utilising a Millipore filtration apparatus (Millipore Corporation, Bedford, Mass. USA). GF/C (25mm) microfibre filters (Whatman International Ltd, Kent. UK) were placed rough side upwards onto the filter holder, after being moistened with the 10% TCA. The funnel assembly was secured tightly on top and the contents of the tubes added to individual funnels. Each was rinsed with TCA (1% w/v TCA, 1% w/v Na pyrophosphate) 6 times, under gentle suction pressure. The filters were then removed, and pinned onto a foil covered card with a yellow Gilson tip beneath to prevent them sticking before being oven dried. The filters were placed into scintillation vials (20ml, Packard Instrument Company, USA) containing 10ml scintillation fluid (Wallac Scintillation Products, Finland), the vials capped and then shaken. Four standards (S) containing 10µl of the 600µM NAD^+ + [^{32}P] NAD^+ solution were prepared and 10ml scintillant added. All of the vials were counted on the β scintillation counter (Wallac Scintillation Products) for 2 minute.

Calculation of the results

Initially the number of counts which represented 1pMol NAD^+ was calculated. The mean of the four standards (S_M) was utilised: $\frac{S_M}{6000}$ = number of counts/pMol NAD^+

The pMol NAD^+ is representative of the level of (ADP-ribose) polymer formed. Therefore, the pMol NAD^+ present in each of the samples (P) after a 5 minute incubation was calculated.

First, the mean of T_0, T_{0M} was determined and subtracted from each of the sample counts, thereby removing non specific background counts: $P - T_{0M} = P_O$

The values of P_O obtained were then divided by the number of counts representitive of 1pmol NAD^+ (C) to give:

$\frac{P_O}{C}$ = pMol NAD^+ in each sample (C_S)

As the volume of cells added to each sample was 300µl. C_S represents the pMol NAD^+ in 300µl of the cell suspension. To determine the pMol NAD^+ in 1ml of cell suspension. C_S was multiplied by 3.33. A cell count taken prior to the experiment. was then utilised to allow the presentation of the final result as:

pMol NAD^+ incorporated /10^6cells/5 minute

The ability of a compound to inhibit PADPRP was expressed as a percentage inhibition of control:

$$1 - \frac{(+\text{Inhibitor})}{\text{control}} \times 100\% = \% \text{ inhibition}$$

However, in certain cases the PADPRP activity was expressed as:

pMol NAD^+/mg protein.

In this case, a sample of the permeabilised cells was removed for protein estimation (see Section 2.15).

2.9 DETERMINATION OF THE CELL GROWTH RATE

The number of parental and TZR L1210 cells were determined utilising a coulter counter (Coulter Electronics Ltd, UK), which is an electronic counting machine. The cells, suspended in an electrolyte, are drawn through a small orifice, across which a current flows between two immersed electrodes. As each cell enters the aperture, it displaces its own volume of the electrolyte and so modulates the basic impedance of the current. This is detected as a voltage pulse that is proportional in height to the volume displaced, and therefore also to the magnitude of the particle size. The pulses in voltage are monitored and a rapid, accurate determination of the cell number is obtained. The counter settings used were, a lower threshold (T_L) of 11µm and an upper threshold (T_U) of 20µm (the threshold settings determine the size range of the particles which are to be counted)

The cells, seeded at $1x10^4$/ml into 24-well multi-dishes, were incubated, and at 24 hour intervals the cells were pipetted up and down using a Gilson pipette, to ensure a single cell suspension, and the number in quadruplicate wells was determined. The results,

plotted as the cell number against time (hours) enabled the doubling time of the cells to be calculated.

2.10 GROWTH INHIBITION ASSAY

Growth inhibition assays are a measure of the cytostatic effect of a drug, or combinations of drugs, on the growth rate of cells. L1210 cells were seeded as described in Section 2.9 and incubated for a 24 hour period prior to the addition of drug combinations (see Figure legends for details of the concentrations and combinations used). In experiments in which PADPRP inhibitors were utilised in conjunction with drugs, e.g. temozolomide (TM), the inhibitors were added first.

For each required drug concentration, triplicate wells were prepared, along with three wells designated for the day zero cell count (N_0), which were counted immediately after drug addition. Following a 48 hour incubation, the treated (N_T) and untreated (N_C) cells were counted as described in Section 2.10, and results expressed as the growth of the treated cells as a percentage of control cell growth utilising the following equation:-

$$\frac{(N_T - N_0)}{(N_C - N_0)} \times 100\% = \% \text{ growth inhibition}$$

The growth inhibition in experiments utilising cells treated with drug combinations were calculated using a control treated with either TM or inhibitor alone (see Figure legends for details).

2.11 CLONOGENIC ASSAY

A colony forming assay was used to determine cell survival after treatment with cytotoxic drugs (Sebolt-Leopold & Scavone 1992). Only cells that maintain their viability are able to proliferate and so enter through the 5-6 doublings required to produce a visible colony.

L1210 cells were seeded into 25cm^2 flasks from a suspension of between 6-

$7.5x10^5$cells/ml to give a final cell number of $3x10^5$cells/flask. The cells were then incubated for between 5-6 hour. The dosing schedules were as described in the Figure legends. In the experiments using either BZ or 3AB. the initial volume of cell suspension in the flasks was 4ml, and this was made up to 5ml with inhibitor to give the required concentration. Dilutions of stock NU1025 and PD 128763 were prepared and added to 5ml of cell suspension directly, in 50µl quantities.

Following a 16 hour or 24 hour incubation (see Figure legends), the cells were suspended thoroughly, to ensure a single cell suspension and then counted by haemocytometer. For each individual flask, a minimum of 80-100 cells were counted. The cells were then diluted up to 1000-fold (3x10 fold serial dilutions) into RPMI medium and the number of cells to be seeded determined. Set volumes of cells were added to plastic tissue culture tubes, e.g. $200\mu l_{1000X}$, $400\mu l_{1000X}$, $800\mu l_{1000X}$, $160\mu l_{100X}$ etc, to give a cell number that would produce a countable number of colonies, e.g. between 50-200 colonies. The volume was then made up to 1ml with medium, and to each tube 5ml of the previously prepared agarose/medium (see below) was added. The tube contents were mixed and then incubated for 7 days to allow for colony formation.

Preparation of the agarose

0.12g of SeaKem ME agarose was boiled in 10ml Dul A, until dissolved. This procedure also ensured sterility. The agarose was then added to growth medium, prewarmed to 37^0C to give a final volume of 100ml, and this was kept at 37^0C until required.

Determination of colony formation

For each experiment, MTT was freshly prepared to a concentration of 0.5mg/ml using sterile water and then stored wrapped in foil at $+4^0$C until use, to protect from the light. 1ml MTT was aliquoted into labelled plastic tissue culture dishes. The agarose containing

the colonies was then added (gentle transfer from the tube to the plate does not disrupt the integrity of the colonies) in a flow hood to maintain sterility and the colonies were incubated overnight at 37^0C, 5% CO_2 to allow for the development of colour. Formation of colour is due to the tetrazolium compound of the MTT being reduced by the mitochondrial reductases in the cells, so forming a blue formazan product. Colonies were counted by eye using a gridded light box, with magnifying lens, as an aid and the results were calculated as follows:-

Initially the Colony Forming Efficiency, CFE for each plate was calculated:-

$$\frac{\text{number of colonies counted}}{\text{number of cells seeded}} \times 100\% = \text{CFE}$$

Then, the treated samples were expressed as a % of the control to give the % survival:-

$$\frac{\text{CFE}_{\text{treated}}}{\text{CFE}_{\text{control}}} \times 100\% = \% \text{ survival}$$

The control CFE was routinely above 95%.

2.12 DNA STRAND BREAK ASSAY

Alkaline elution, as described by Kohn *et al* (1981), is a technique that utilises polycarbonate filters to separate single strands of DNA on the basis of size. The filters, rather than adsorbing the DNA, impede the passage of the longer DNA fragments, and so the rate of elution of the DNA from the filter is relative to the size of the DNA strand. Thus, the elution rate is proportional to the DNA strand break frequency. Adaptations of the technique enable the measurement of single strand and double strand breaks, alkali labile sites, DNA-protein cross links and DNA interstrand crosslinks. The sensitivity of the assay has been previously calculated to be 1 DNA lesion/10^7 nucleotides. Assay precision was increased by the coelution of the treated samples with an internal standard (see below and Section 4.3). The internal standard defines a corrected time scale so eliminating intersample differences.

Preparation of the filters

Polycarbonate filters (Whatman International Ltd, UK), moistened in ice-cold phosphate buffered saline (PBS) (Gibco), pH 7.2 were placed shiny side up onto 16x25mm polyethylene filtration funnels (Millipore Corporation, USA). A rubber gasket was then centralised over the filter to prevent leakage of the system, and a filtration funnel top screwed on tightly. Subsequently, they were covered in foil, to prevent the induction of light induced breaks in the DNA strands, and then placed onto a numbered stand. Two volumes of 2.5ml PBS was passed through the filters, and then to each a 50ml syringe (Becton Dickinson, Ireland) was connected. 15ml PBS was added and the filters were allowed to drip freely. Any of the filters that did not drip had the airlock freed by sucking out the trapped air with a 5ml Gilson. In each of the barrels ~ 10ml PBS was left, in readiness for the cell additions and the apparatus was stored at $+4^0$C.

Sample preparation

Sample and internal standard cells were seeded at $2.5x10^4$cells/ml into 80cm^2 flasks at 40ml/flask and incubated for 24hr. The sample and internal standard cells were labelled with [^{14}C]thymidine (0.016μCi/ml) and [^{3}H]thymidine (0.1μCi/1ml), respectively, for 24hr. Any unincorporated label was removed by centrifuging the cell suspension at 400g for 5 minutes at 20^0C, and the cells were resuspended in fresh medium. A further incubation of approximately 4 hours ensured that the label was chased into the high molecular weight DNA.

After the sample cells had been aliquoted into 6 well multi dishes, 2ml/well, they were treated with varying drug combinations (see Figure legends for the details). The cells were then harvested by centrifugation, as described above and resuspended in 2ml ice-cold PBS. The internal standard cells were resuspended in 10ml of PBS before being irradiated with 300 cGray (see Section 4.3 for the rationale for use of an internal standard). (The majority of these irradiations were performed using a Marconi X ray machine, but due to changes in circumstances, the final experimental series were carried

out using a Gammacell 1000 elite, Nordian International Inc., Canada). All the above procedures, from the point of harvesting the cells until the lysis of the cells (see below), were carried out in the dark and on ice as this prevented any further light induced strand breakage and stopped repair processes within the cells.

Elution protocol

The sample cells were loaded onto previously prepared filters, and an equal number of internal standard cells, as determined by haemocytometer count, added to each. The cells were washed with 2ml lysis buffer (69mM SDS, 25mM EDTA, adjusted to pH 10 using 1M NaOH), then the clips on the filtration funnels were fastened and the cells treated for 1hr with 1.5ml lysis buffer containing proteinase K (0.5mg/ml), before being washed twice with 20mM EDTA, pH 10. The filtration funnels were then attached to the appropriate tubes on a fraction collector and the syringes connected. To each, 25ml of elution buffer (2mM EDTA-acid form, 5M tetrapropylammonium hydroxide, adjusted to pH 12.2 using the tetrapropylammonium hydroxide) was added, and all were covered to prevent the entrance of light. The fraction collector (2211 Superfrac, Pharmacia LKB Technology, Sweden) was then programmed for 12 hours, 90 minutes for each of 8 fractions and the pump (202U/AA, Watson-Marlow, UK) started. This utilized solvent resistant manifold tubing (Watson-Marlow) and was set at a slow and constant speed, operating at 0.033ml/min. The fractions were delivered to the fraction collector via polythene tubing (1.0mm internal diameter, Pharmacia LKB Technology) and collected into scintillation vials containing 15ml liquid scintillation cocktail. After 12 hours, the pump was continued for a further 20 minutes to collect into fraction 9 any sample remaining in the tubes. The tubing was then disconnected from the filtration funnels and 0.4mM NaOH pumped through for another 20 minutes to flush the system of any remaining counts. At this point, the filtration funnels were dismantled and the filters placed on the bottom of fresh scintillation vials. To each, 0.4ml 1M HCl was added, the vials capped and then baked for 1 hour at 60^0C to depurinate the DNA. 2.5ml 0.4mM

NaOH was then added, the vials shaken and left at room temperature for 1 hour. This resulted in the conversion of the apurinic sites to strand breaks, thereby the DNA was fragmented and released from the filter. 15ml of scintillation fluid was added to each vial. This vial allows computation of the counts retained by the filter. All of the vials were then capped, shaken and placed in racks onto a 1410 scintillation counter (Wallac Scintillation Products). The levels of ^{3}H (fraction of internal standard DNA) and ^{14}C fraction of sample DNA) present in each of the vials was determined using a 1minute dual label count.

Calculation of the results

For each sample, the total amount of either ^{3}H or ^{14}C (T) initially placed onto the filter was calculated by addition of counts from each of the 10 fractions (9 "fractions" + a vial containing the filter). Then, using cumulative addition, the number of counts remaining on the filter after each fraction had eluted (R) was determined:-

$T - V_1 = R_1$ where V = the number of counts in the vial

$T - (V_1 + V_2) = R_2$

$T - (V_1 + V_2 + V_3) = R_3$.....

This was converted to the fraction of counts remaining using the following equation:-

R_1 / T = Fraction of counts remaining after vial 1

R_2 / T = " " " 2

R_3 / T = " " " 3.....

To aid the computation of the results, the computer software programme, Quatropro 4.0 (Borland) was utilised. The calculated data was plotted as the fraction of [^{14}C]-dThd remaining on the filter against the fraction of [^{3}H] Thd using a double log scale to form an elution profile. Example profiles are given in Figures 4.5 & 4.6. To summarise the data, the relative elution (R.E.) value was calculated. R.E. represents the ratio of the rate of elution of the DNA after treatment compared to untreated control. Initially, utilising

the software programme, "Graphpad Inplot tm" (San Diego, CA. USA) the relative retention (R.R.) values of each elution profile were determined by regression analysis. R.R. is the fraction of sample DNA retained on the filter when 50% of the internal standard DNA has eluted. The following equation was subsequently used to give the R.E. values:- R.E. = (log.R.R. sample DNA) - (log.R.R. control DNA)

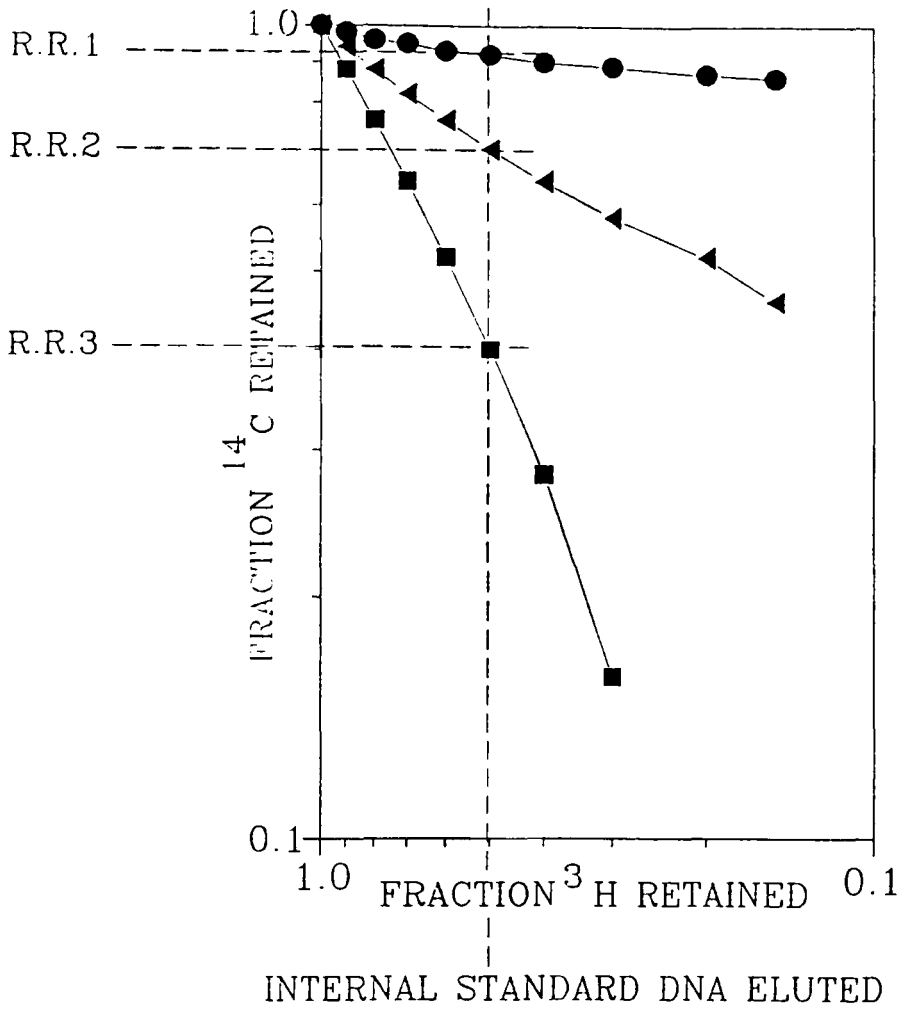


Figure 2.1 Method for calculation of the relative retention values.

2.13 DETERMINATION OF CELLULAR NAD^+ LEVELS

The cellular NAD^+ concentration in L1210 cells was determined utilising a colourimetric, enzyme cycling assay. For assay principles see "Measurement of the cellular NAD^+".

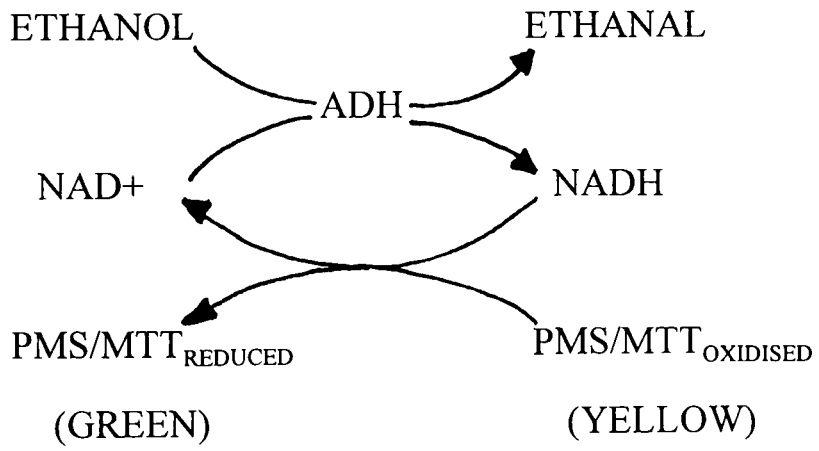
Sample preparation

L1210 cells were seeded into $25cm^2$ flasks at a density of $1.5x10^5$cells/ml, 10ml/flask, and were incubated overnight before being exposed to drug combinations (see Figure

legends for details). All drug treatments were performed in triplicate. The cells, after being harvested and washed once with Dul A, were repelleted and resuspended into 1ml 50% v/v ethanol. At this stage of the protocol the cell samples could be stored at -20°C. Each sample was sonicated (MSE Soniprep 150. Fissons) on ice for 3x10 second bursts at an amplitude of 15. A 200µl aliquot of this suspension was then transferred to a fresh 1.5ml Eppendorf (Starsted Ltd, UK) and stored at -20°C to await protein estimation. The remaining sample was centrifuged for 10 minutes at 17000g using a microfuge (Biofuge 15, Heraeus Sepatech GmbH, Germany) and the supernatant was carefully decanted from the pellet and stored, also at -20°C, for NAD assays.

Determination of NAD^+ concentration

The protocol was performed as described by Nisselbaum & Green (1969). The assay is termed a "cycling assay" due to the following reaction sequence:-



The alcohol dehydrogenase (ADH) reduces the ethanol and NAD^+ to give the corresponding aldehyde and NADH. The NADH is then chemically oxidised by the PMS and MTT, with the reduction of MTT resulting in a colour change as described in Section 2.11. The colour change from yellow to green is monitored spectroscopically and is proportional to the NAD^+ concentration. Each assay is standardised using NAD^+ of a known concentration. All the reagents are present in vast excess over the NAD^+.

A 100mM Triethanolamine HCl buffer, pH 7.4, containing 2.7% ethanol, was prepared

using a 500mM triethanolamine stock that had been dissolved in distilled water, autoclaved and stored at +4^{0}C. MTT (12.1mM) and PMS (2.6mM) were freshly prepared in distilled water, for each experiment, and due to light sensitivity were covered in foil for protection. The ADH (which comes as a solution) (30mg/ml) was diluted 3 fold with distilled water. All the reagents were maintained on ice until required.

The components of the cocktail, (53mM triethanolamine (1.7ml), 0.83mM PMS (1ml), 0.19mM MTT (0.05ml), 0.1mg ADH (0.1ml)), were added to give a final volume of 2.85ml in 4.5ml cuvettes and warmed to 37^{0}C, prior to the addition of the NAD^+ sample.

Construction of the standard curve

The concentration of NAD^+ present in the prepared cell extracts was determined by reference to a standard NAD^+ curve. A stock solution of NAD^+ was prepared at 0.1mg/ml in sterile water and stored aliquoted at -20^{0}C. To determine the absolute concentration of this stock, a 10 fold dilution was made with the triethanolamine buffer and the absorbance at 260nm recorded, utilising the UV lamp and quartz cuvettes. The Beer Lambert law is used to determine the absolute concentration of the 0.01mg/ml NAD stock:-

$$A = \varepsilon.c.\ell$$

where A = absorbance of the NAD stock, ε = molar extinction coefficient (NAD = 18000), c = unknown concentration and ℓ = pathlength (cm). The pMoles NAD/ml for each point on the standard curve can be calculated.

Based on these data, a working NAD^+ standard of 0.01mg/ml was prepared using the sample buffer. Prior to the addition of NAD^+, up to 300µl of 50% ethanol was added to each cuvette (4.5ml, Fisons) to maintain a constant volume. The reaction was initiated by NAD^+ addition and the cuvettes were inverted several times to ensure thorough mixing before being placed into a PU 8610 UV/VIS kinetics spectrophotometer (PYE Unicam Ltd, UK). The spectrophotometer, used in the "kinetics mode", was heated using a cell temperature controller (PYE Unicam Ltd) to 37^{0}C, and the change in absorbance at

556nm monitored over a period of 5 minute, at intervals of 1 minute. A "blank", containing the reaction cocktail minus the NAD^+ was utilised for each standard curve. Similarly, for the samples, 300µl of cell extract was added to the cocktail, mixed and the change in absorbance recorded as described for the standard curve. For each set of samples a separate blank containing 300µl 50% ethanol was included.

Calculation of Results

For each standard/sample the absorbance readings were recorded over 5 cycles as described above. The blank of each cycle was subtracted from the standard/sample reading and the amended absorbances were then entered into the following equations:-

$$(S_{Abs2} - S_{Abs1}) = \Delta \text{ Abs}$$

$$(S_{Abs3} - S_{Abs2}) = \Delta \text{ Abs}.....$$

Where s = standard/sample and ΔAbs = the change in absorbance. The mean change in absorbance over a one minute interval was then calculated (ΔAbs_M) .

Construction of the standard curve

The NAD^+ concentration of triplicates of the prepared solution, calculated as shown above were plotted against the change in absorbtion. An example curve demonstrating a linear relationship is shown in Figure 2.2.

Determination of the NAD^+ concentration in the cell extracts

To aid the computation of results the computer programme, Quatropro was utilized. The gradient of the standard curve was calculated by linear regression and this value was termed the x-coefficient. The NAD^+ concentration of each sample was then determined by:-

$$\frac{\Delta Abs_M}{\text{X coefficient}} = [NAD^+]$$

The NAD^+ concentration was then multiplied by the appropriate dilution factor to give the final result in pMoles NAD^+/ml cell extract.

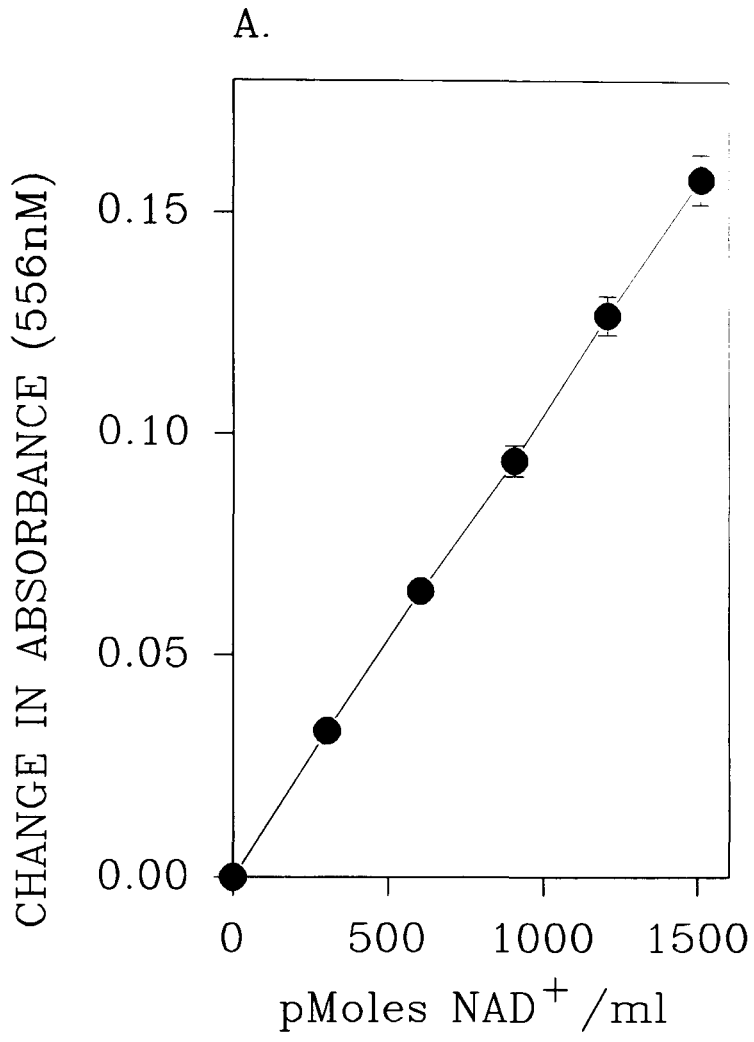


Figure 2.2: A. Example NAD^+standard curve. Each point is representative of triplicate samples +/− SE. B. NAD^+ absorbance over a 5 minute incubation period, ● 604pMoles NAD^+/ml, ■ 1208pMoles NAD^+/ml.

The NAD^+ concentration was then divided by the protein concentration of the sample (see Section 2.15) to give:-**pmoles NAD^+/mg protein**

2.14 DETERMINATION OF CELLULAR NMNAT LEVELS

The final reaction in the biosynthesis of cellular NAD^+ is catalysed by nicotinamide mononucleotide adenyl transferase (NMNAT):-

$$NMN + ATP \rightarrow NAD^+ + PP_i$$

A methodology for assaying NMNAT in murine tumour tissue has been published (Ahluwalia *et al*, 1984). A 20 minute incubation of tissue extract with exogenous NMN and ATP formed NAD^+. Following termination of the reaction, the concentration of NADH present in the assay mixture was determined by measurement of the absorbance at 340nm. The NAD^+ that had formed was subsequently reduced to NADH during a 30 minute incubation in the presence of ADH. The absorbance at 340nm was then reevaluated and the difference between the absorbtion readings was equivalent to the NMNAT activity, described as:

The amount of enzyme that cayalyses the transformation of

1 mol NMN to 1 mol NAD^+/second.

Initial trials for the conversion of the above methodology for use with tissue culture cells exhibited poor sensitivity, even when $1x10^7$cells per sample were used. Due to the formation of NAD^+ during the first incubation period of the NMNAT assay, I developed a method that coupled together the beginning reactions of the NMNAT assay with the enzymic cycling NAD^+ assay (described in Section 2.13). The increase in sensitivity of the coupled assay was determined using the molar extinction coefficient for NADH at 340nm, (ε = 6220). Utilising the Beer Lambert law, the NADH concentration required to give an optical density (OD) unit of 0.1, was calculated to be 16μM. However, utilising the NAD^+ standard curve shown in Figure 2.2, a change in absorbance of 0.1 OD required ~950pMoles NAD^+/ml in the coupled colourimetric assay, representing a

concentration of ~1μM, and an ~16 fold increase in sensitivity.

Cell extract preparation

Treated or untreated L1210 cells, (see Figure legends for details of the concentrations) were counted by haemocytometer, and samples containing $1x10^7$cells prepared (~20mls cell suspension at $6x10^5$cells/ml). The following procedures were performed on ice to prevent enzyme activity. The cells were washed once in ice cold Dul A and the pellet was resuspended into 0.5ml resuspension buffer (20mM Tris-HCl, 1mM DTT (the DTT was added prior to use from a 100mM stock) adjusted to pH 7.4 using HCl) before being transferred to labelled Eppendorfs. The cells were then sonicated for 20 seconds at an amplitude of 15, which resulted in the total disruption of the cell integrity as observed by the light microscope. In this respect, the cell extract differes from Ahluwalia *et al* (1984), as this is a whole cell extract as opposed to a nuclear suspension.

Assay protocol

To previously prepared assay buffer (0.2mM nicotinamide, 25mM glycyl glycine, 7.5mM $MgCl_2$, stored frozen in 20ml aliquots), ATP was added to a final concentration of 3mM, together with either 10mM glycyl glycine buffer pH 7.6, or NMN, to final concentrations of 0.1mM and 8mM, respectively. These buffers were termed "-" and "+", respectively. 0.5ml of the prepared assay buffer was aliquoted into fresh Eppendorfs. For each sample, an Eppendorf containing the "-" assay buffer and an Eppendorf containing the "+" assay buffer was required. The cell extract contains modest quantities of both NMN and NAD^+, of which the NMN would be converted to NAD^+ during the reaction time, which would subsequently be measured spectroscopically. So, to determine the activity of the NMNAT due solely to added NMN, a "blank" containing no NMN, ie "-" was prepared for each sample. To each of the Eppendorfs ("-" and "+"), 200μl of the cell extract was added, and the reaction initiated by incubating in a water bath for 30 min, at 37^0C. The remaining cell sample was stored at -20^0C to await protein quantification. The reaction was terminated by plunging the Eppendorfs into a boiling water bath for 1 minute before

centrifuging at 3000g for 5 minutes using the microfuge. 500µl of the supernatant was transferred to fresh Eppendorfs before being stored at -20^{0}C until assaying of the NAD^+. The NAD^+ assay was as described in Section 2.13 with minor modifications. 150µl of cell extract was added to the cocktail, together with 150µl 50% ethanol. The ethanol is an essential component of the cycling reaction, therefore its presence was maintained in the cocktail.

Expression of the results

The results were calculated as previously described in Section 2.13. The concentration of NAD^+ formed in 1 minute ($[NAD^+]_F$), due solely to the added NMN was calculated as follows:-

$$\frac{([NAD^+]_{+NMN} - [NAD^+]_{-NMN}) = [NAD^+]_F}{30 \text{ minute}}$$

Any dilutions made throughout the assay were taken into account by multiplying the $[NAD^+]_F$ value with the dilution factor, to give the final NAD^+ concentration. The quantity of NAD^+ formed in 1 minute was then divided by the protein concentration to give activity of the NMNAT, expressed as:-

pMoles NAD^+ formed/mg protein/min

2.15 ESTIMATION OF PROTEIN CONCENTRATION

The protocol utilised was based on the Bradford method (Bradford 1976). Previous to this, assays that determined protein concentration were described in the literature as insensitive and suffering from interference with a wide range of compounds, e.g. EDTA, Tris. The Bradford method eliminates the majority of these disadvantages and can be used to screen large numbers of samples. The assay utilises Coomassie blue, existing in two colour forms, red and blue. The red dye, on binding to the protein, results in the formation of a blue protein dye complex, and due to the high extinction coefficient of this complex and its stability over 1hr, the assay is highly sensitive and does not require critical timing. As the assay is slightly non linear, due to overlap of the two dye

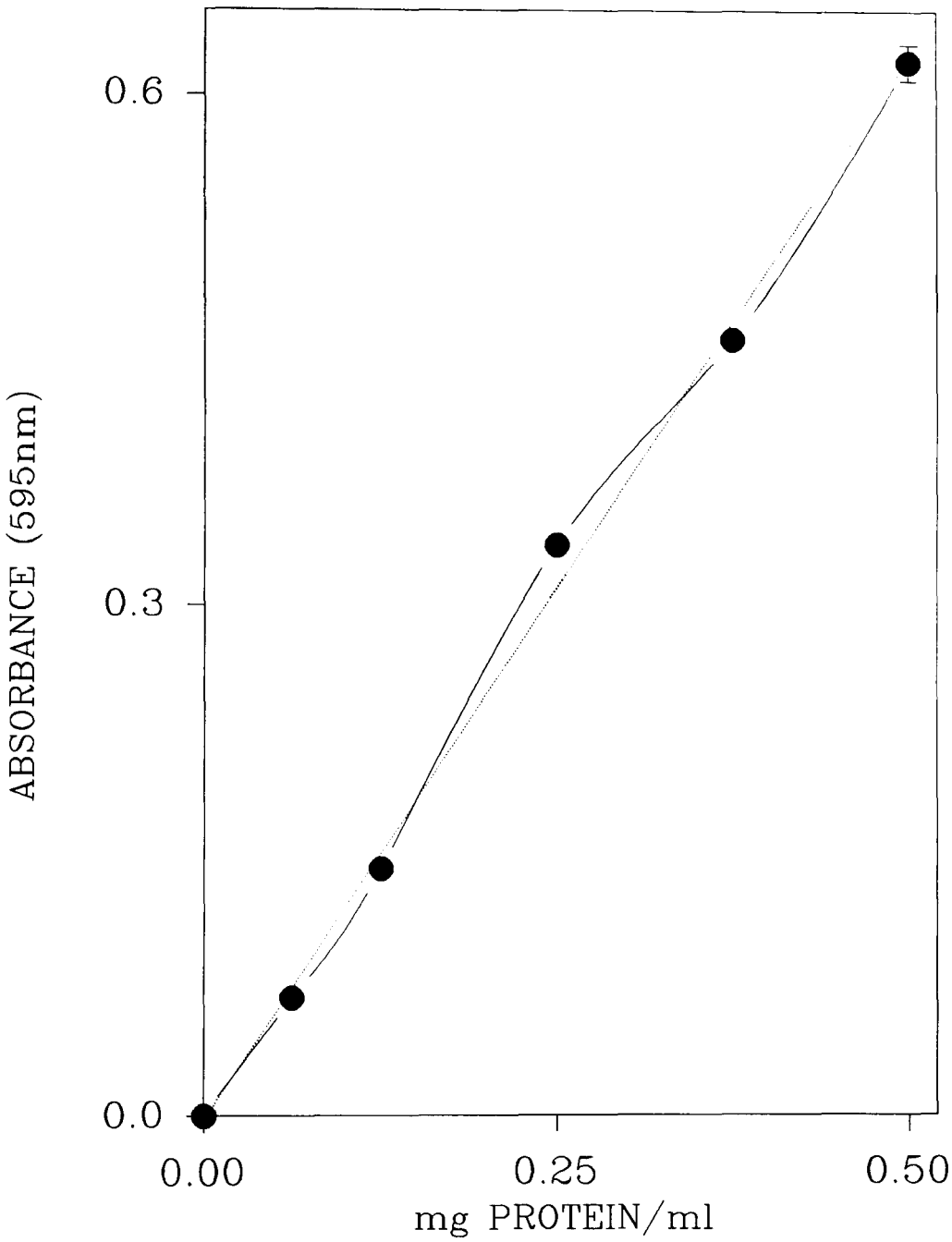


Figure 2.3: Example protein standard curve. Each point represents the mean of triplicate samples +/− SE.

spectrums, a standard curve is constructed for each assay. but this problem decreases as complex formation increases.

The standard protein samples were prepared over a concentration range of 0.0625 - 0.5mg/ml from a BSA stock at 2mg/ml using the sample buffer (50% ethanol or the resuspension buffer for the NAD and NMNAT assays respectively. see Sections 2.13 and 2.14).

50µl of the prepared standards and 2.5ml Coomassie protein assay reagent (the bottle was inverted several times to ensure the contents were well mixed) were added to each tube. The tube contents, after being thoroughly vortexed were transferred to 1.6ml plastic cuvettes (Fisons) and the absorbance at 595nm measured using the spectrophotometer. A blank consisting of only the buffer and Coomasie reagent was used with each run. For the unknown samples, the protocol was as described for the standards, although dilutions were performed as required, if the protein present was too concentrated.

Calculation of results.

The absorbance of the blank was subtracted from the sample absorbance to give the corrected reading. A standard curve (see Figure 2.3 for an example standard curve) was constructed with protein concentration plotted against the absorbance reading, again using the linear regression analysis function on Quatropro, and the concentration of protein in the samples determined by division of the x coefficient. Any dilutions made were taken into account by multiplying the determined protein concentration with the dilution factor.

2.16 STATISTICAL ANALYSES

Mean (x): The experiments presented in this thesis were performed, generally in triplicate and the mean was used to calculate the representative value. The definition of the mean is:- The sum of the observations, i.e. sample repeats (x) divided by the total number of observations (n). $x = \sum x/n$

Standard deviation (S): The amount of scatter of each observation from the mean is

expressed as the DEVIATION from the mean distribution. However, the square of the deviation must be calculated (sum of squares), as when the average deviation is calculated this is equal to zero, due to the influence of the sign, i.e. + or -. An average of the square deviation is determined by division by n-1 (degrees of freedom), rather than n, as an estimation of the scatter from the original population is required. This value is the VARIANCE (S^2).

$$S^2 = \sum(x - \bar{x})^2 / n-1$$

Due to the units of S^2, the square root of the variance is calculated to give the STANDARD DEVIATION (S). This has the same units as the initial observations.

$$S = \sqrt{S^2}$$

Standard error of the mean (SE): is an estimate of the standard deviation calculated if repeated samples from the same population were taken for analysis.

$$SE = S / \sqrt{n}$$

Conventionally, SE is used when the concern is with precision of the estimates, whereas S is utilised when the variability of the population or distibutions requires consideration. For the experimental data presented in this thesis, SE was utilised to express the error on the data.

Regression: demonstrates how one variable, y is related to changes in another variable, x and is a criterion for judging the line of best fit through experimental data points. The deviations of the points from the line in the y direction represents the variability of y not explained by x. The chosen line of best fit makes the sum of the deviations around the line a minimum. This gives the METHOD OF LEAST SQUARES. The equation of the least squares linear regression line is:

$$Y = a + bX$$

The slope of the line, b is calculated:

$$\sum(x - \bar{x})(y - \bar{y}) / \sum(x - \bar{x})^2$$

and the line must pass through the mean of the data, x and y. Regression analysis was utilised in calculating the NAD^+ and protein standard curves (see Chapter 3 and 6). the R.R. values from the alkaline elution profiles (see Chapter 4, 5 and 6) and the regression analysis for the increase in DNA single strand break levels following TM treatment in the presence of increasing concentrations of PD 128763 (see Chapter 5).

Non linear regression: fits a particular equation to the data points to give the line of best fit. A sigmoid curve analysis with the resulting curve smoothed was utilised to calculate IC_{50} and DEF_{10} values in growth and clonogenic survival curves (see Chapter 3). Graphpad Inplot tm, San Diego, CA software package was used for these calculations.

Student's two tailed, unpaired t test: A significance test asks whether the difference observed between two values was small enough to have occurred by chance.

A t distribution is the mean of a sample with a normal distribution but unknown variance. Normal distribution is assumed but this varies according to the associated degrees of freedom i.e. n-1. t distributions are utilised for the analysis of small populations.

$$t = x - \mu / \sqrt{(S^2 /n)}$$

where μ = population mean.

"Two tailed" allows for the possibility of the difference being greater or lesser than the given value. "Unpaired" is the comparison of two independent groups of data. The variability between subjects is of importance as well as the mean difference between the groups.

A two tailed, unpaired student's t test was used to determine whether the values calculated for the DNA single strand break level in TM alone treated cells was significantly different to the DNA single strand break level due to TM + specified concentrations of a PADPRP inhibitor (see Chapter 5), and also to determine whether the

difference between NMNAT activity in control or TM treated L1210 cells was significantly different (see Chapter 6)

CHAPTER 3 : EFFECT OF THE PADPRP INHIBITORS ON TM INDUCED GROWTH INHIBITION, CYTOTOXICITY AND NAD^+ DEPLETION.

3.1 INTRODUCTION

Most of the treatments for cancer rely on the use of chemotherapeutic drugs that interfere with proliferation of tumour cells. Unfortunately, this activity also affects normal tissue cells, resulting in toxicity, especially in highly proliferative cell populations, e.g. lymphocytes, epithelial cells. These toxicities manifest over the period chemotherapy treatment is administered and include myelosuppression, mucositis, and damage to critical organs such as the kidneys, lungs and the central nervous system. Such toxicities can therefore limit both the dose, and frequency of administration of the antineoplastic agent, which makes for a poorer prognosis.

A primary goal of cancer research is the discovery of novel compounds that possess increased antitumour activity, but lack systemic cellular toxicity. However, a further avenue of research is to identify compounds that would attenuate the toxic effects of the agents already in clinical usage. Such "chemoprotectants" or "resistance modifying agents" would themselves be inherently non-toxic, but raise the therapeutic index. The therapeutic index is a measure of the antitumour activity of a chemotherapeutic agent in relation to the level of normal tissue damage induced. A good strategy, rather than isolating chemoprotectants specific for individual anticancer agents, is to target the common mechanisms of action of these agents. One such action is the production of either direct or indirect damage to the DNA, with the ensuing stimulation of the cellular DNA repair mechanisms. The base excision repair (BER) process recognises damaged and missing bases that result from treatment with alkylating and oxidative agents, as well as ionising radiation. Inhibition of BER would therefore increase the cytotoxicity of many clinically relevant chemotherapeutic agents, as well as ionising radiation.

Resistance is another major problem associated with the clinical use of chemotherapeutic agents which could also benefit from the modification of the repair processes. The development of resistance, e.g. the multi-drug resistance (mdr) phenotype, thymidylate synthase (TS) gene amplification, to the chemotherapeutic agents leads to an associated decrease in the level of antitumour activity of the drugs employed. One possible mechanism of resistance that has been demonstrated is that cells exhibit an increased proficiency to repair damage to the DNA (e.g. Masuda *et al*, 1988 and 1990, Institoris *et al*, 1992). By inhibiting the repair process, the antitumour activity of the agent would be restored. Institoris *et al* (1992) found that P388 leukaemia cells resistant to 1,2:5,6-dianhydrogalacticol (DAG) undergo DNA excision repair of the intrastrand crosslinks, resulting in the formation of DNA strand breaks. In the presence of BZ, the cytotoxicity of DAG was potentiated, and the DNA strand break levels were increased, consistent with an inhibitory effect on repair. In the sensitive P388 cell line, DAG cytotoxicity was not potentiated by BZ and showed no evidence for DNA strand break formation.

PADPRP is involved in BER (see Section 1.5). The potential for inhibitors of PADPRP to act as resistance modifying agents was initially suggested by Durkacz *et al*, in 1980, for use in the treatment of human leukaemia. In the presence of 3AB, the cytotoxicity of the alkylating agent DMS was enhanced 4-fold. Subsequent reports in the literature have shown this potentiation of cytotoxicity to occur with other alkylating agents (e.g. Nduka *et al*, 1980, Lunn & Harris, 1988), oxidising agents (e.g. Huet & Laval, 1985) and ionising radiation (e.g. Lunec *et al*, 1984), in the presence of several PADPRP inhibitors e.g. 3AB, 3AAB, BZ (see Table 1.1). The level of cytotoxic enhancement correlated with the potential of the compounds to inhibit PADPRP (Nduka *et al*, 1980). A further indicator for the PADPRP inhibitors to act as resistance modifiers is that the cellular NAD^+ concentration in tumour cells is reported to be reduced in comparison to that found in their normal parental counterparts (Jedeikin & Weinhouse, 1955; Branster & Morton, 1956; Glock & McLean, 1957; Morton, 1958). As NAD^+ is the substrate for PADPRP,

the potency of PADPRP inhibitors which act in a competitive manner will be dependent on the NAD^+ concentration. Therefore, in tumour cells, low NAD^+ levels would create an advantageous situation for the action of the PADPRP inhibitors. Solid tumours have been predicted as containing up to 10-20% hypoxic cells, which exhibit an increased reductive potential, i.e. the NADH:NAD^+ ratio is increased (Kennedy *et al*. 1980). NADH also acts as a potent inhibitor of PADPRP, with a K_i comparable to that of 3AB, of 5μM (Ueda *et al*, 1982). Together, the increased cytotoxicity due to the inhibition of repair, with the reduced cellular NAD^+ pool in the tumour cells could lead to an increase of the therapeutic index for many of the antineoplastic agents used clinically when used in conjunction with PADPRP inhibitors. However, a major problem for exploiting this potential was the lack of potent PADPRP inhibitors with specificity towards the enzyme. The nicotinamide analogues, i.e. benzamide and the 3′-substituted benzamides, especially at high concentrations, were found to affect other metabolic processes, e.g. glucose and purine metabolism (Milam & Cleaver, 1984). Therefore, a number of programmes were recently initiated with the aim of developing novel PADPRP inhibitors that exhibited increased potency, and that could eventually be entered into clinical trials (Suto *et al*, 1991; Banasik *et al*, 1992, Griffin *et al*, in press).

3.2 AIMS

A vital assessment of any novel compound that possesses the potential to inhibit a metabolic reaction is the ability of that compound to act in an intact cell situation. The initial *in vitro* screening of possible compounds utilises purified enzymes or conditions that allow the facilitated access of the compound to its target, e.g. in the case of PADPRP, a permeabilised cell assay. This type of study solely provides a measure of the maximum inhibitory potential of the compound. The cellular influx and efflux rates, the cellular concentrations achieved, the interactions with cellular components other than the intended target, and the intracellular metabolism of the compound are all important

considerations that will influence the inhibitory potential of a novel compound. Therefore, intact cell studies which will take into account such reactions, are an essential step in the progress of a compound into clinical trials.

The aim of this initial Results Chapter was to assess the ability of the two novel compounds, 8-hydroxy-2-methylquinazolin-4[*3H*]-one (NU1025) and 3,4-dihydro-5-methoxy-I[*2H*]-isoquinolinone (PD 128763) to act as PADPRP inhibitors in intact cells. A comparative study with the classical inhibitors, 3-aminobenzamide (3AB) and benzamide (BZ) was carried out.

PD 128763 was the lead compound in a series of 5′substituted dihydroisoquinolinones developed by Leopold and colleagues, which was shown to exhibit a 60-fold increase in potency towards PADPRP as compared to 3AB (Suto *et al*, 1991). 500μM PD 128763 in combination with the monofunctional alkyating agent streptozotocin, exhibited a 7-fold potentiation of streptozotocin cytotoxicity in L1210 cells, whereas a similar study utilising 5mM 3AB produced only a 2.5 fold enhancement (Sebolt-Leopold & Scavone, 1992). Due to the structural similarities of NU1025 and PD 128763 (see Figure 1.9) a comparable level of potency was anticipated.

The activation of PADPRP in response to DNA damage induced by monofunctional alkylating agents is extensively reported in the literature. Therefore, the biological endpoints chosen for the comparative study assessed the cytotoxicity of the clinically relevant monofunctional alkyating agent TM in L1210 cells (see Section 1.4.3a) via both growth inhibition studies (see Section 2.10), and clonogenic survival assays (see Section 2.11). The effects of an incubation with the PADPRP inhibitors alone, or in coincubation with TM were considered. An indirect measure of PADPRP inhibitory ability was also utilised in the study, with measurement of ability of the PADPRP inhibitor to prevent the TM induced NAD^+ depletion.

3.3 THE *IN VITRO* INHIBITION OF PADPRP

The data presented in Figure 3.1, reproduced with the kind permission of J.K. Porteous and K.J. Bowman (Cancer Research Unit, The University of Newcastle Upon Tyne) displays the percentage inhibition of PADPRP activity versus the concentration of the PADPRP inhibitors, 3AB, BZ, NU1025 and PD 128763, as determined utilising an *in vitro* permeabilised cell assay (see Section 2.8). Each compound inhibited the activity of PADPRP in a concentration-dependent manner (see Figure 3.1), and as predicted from their similar structural forms, PD 128763 and NU1025 exhibited comparable potency. The concentrations of the compounds required to inhibit the activity of PADPRP by 50% (IC_{50}) are shown in Table 3.1, together with their potencies in relation to 3AB. (The relative potency of 3AB has been set as 1). The relative potencies calculated for 3AB and PD 128763 are in excellent agreement with those established by Suto *et al*, (1991). Both NU1025 and PD 128763 were found to be more than an order of magnitude more potent than 3AB and BZ, i.e. the IC_{50} values for NU1025 and PD 128763 were 0.44μM and 0.36μM respectively, which represent an ~43 and ~53 fold increase in potency as compared to 3AB (IC_{50} = 19.1μM). Therefore the increased level of potency exhibited by NU1025 and PD 128763 against PADPRP would indicate a potential for use as chemoprotectants/resistance modifiers.

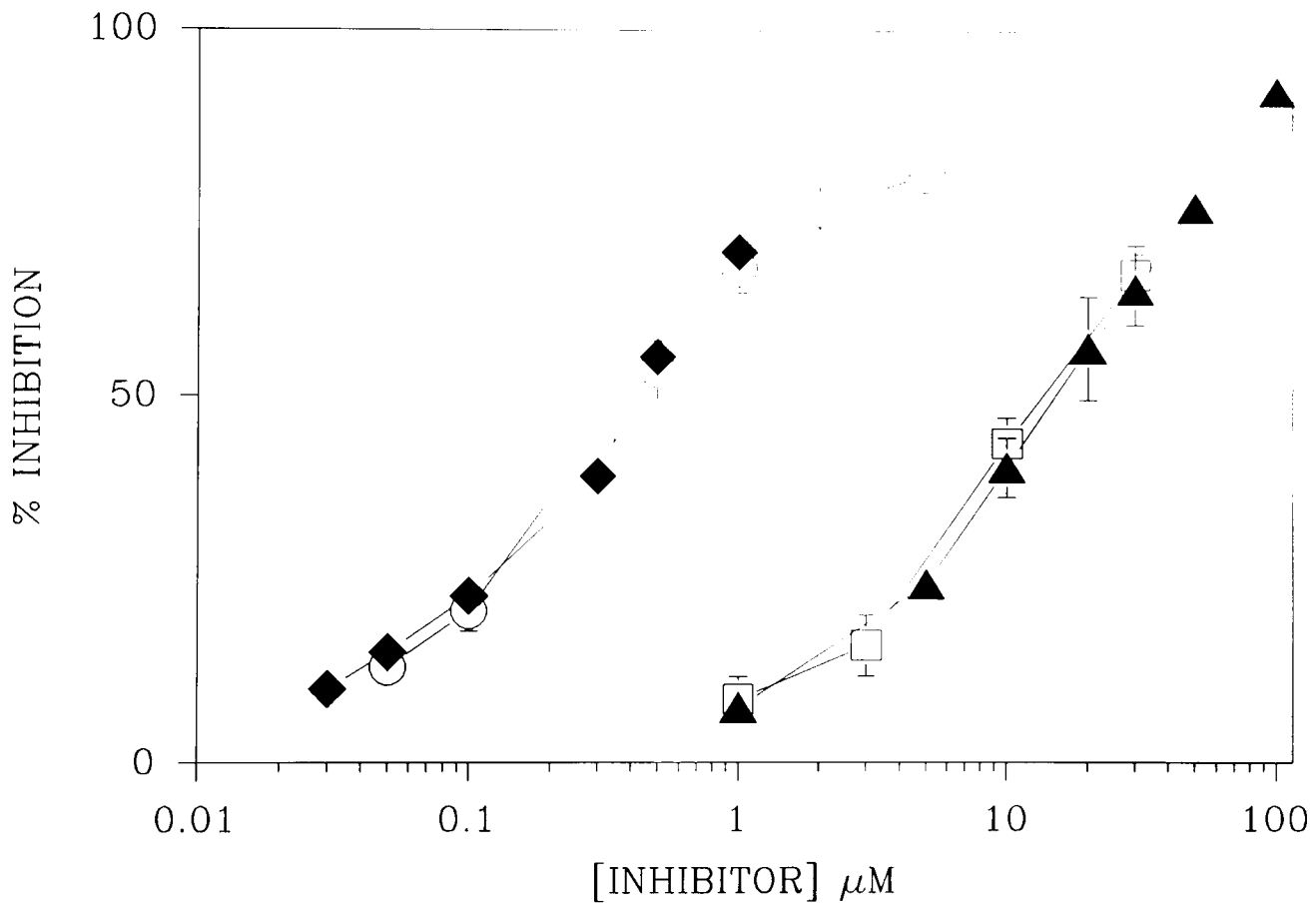


Figure 3.1: A comparison of the relative potencies of the compounds as inhibitors of PADPRP.
◆ PD 128763; ○ NU1025; ▲ 3AB; □ BZ.
The points represent the mean of at least four independent experiments.

TABLE 3.1

INHIBITOR	IC_{50} VALUE ± SE	RELATIVE POTENCY
3AB	19.1μM ± 6.9μM	1.0
BZ	13.7μM ± 6.9μM	~1.0
NU1025	0.44μM ± 0.13μM	~43
PD 128763	0.36μM ± 0.01μM	~53

Table 3.1: Permeabilised L1210 cells were incubated with increasing concentrations of the PADPRP inhibitors, and PADPRP activity was measured as a % of untreated control. PADPRP was activated utilising a blunt ended oligomer. The IC_{50} values were calculated utilising the non-linear regression analysis of "Graphpad Inplot tm. San Diego, CA" software and are the average of at least three independent experiments ± SE.

3.4 THE EFFECT OF THE PADPRP INHIBITORS *PER SE* ON L1210 CELL GROWTH AND SURVIVAL.

An effective resistance modifier should lack inherent cellular toxicity. The literature indicates that both 3AB and BZ, over the concentration range required to enhance the cytotoxicity of DNA damaging agents, i.e. up to 5mM, exhibited little or no effect themselves on cell survival (James & Lehmann, 1982; Brown *et al*, 1984; Huet & Laval, 1985; Boorstein *et al*, 1987; Moses *et al*, 1988). Therefore, a study was required that determined whether the two novel compounds were themselves cytotoxic to cells and how they compared to 3AB and BZ.

Initially the cytostatic effect of all four compounds was investigated. Figure 3. 2 shows that the growth of L1210 cells over a 48 hour incubation with each of the PADPRP inhibitors was inhibited in a concentration dependent manner. Cell growth was inhibited by 50% at concentrations of 0.41mM NU1025 and 0.45mM PD 128763, whereas 6.7mM

3AB and 2.5mM BZ were required to produce similar levels of growth inhibition. This represents a 20-fold reduction in the concentrations of NU1025 and PD 128763 as compared to 3AB, and a 5-fold decrease in concentration compared to that of BZ. The IC_{50} values are summarised in Table 3.2.

The subsequent analysis performed assessed the cytotoxic effects of the four PADPRP inhibitors on L1210 cell survival. The exposure time for the clonogenic assays was over a 24 hour period, as opposed to the 48 hour incubation for the growth inhibition evaluation. Figure 3.3 reveals biphasic survival curves for 3AB, BZ and NU1025. The initial shoulder observed indicates that the lower concentrations of inhibitors had little effect on the cloning ability of the L1210 cells. However, the steady increase in the concentration, initiated a concentration dependent decrease in cell survival. In the presence of PD 128763, the survival curve appeared to lack the initial shoulder and cell survival was immediately concentration dependent.

A biphasic curve would indicate that as the concentration of the PADPRP inhibitors increases initially, there is little or no cytotoxic effect, but the attainment of a critical concentration initiates a reduction of cellular survival.

The calculated LD_{50} values (the dose required to reduce survival to 50% of control) for 3AB, BZ, NU1025 and PD 128763 were 14.9mM ± 1.0, 5.7mM ± 1.5, 1.6mM ± 0.08 and 0.99mM ± 0.18 respectively with the same order of relative potencies as observed for the growth inhibition and *in vitro* PADPRP activity studies (see Table 3.2). However, the LD_{50} values were increased 2-fold when compared to the IC_{50} values calculated for the cytostatic effect of 3AB, BZ and PD 128763 on L1210 cells.

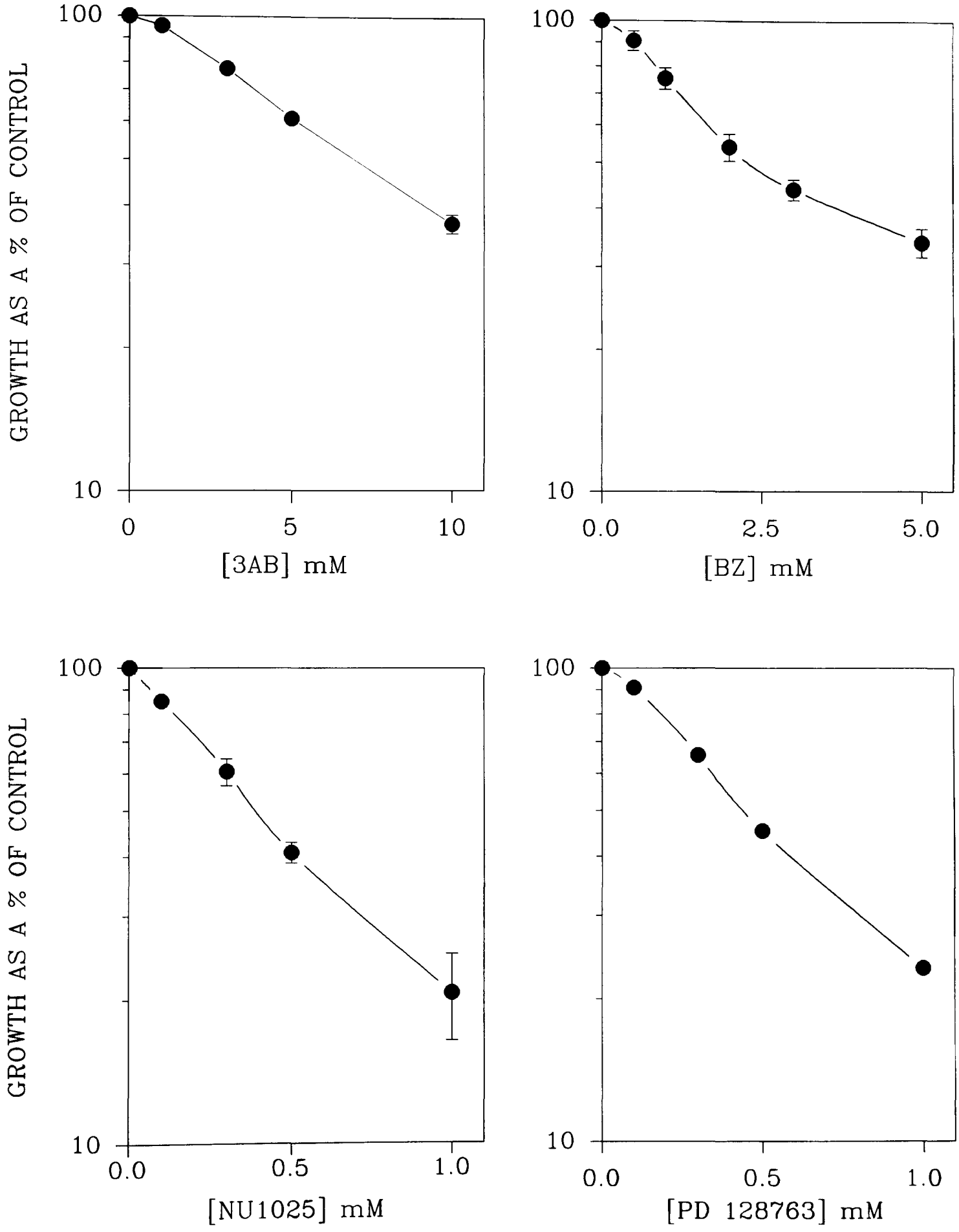


Figure 3.2: The growth inhibitory effect of a 48 hour continuous exposure to an increasing concentration of the PADPRP inhibitors. The points are the average of at least six samples taken from three independently dosed experiments.

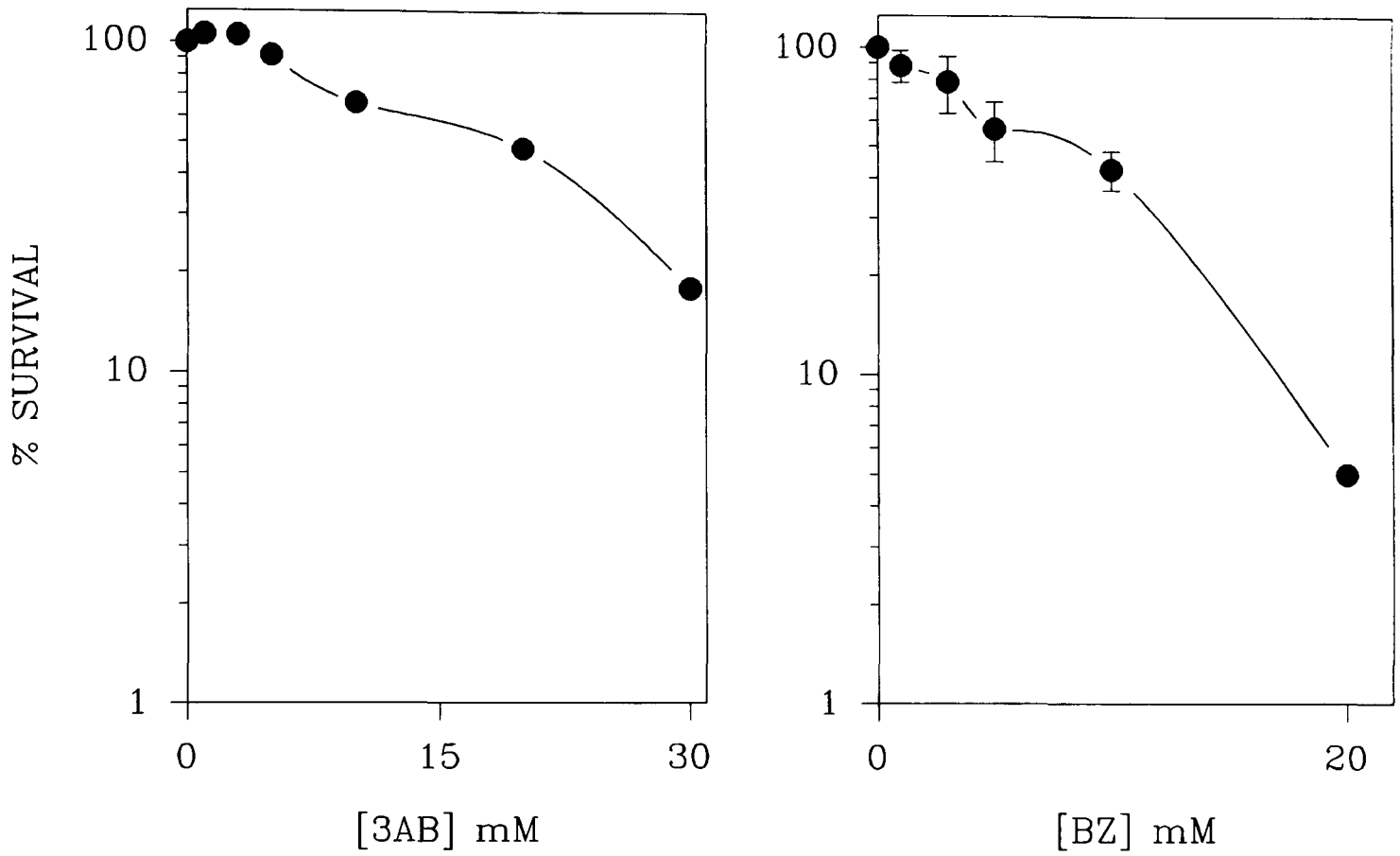


Figure 3.3 The cytotoxic effect of a 24 hour continuous exposure to an increasing concentration of PADPRP inhibitors *per se*. The cells were subsequently plated for survivors in the absence of the inhibitors. The points represent the mean of three independent experiments.

TABLE 3.2

INHIBITOR	GROWTH INHIBITION IC_{50} (mM)	RELATIVE POTENCY	CYTOTOXICITY LD_{50} (mM)	RELATIVE POTENCY
3AB	6.7 ± 0.2	1.0	14.9 ± 1.0	1.0
BZ	2.5 ± 0.3	~3	5.7 ± 1.5	~3
NU1025	0.41 ± 0.06	~16	1.6 ± 0.08	~9
PD 128763	0.45 ± 0.01	~15	0.99 ± 0.18	~15

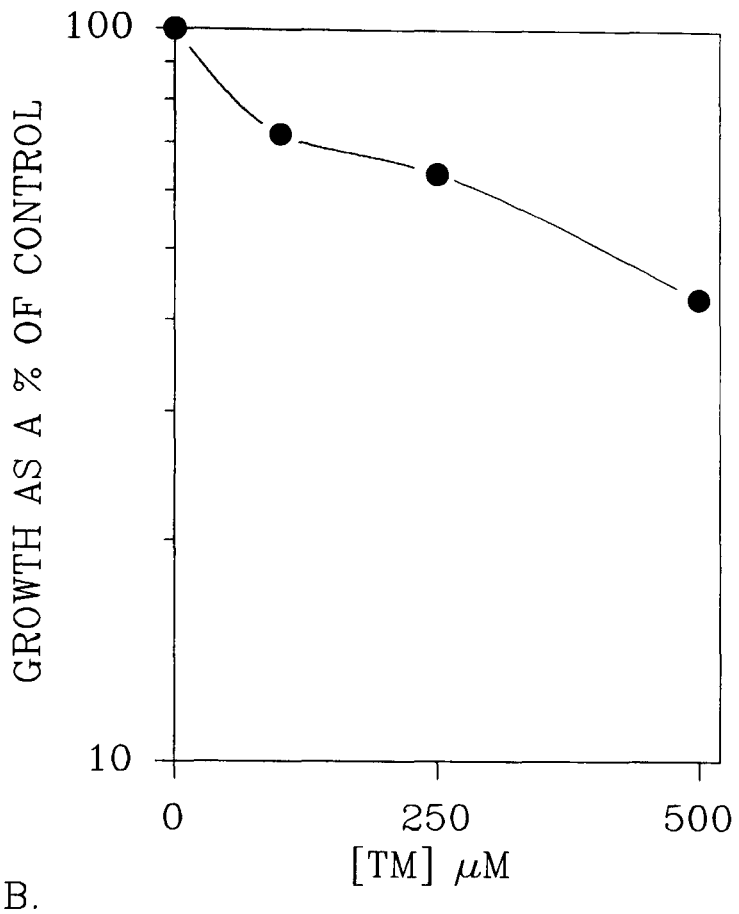
Table 3.2: Comparison of IC_{50} and LD_{50} values resulting from 48 and 24 hour continuous exposures of L1210 cells to increasing concentrations of the PADPRP inhibitors respectively. Both the IC_{50} and LD_{50} values were calculated utilising the "smooth curve" function on the non linear regression analysis of "Graphpad Inplot tm". The values represent the mean of at least three independent experiments ± SE.

3.5 THE CYTOSTATIC AND CYTOTOXIC EFFECT OF TM ON L1210 CELLS.

The active biological species of TM is the monofunctional linear triazene, MTIC. TM is chemically degraded in biological mileau to MTIC, which then methylates the bases of the DNA, e.g. 3meA, 7meG and O^6meG, via a methyl diazonium ion.

A 48 hour continuous exposure of L1210 cells to an increasing concentration of TM resulted in a concentration dependent inhibition of cell growth (see Figure 3.4) with an IC_{50} value of 361μM ± 25μM. Over a similar TM dose-range, but with just a 16 hour continuous incubation period, the cytotoxic effect on L1210 cells also increased (see Figure 3.4). The LD_{50} was calculated as 251μM ± 13μM.

A.



B.

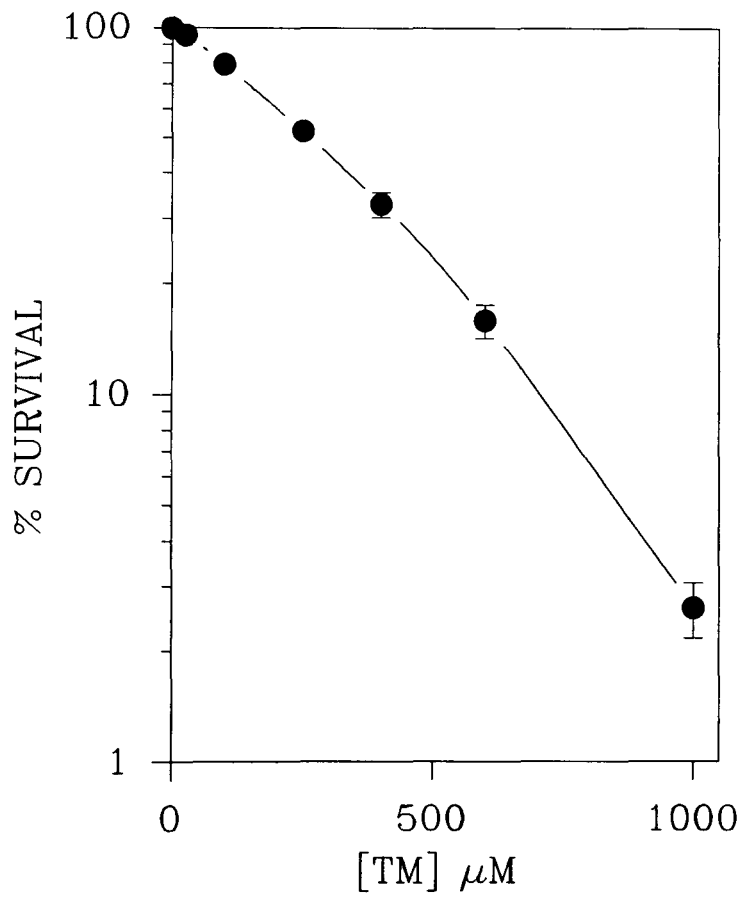


Figure 3.4: Effect of A. a 48 hour exposure to TM on the growth of L1210 cells, and B. a 16 hour exposure to TM on L1210 cell survival. Points represent the mean of at least eight samples, taken from at least three independent experiments.

3.6 CHEMOPOTENTIATION OF TM CYTOTOXICITY BY INHIBITORS OF PADPRP

3.6.1 The effect of a fixed dose of TM on the growth inhibitory effect of the PADPRP inhibitors on L1210 cells.

The growth inhibition, of L1210 cells, following a 48 hour exposure to a fixed dose of TM in the presence of increasing concentrations of PADPRP inhibitors was investigated. Two criteria were essential when considering the TM concentration to be used:- (1) the concentration should show only moderate growth inhibition itself, and (2) the concentration should induce a biologically measurable level of DNA damage, thereby allowing subsequent comparisons with DNA strand break analyses. A TM dose of 100μM was chosen, which itself reduced the cell growth by ~26% (see Figure 3.4), and for this series of experiments the growth inhibition due to the TM alone was as used as the control (i.e. normalised to 100% survival).

All four of the PADPRP inhibitors inhibited cell growth when co-incubated with 100μM TM (see Figure 3.5). An analysis of the growth curves in Table 3.3A and B shows that in the presence of 100μM TM, the concentrations of NU1025 and PD 128763 required to inhibit cell growth by 50% were reduced from 0.41mM to 0.04mM, and 0.45 to 0.023 respectively, representing a 10-20-fold decrease in the inhibitor concentration. However, the difference between the IC_{50} concentrations of 3AB and BZ alone, or in association with the TM was only 2-3 fold, with values of 6.7mM to 2.5mM for 3AB, and 2.5mM to 0.84mM for BZ respectively. This represents a much closer overlap of the growth inhibitory effects between both of these inhibitors alone, and in the presence of TM. However, a statistical analysis, utilising a two-tailed, unpaired students T test showed the difference between the inhibitor alone, and in the presence of 100μM TM to be significant for each of the four inhibitors (P values: 3AB, <0.0001; BZ, 0.0184; NU1025, 0.0056; PD 128763, <0.0001).

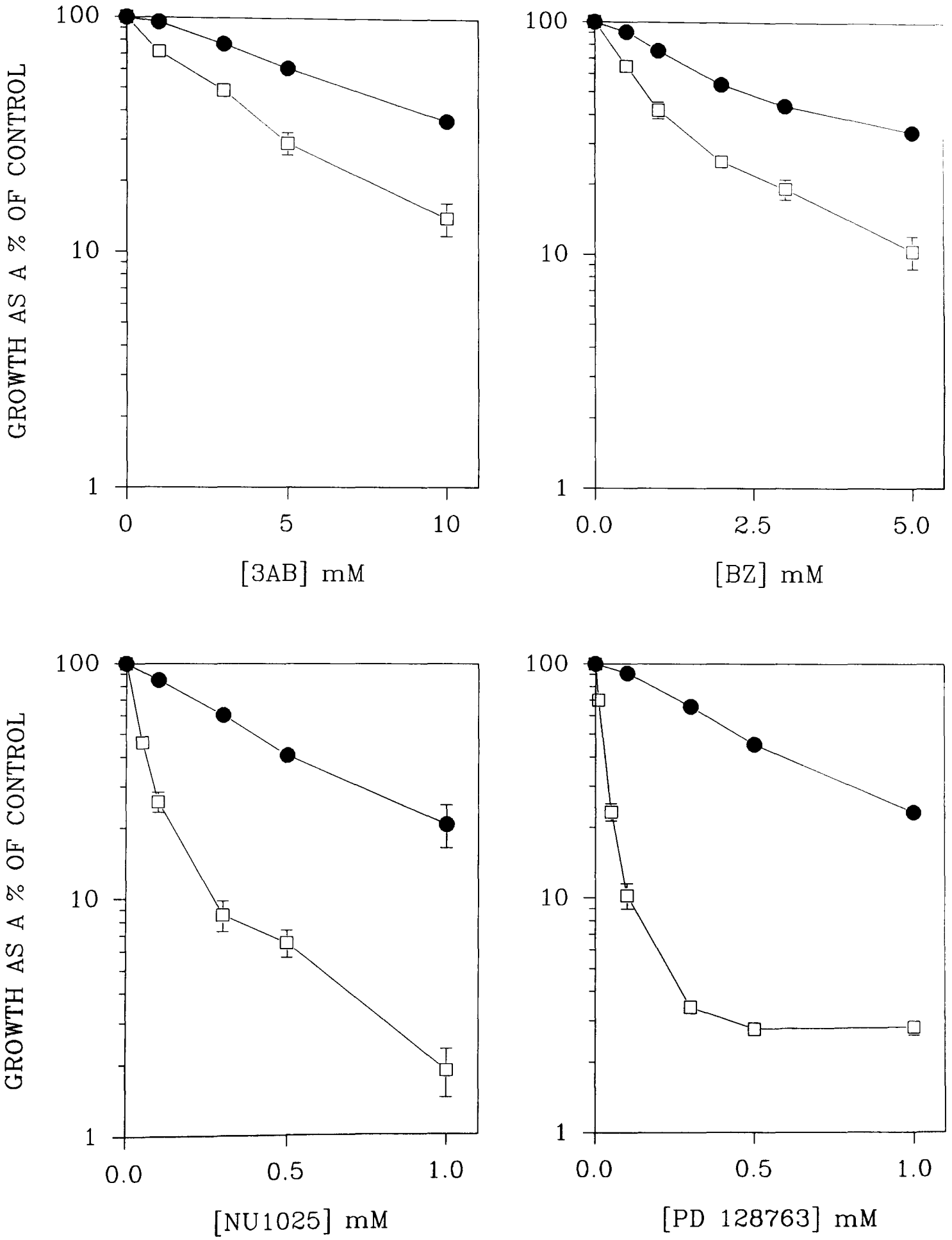


Figure 3.5: The growth inhibitory effect of a 48 hour continuous exposure to an increasing concentration of the PADPRP inhibitors in the absence ● , or presence □ of 100μM TM, The points represent the mean of at least nine individually dosed samples, taken from three independent experiments.

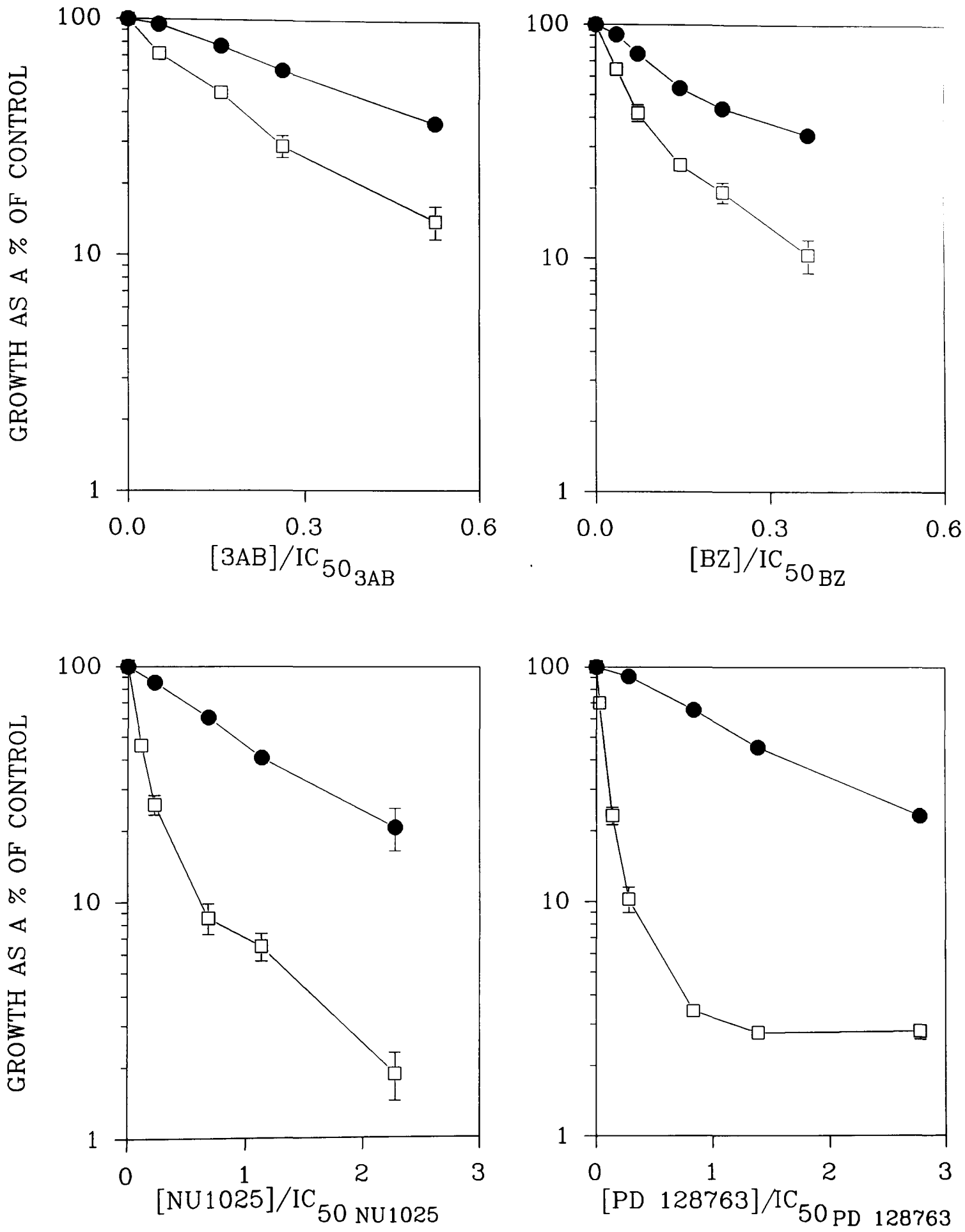


Figure 3.5A: The graphs presented in Figure 3.5 have been replotted. The X axis has been altered to express the inhibitor concentration as a ratio: [inhibitor]/IC50 inhibitor, with the IC50 value derived from the *in vitro* cell assay (see Section 3.3 and Table 3.1).

TABLE 3.3A

INHIBITOR	IC_{50} (mM) ± SE INHIBITOR ALONE	[INHIBITOR](mM) / $IC_{50\ INHIBITOR}$
3AB	6.7 ± 0.2	0.37 ± 0.01
BZ	2.5 ± 0.3	0.21 ± 0.03
NU1025	0.41 ± 0.06	0.93 ± 0.05
PD 128763	0.45 ± 0.01	1.2 ± 0.05

TABLE 3.3B

INHIBITOR	IC_{50} (mM) ± SE INHIBITOR + 100μM TM	([INHIBITOR](mM) + 100μM TM) / $IC_{50\ INHIBITOR}$
3AB	2.5 ± 0.1	0.121 ± 0.009
BZ	0.84 ± 0.12	0.062 ± 0.005
NU1025	0.04 ± 0.003	0.106 ± 0.007
PD 128763	0.023 ± 0.002	0.057 ± 0.001

Tables 3.3A and 3.3B: L1210 cells were continuously exposed for 48 hours to increasing concentrations of the PADPRP inhibitors, in the absence or presence of a fixed concentration of TM, and the level of growth inhibition assessed. The IC_{50} values were calculated utilising the smooth curve analysis of "Graphpad Inplot tm", and are the average of three independent experiments ± SE. The ratio: [inhibitor](mM) +/- TM / $IC_{50\ inhibitor}$ was also calculated by smooth curve analysis utilising the inhibitor IC_{50} values determined in the *in vitro* PADPRP assay (see Section 3.3 and Table 3.1).

Further analysis of the data, resulting from the division of the inhibitor concentration by the inhibitor IC_{50}, as calculated in the *in vitro* PADPRP assay (see Chapter 2, Section 2.8 and Chapter 3, Section 3.3) provides an indication as to whether each inhibitor acts on the same target. Table 3.3A reveals that following division by the inhibitor IC_{50} the difference between the ratios for the different inhibitors is reduced to ~5 fold as opposed to ~20 fold when the IC_{50} concentrations are compared, (e.g. [PD 128763]/IC_{50} = 1.2 ± 0.05; [3AB]/IC_{50} = 0.37; whereas IC_{50} PD 128763 = 0.45 ± 0.01mM; IC_{50} 3AB = 6.7 ± 0.2mM). When the cells were co-treated with 100μM TM the values for the ratios were reduced even further from ~120 fold to ~2 fold, (e.g. IC_{50} 3AB = 2.5 ± 0.1; IC_{50} PD 128763 = 0.023 ± 0.002 whereas ([3AB] + TM)/IC_{50} = 0.121 ± 0.009; ([PD 128763] + TM)/IC_{50} = 0.057 ± 0.001). The similarity between the ratio values for each of the four inhibitors in the presence or absence of TM would suggest that the four inhibitors act on the same cellular target.

3.6.2 The growth inhibitory effect of increasing concentrations of TM in the presence of fixed concentrations of the PADPRP inhibitors on L1210 cells.

In Section 3.5, a dose-dependent decrease of L1210 cell growth following a 48 hour continuous exposure to TM was presented. Subsequently, the ability of the PADPRP inhibitors to potentiate this growth inhibition was investigated. The concentrations of inhibitor involved caused minor growth delays themselves but this was corrected for by normalising the growth in the presence of inhibitor alone to 100%.

A 48 hour continuous exposure to an increasing concentration of TM in the presence of fixed doses of each of the four PADPRP inhibitors potentiated the inhibition of cell growth in a concentration dependent manner (see Figure 3.6). The determined IC_{50} values are shown in Table 3.4, together with the enhancement factor, which represents, the ratio

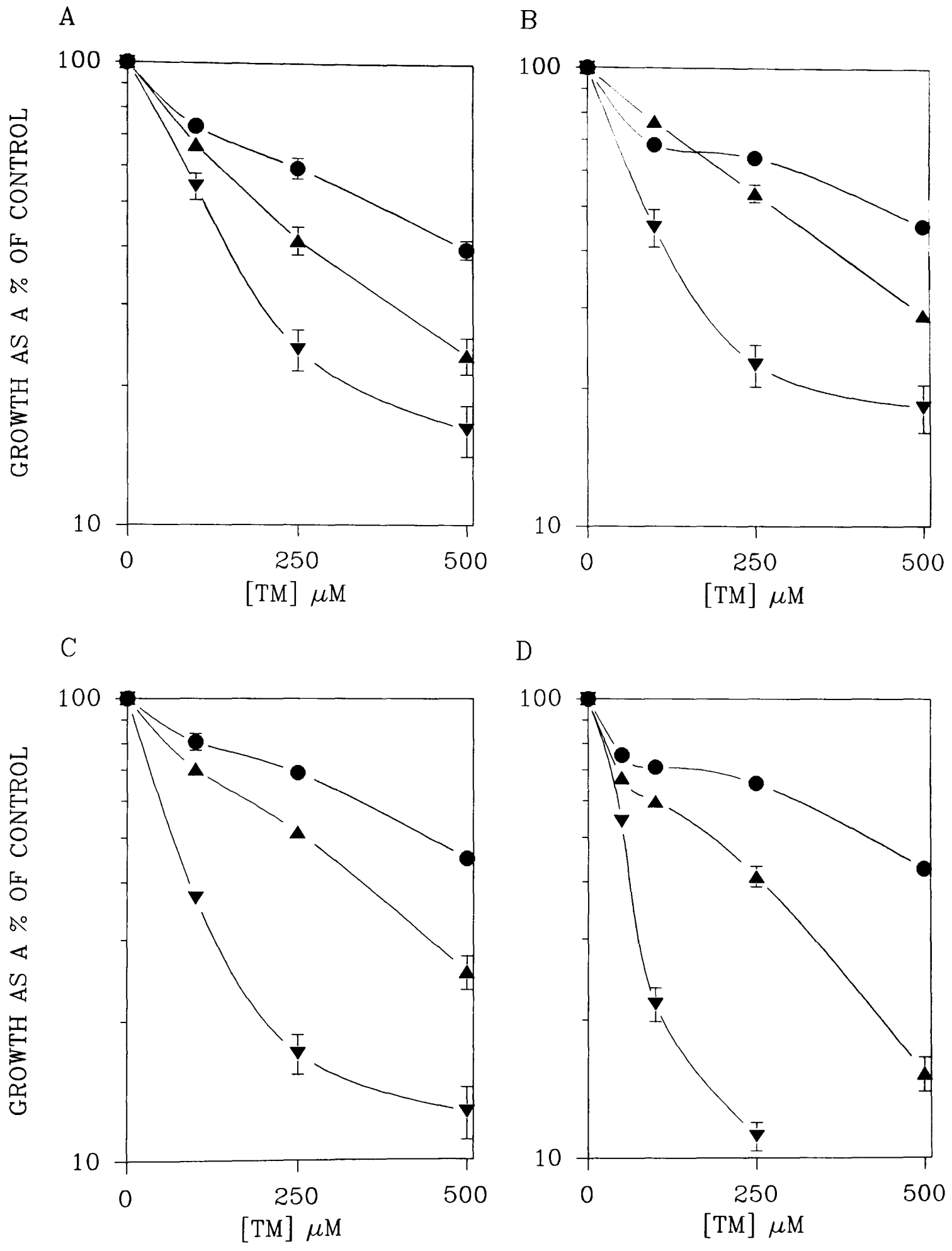


Figure 3.6 The growth inhibitory effect of a 48 hour continuous exposure to an increasing concentrationof TM alone ● , or in the presence of fixed concentrations of the PADPRP inhibitors. A. ▲ 1mM 3AB, ▼ 5mM 3AB. B. ▲ 0.3mM BZ, ▼ 3mM BZ. C. ▲ 10μM NU1025, ▼ 100μM NU1025. D. ▲ 10μM PD 128763, ▼ 50μM PD 128763 The points represent the mean of at least nine individually dosed samples, taken from three independent experiments.

of the concentration of TM alone which reduces growth to 50% of control, divided by the concentration of TM that inhibits growth by 50% when coincubated with a fixed concentration of PADPRP inhibitor. The enhancement factor was calculated using the IC_{50} value of TM alone of 361μM (averaged from 14 individual experiments).

An ~100 and ~30 fold decrease in the concentration of NU1025 was required to exhibit a corresponding cytostatic effect as observed for 3AB and BZ respectively, during a co-incubation with TM, e.g. 1mM 3AB and 10μM NU1025 both showed enhancement factors ~2. However, a 2-fold lower concentration of PD 128763 (50μM, EF = ~6.7) gave an an almost 2-fold higher enhancement as compared to NU1025 (100μM, EF = ~4.1).

TABLE 3.4

INHIBITOR	CONCENTRATION	IC_{50}μM ± SE	ENHANCEMENT FACTOR
3AB	1mM*	177.6 ± 3.9	~2
	5mM	147.4 ± 5.2	~2.5
BZ	0.3mM#	250.1	~1.4
	3mM	114.6 ±21.3	~3.1
NU1025	10μM*	223.2 ± 5.5	~1.6
	100μM	87.9 ± 9.1	~4.1
PD 128763	10μM	140.5 ± 3.2	~2.6
	50μM	53.6 ± 3.2	~6.7

Table 3.4: L1210 cells were incubated continuously for 48 hours with increasing concentrations of TM in the presence of fixed concentrations of the PADPRP inhibitors, and the level of growth as compared to control cell growth assessed. The IC_{50} values were calculated utilising the smooth curve analysis as described for Table 3.2. The values were

averaged from three independent experiments ± SE. unless otherwise indicated.

* IC_{50} value determined from two individual experiments. # Single experiment.

3.6.3 The cytotoxic effect of an increasing concentration of TM in the presence of fixed concentrations of the PADPRP inhibitors on L1210 cells.

Figure 3.7 shows that in the presence of fixed doses of all four of the PADPRP inhibitors, the dose-dependent cytotoxicity of TM was enhanced. The magnitude of the potentiation was observed to increase as the concentration of each inhibitor was raised. Both NU1025 and PD 128763 at concentrations of just 10μM exhibited significant potentiation of TM cytotoxicity, with concentrations of 50μM and 100μM showing a maximal effect (see Graphs C & D, Figure 3.7). In comparison, 1-5mM 3AB and 1-3mM BZ was required to potentiate TM cytotoxicity to a similar extent (see Graphs A and B, Figure 3.7).

Potentiation of TM cytotoxicity was quantified utilising dose enhancement factor values calculated at a survival of 10% (DEF_{10}). DEF_{10} values are defined as a ratio of the concentration of TM alone that reduces cell survival to 10%, divided by the dose of TM that exhibits 10% survival when co-incubated with a fixed dose of a PADPRP inhibitor. Table 3.5 shows the DEF_{10} values for each of the four inhibitors. As the concentration of the inhibitor increased, so too did the DEF_{10} values.

10μM NU1025 and PD 128763 resulted in similar DEF_{10} values of 2, but these concentrations were decreased 100 and 50-fold in comparison to the concentrations of 3AB and BZ required respectively for a similar DEF_{10}, e.g. 1mM for 3AB. This represents a similar concentration difference to that which was observed in the growth inhibition results (see Section 3.6.2). The DEF_{10} values therefore give an excellent measure of potency of the compounds against the activity of PADPRP, with the same order of potency as in the *in vitro* PADPRP activity (see Section 3.3) and growth inhibition studies (see Section 3.6.1 and 3.6.2).

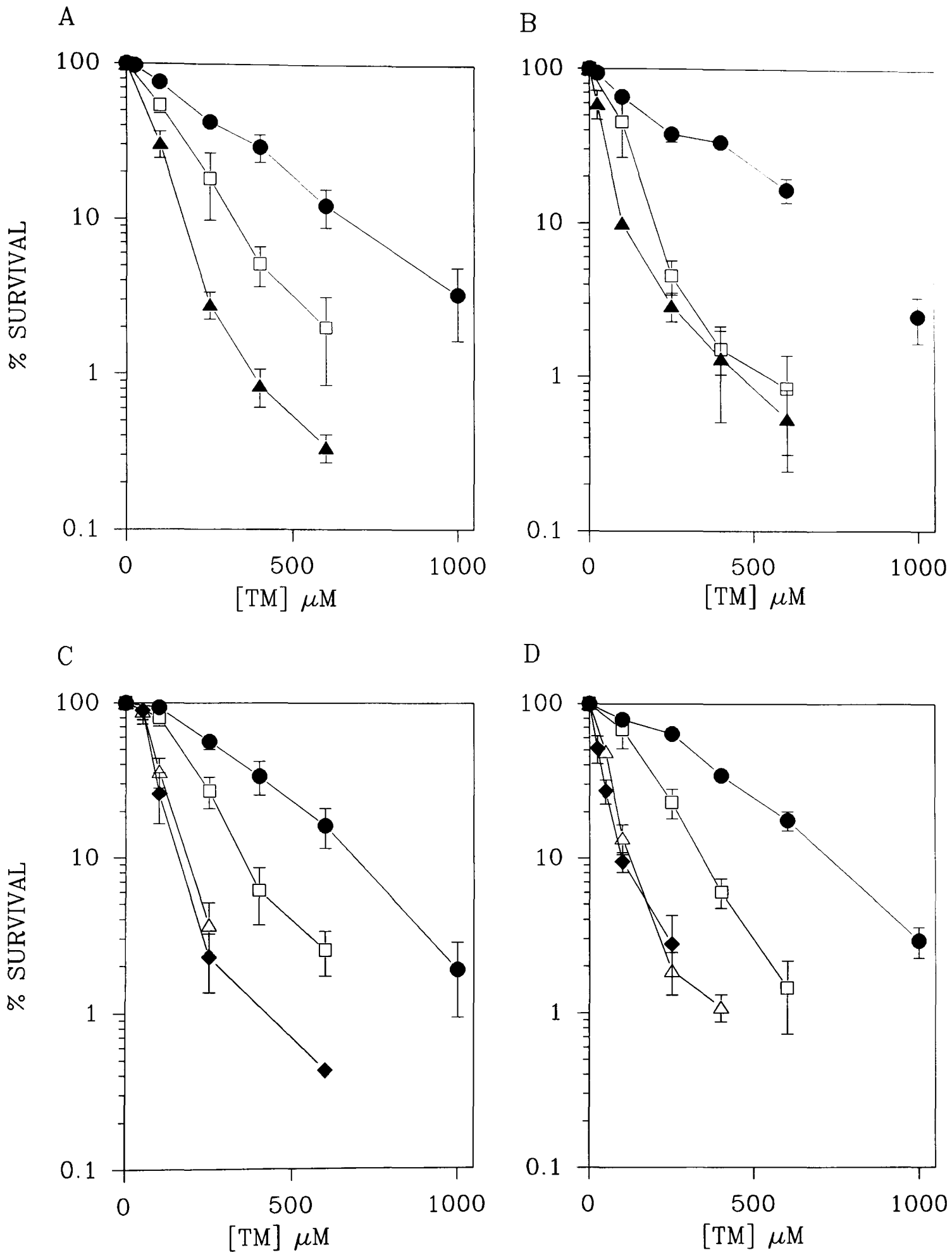


Figure 3.7: The cytotoxic effects of a 16 hour continuous exposure to increasing concentrations of TM alone ● ,or in the presence of a fixed concentration of PADPRP inhibitors. A. □ 1mM 3AB, ▲ 5mM 3AB. B. □ 1mM BZ, ▲ 3mM BZ. C. □ 10μM NU1025, △ 50μM NU1025, ◆ 100μM NU1025. D. □ 10μM PD 128763, △ 50μM PD 128763, ◆ 100μM PD 128763.

The points represent the mean of at least three independent experiments.

TABLE 3.5

INHIBITOR	CONCENTRATION	$DEF_{10} \pm SE$
3AB	1mM	2.4 ± 0.3
	5mM	4.1 ± 0.4
BZ	1mM	4.0 ± 0.7
	3mM	6.9 ± 0.2
NU1025	10μM	2.0 ± 0.2
	50μM	4.0 ± 0.5
	100μM	5.1 ± 0.7
PD 128763	10μM	2.0 ± 0.1
	50μM	6.0 ± 0.5
	100μM	7.1 ± 0.4

Table 3.5: L1210 cells were continuously exposed for 16 hours to increasing concentrations of TM in the presence of fixed concentrations of the PADPRP inhibitors, and cell survival was determined by colony formation. The DEF_{10} values were calculated utilising a smooth curve analysis as described in Table 3.2. Each value is calculated from 3 independent experiments.

3.7 CELLULAR NAD^+ STUDIES

3.7.1 The effect of TM on cellular NAD^+ levels

Some of the work described in this Section was carried out by S. Jones and J.K. Porteous, Cancer Research Unit, The University of Newcastle Upon Tyne, and has been included because of its relevance to the research presented in this thesis.

PADPRP is activated in response to DNA strand breaks with the resulting formation of the homopolymer, (ADP-ribose). Cellular NAD^+ is the substrate for PADPRP with the

ADP-ribose moiety utilised for the polymer formation. Therefore, the extent of depletion of the cellular NAD^+ pool following DNA damage gives an indication of PADPRP activation.

In the previous Sections, the cytostatic and cytotoxic effects of TM on L1210 cells was demonstrated. As the concentration of TM increased, growth was inhibited and cell survival decreased. TM related DNA damage activates cellular repair processes, with the consequential activation of PADPRP. The magnitude of activation can be indirectly determined by measuring cellular NAD^+ pool modifications. Hence, following a 4 hour incubation of L1210 cells in the presence of increasing concentrations of TM, the NAD^+ levels were recorded. Over this period, ~95% of the TM will have degraded to the active MTIC. Figure 3.8B shows that NAD^+ levels were depleted in a concentration dependent manner, and that a ~60% depletion was observed with a concentration of 2mM TM. However, this represented a ~10 fold higher TM concentration required in comparison to the doses of TM required to achieve significant growth inhibition e.g. IC_{50} = 361μM and cytotoxicity e.g. LD_{50} = 251μM (see Section 3.5).

An important determination was the effect of time on the depletion of the cellular NAD^+ levels following an incubation with TM. Previous data in the literature reports that, following administration of fixed doses of the mono-functional alkylating agents, MNU and DMS, there was a rapid depletion of the cellular NAD^+ within 2 hours. The extent of the depletion was concentration dependent. However, following a 14 hour incubation, control NAD^+ levels were recovered at each concentration, apart from those which reduced the NAD^+ to less than 10% of control (Skidmore *et al*, 1979; Durkacz *et al*, 1980). A possible explanation proposed is that in circumstances of extreme damage to the cell, the severe NAD^+ depletion would act as a suicide mechanism, arresting ATP synthesis and thus halting metabolic processes essential for the existence of the cell, e.g. DNA replication, glucose metabolism, (Sims *et al*, 1982,1983).

As the above agents act immediately, e.g. MNU has a $t_{1/2}$ of 10 minutes (Skidmore *et*

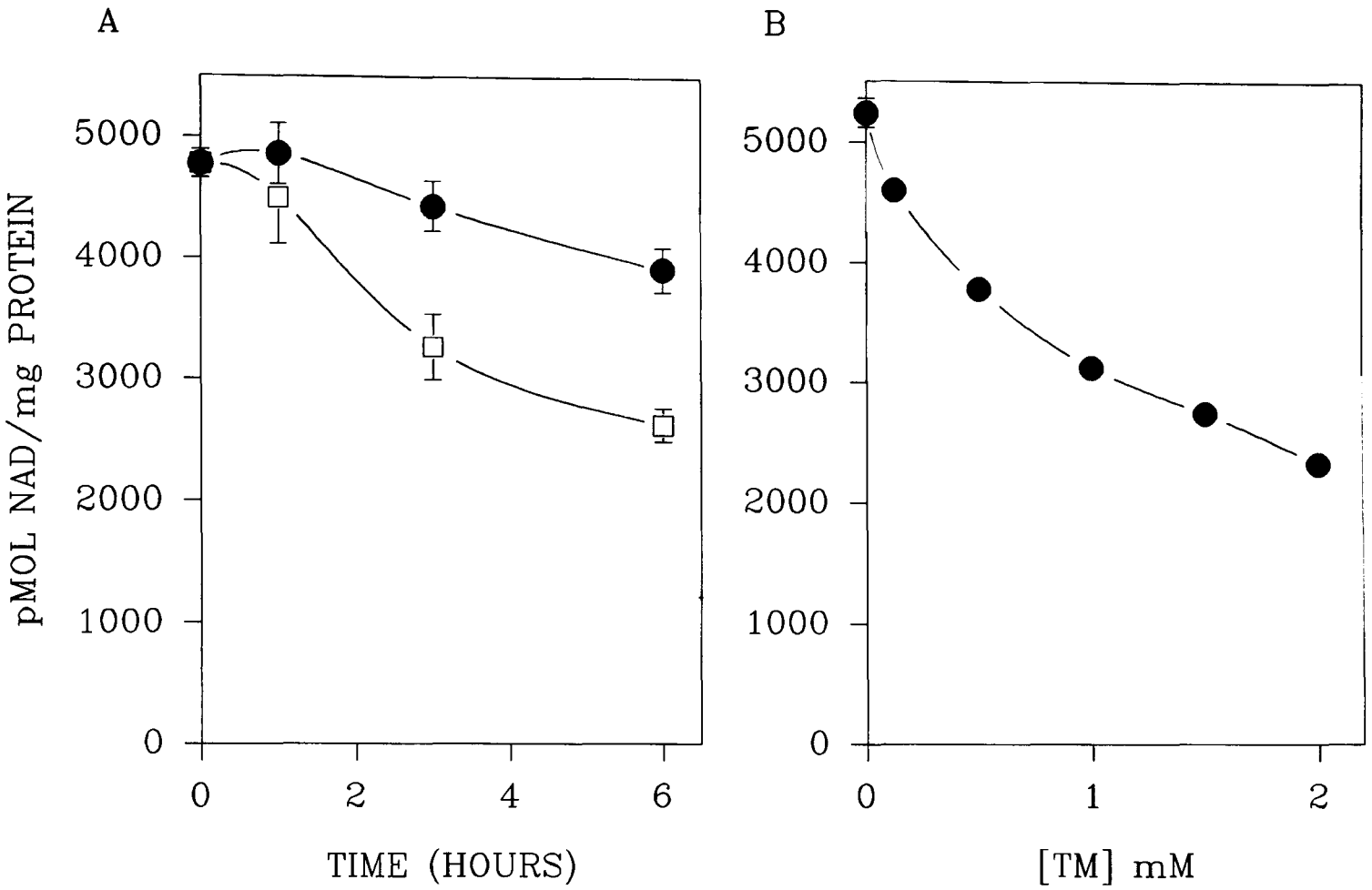


Figure 3.8: The effect of TM and the PADPRP inhibitors on the cellular NAD levels in L1210 cells. A. The effect of ● 1mM TM or □ 2mM TM over 6 hours. B. Cells were treated with an increasing concentration of TM for 4 hours. C. The effect of increasing doses of ◆ PD 128763 and △ 3AB on the NAD levels in cells treated with 2mM TM for a 4 hour incubation. The graphs are representative examples, with the points shown being the mean of triplicate samples, taken from a single experiment.

al, 1979), whereas TM has a prolonged active period of upto 6 hours, it was of interest to determine how the NAD^+ pool size varied with time after TM treatment. 1mM and 2mM TM, which reduced the cellular NAD^+ levels by ~40% and ~60% respectively following a 4 hour continuous incubation (see Figure 3.8B) were incubated for various times with the L1210 cells. The NAD^+ was depleted in a time dependent manner for both TM doses, with a 50% NAD^+ loss only observed following a 6 hour incubation with 2mM TM. However, this reduction remained concentration dependent (see Figure 3.8A).

The active metabolite of TM is MTIC, which has a half life of ~4 minutes (REF) and so represents the effect of a more direct acting DNA damaging agent. A comparison of the effect of MTIC on NAD^+ levels was therefore carried out. Initially, the effect of an increasing concentration of MTIC on the growth of the L1210 cells was assessed to determine a suitable concentration range for use in an NAD^+ study. Figure 3.9 shows that after a continuous 48 hour exposure to concentrations of MTIC between 0-1000μM, L1210 cell growth was inhibited in a concentration dependent manner, with an IC_{50} of 154 ± 2μM.

The intracellular NAD^+ level of L1210 cells treated for either a 2 or 4 hour continuous exposure to MTIC were depleted in a concentration dependent manner with a 50% depletion of the NAD^+ observed after just a 2 hour incubation with 1mM MTIC (5178pMoles NAD^+/mg protein depleted to 2496pMoles NAD^+/mg protein) (see Figure 3.10A). However, Figure 3.10A also shows that after a 4 hour exposure to increasing concentrations of MTIC the NAD^+ levels were beginning to recover, with NAD^+ levels being higher than the 2 hour levels over the entire concentration range of MTIC.

To investigate further the effect of time on the MTIC induced NAD^+ depletion, L1210 cells were incubated with 1mM MTIC for increasing incubation periods of up to 6 hours. Figure 3.10B shows that after just a 30 minute incubation, the NAD^+ was maximally depleted to ~60% of control, and that over the subsequent incubation times of 1-6 hours the NAD^+ levels were recovered in a time-dependent manner. By 6 hours the intracellular

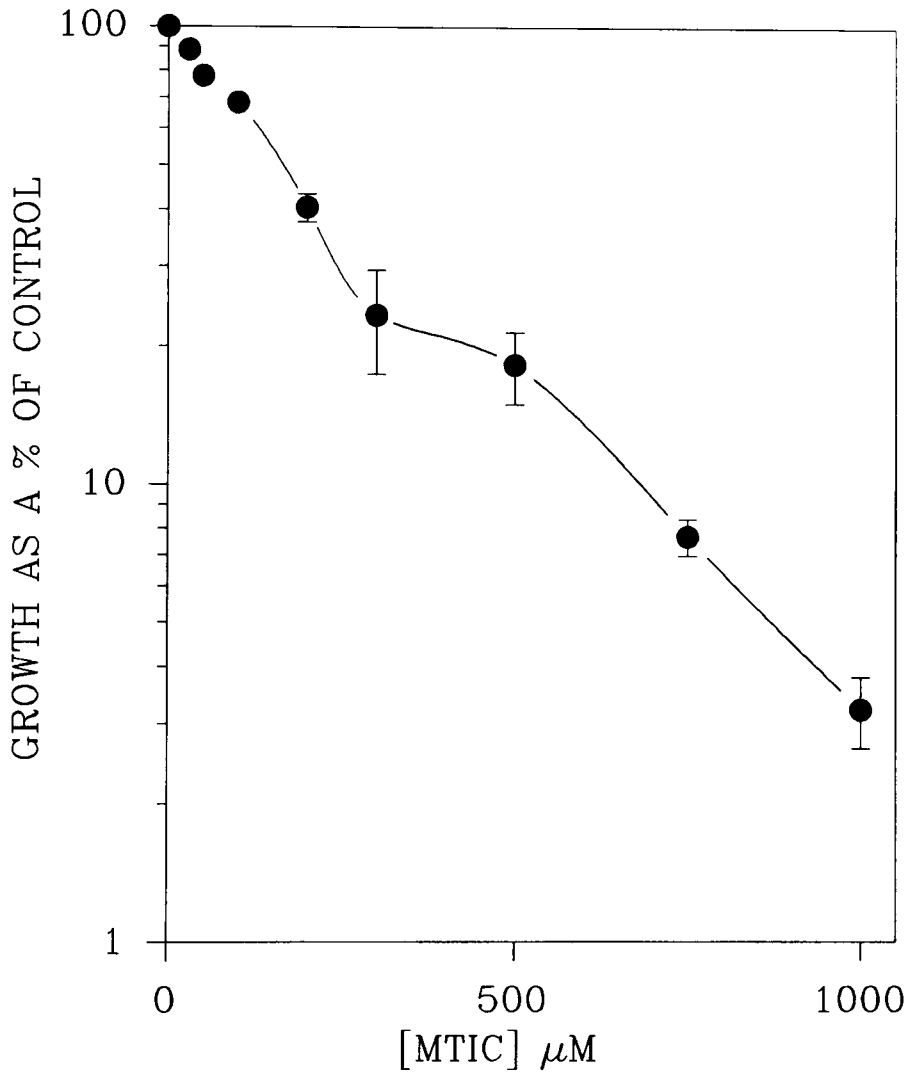


Figure 3.9: Growth inhibitory effects of a 48 hour continuous exposure of L1210 cells to increasing concentrations of MTIC. The points represent the average of quadruplicate samples, taken from two experiments.

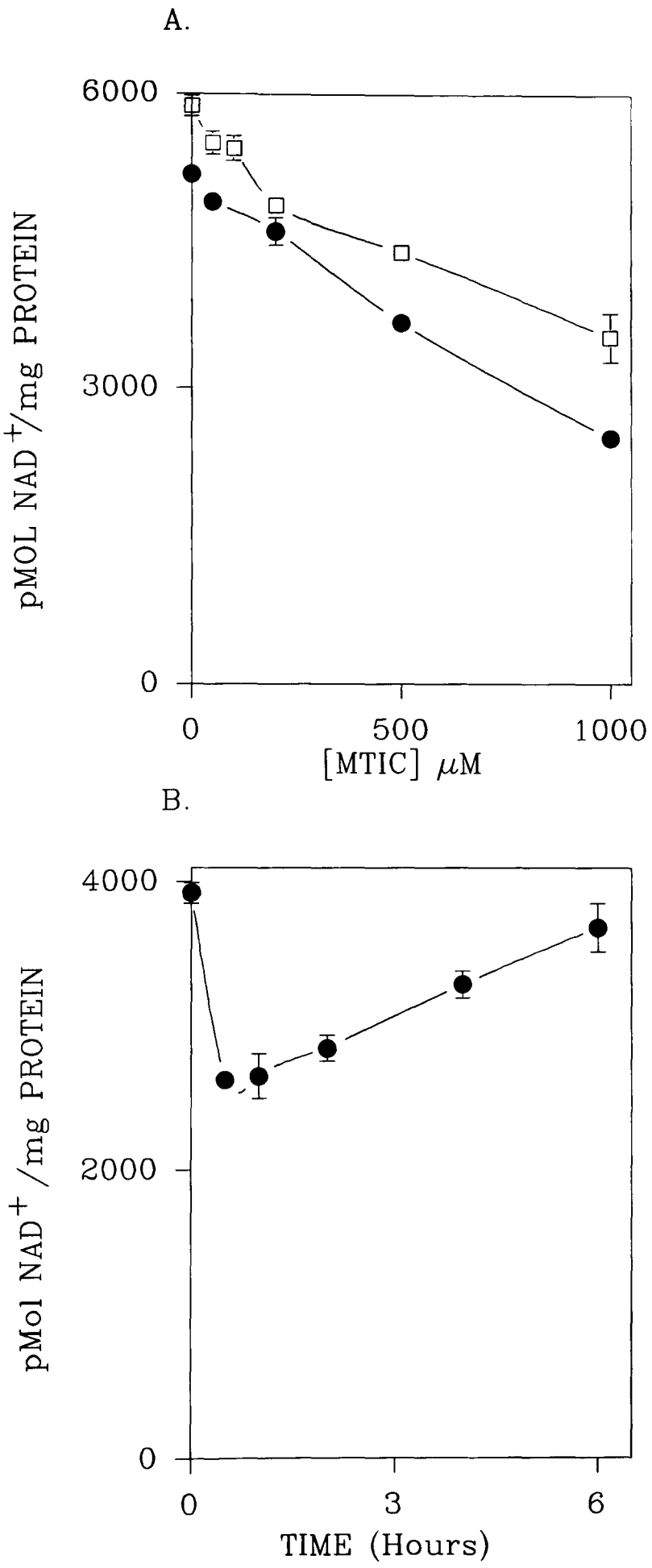


Figure 3.10:Effect of MTIC on the intracellular NAD^+ concentration of L1210 cells. A. The effect of increasing concentrations of MTIC over a ● 2 hour or □ 4 hour continuous exposure.

B. The effect of an increasing exposure time to 1mM MTIC. The points represent the mean of triplicate samples from one experiment.

NAD^+ level had almost returned to control values.

3.7.2 The effect of a co-exposure to TM and a PADPRP inhibitor on the cellular NAD^+ pool in L1210 cells

The prevention of NAD^+ depletion by inhibitors of PADPRP provides evidence that the decrease in intracellular NAD^+ is due to increased PADPRP activity. Previous publications have shown, that prevention of the NAD^+ depletion was dependent on the concentration of the damaging agent, and both the potency and concentration of the inhibitor (Skidmore *et al*, 1979; James & Lehmann, 1985). Therefore, in view of the enhanced cytostatic effects and cytotoxicity of TM in the presence of NU1025 and PD 128763 as compared to 3AB and BZ, the ability of the novel inhibitor PD 128763, compared to 3AB, to prevent the NAD^+ depletion was investigated. L1210 cells were incubated for 4 hours in the presence of 2mM TM in the presence of increasing concentrations of either 3AB or PD 128763, representative examples of a classical and a novel inhibitor. Both inhibitors abrogated the depletion of NAD^+ in a concentration dependent manner as shown in Figure 3.8C. However, whilst a concentration of ~100μM PD 128763 completely prevented the NAD^+ depletion, a concentration of ~1mM was required by 3AB to emulate this effect.

3.8 DISCUSSION

Overall, the results presented in this Chapter clearly demonstrate that the two novel compounds, NU1025 developed by the Drug Development Initiative at this University, and PD 128763 formulated by Leopold and colleagues at the Parke-Davis Division of the Warner Lambert Pharmaceutical Company, act as potent inhibitors of the enzyme, PADPRP, both in *in vitro* and in intact cells. When compared in an *in vitro* PADPRP assay to two of the classically used PADPRP inhibitors, 3AB and BZ, a ~50-fold increase in potency was observed. The ability of the compounds to retain inhibitory activity on

transfer from an *in vitro* situation to the intact cell was of extreme importance, in view of their anticipated use as resistance modifying agents in the clinic.

A major criterion for a compound to act as a resistance modifer requires that inherent cellular toxicity is low. Therefore, the effect of the PADPRP inhibitors *per se* was determined utilising both growth inhibition and cytotoxicity studies (see Section 3.4). Each of the inhibitors reduced cell growth and cell survival in a concentration dependent manner. However, in both studies, the concentrations of NU1025 and PD 128763 required to reduce the cell growth and cell survival by 50% were ~15-fold and ~5-fold lower than required by 3AB and BZ. These data correlated to some extent with the relative potencies of the four inhibitors against *in vitro* PADPRP activity (see Table 3.1 and Table 3.2).

Cytostasis, i.e. growth inhibition following administration of PADPRP inhibitors, has been reported to result in a G1/S phase cell cycle arrest (Schwartz *et al*, 1983; Moses *et al*, 1988). One of the roles proposed for PADPRP is as an early event in DNA replication. The synthesis of the (ADP-ribose) polymer was suggested to relax the chromatin structure thereby facilitating the access of replisomes to DNA. The inhibition of this process could possibly retard replication, and subsequently be manifested as a growth delay of the cell cycle. However, evidence suggested this cell cycle delay to be a transient effect, with the eventual resumption of normal growth patterns (Kidwell *et al*, 1982). If so, the survival of cells treated with the PADPRP inhibitors should not be reduced.

Previous data in the literature found no evidence to imply inhibition of endogenous PADPRP activity as responsible for cytostatic effects. Hunting *et al* utilised a growth inhibition analysis to compare the cellular effects of several PADPRP inhibitors. They found no correlation between the extent of delayed growth and the relative potencies of the compounds as inhibitors of PADPRP, e.g. at concentrations exhibiting comparable potencies against PADPRP in L1210 cells, 1mM 3AAB was not growth inhibitory, 5mM 3AB showed a slight inhibition of growth (80% of control), but 5mM 3meBZ was

extremely growth inhibitory (10% of control) (Hunting *et al*, 1985). Recently, the existence of a knock-out$_{PADPRP}$ mouse has shown PADPRP to be nonessential for cell survival and proliferation (Heller *et al*, 1995). The results presented in this Chapter would also support a nonessential role for PADPRP due to the lack of cytotoxicity at the concentrations which result in a cytostatic effect, e.g. at 0.5mM NU1025, growth was inhibited by 60% of control (see Figure 3.3), but 100% cell survival was observed following a 24 hour exposure (see Figure 3.2).

The work of Cleaver and colleagues proposed that PADPRP inhibitors affected other metabolic reactions. Perturbations of glucose and *de novo* purine & pyrimidine biosynthesis became apparent following the administration of high concentrations of the PADPRP inhibitors, e.g. 10mM 3AB (Milam and Cleaver, 1984). The data presented shows a cytotoxic effect of the inhibitors only at the higher concentrations, e.g. 20mM 3AB and 2mM PD 128763 as compared to the concentrations used in the potentiation of cytotoxicity studies, e.g. 5mM 3AB and 50μM PD 128763. The cytotoxic effects could be due to the secondary effects of the PADPRP inhibitors on other metabolic processes as described above, and are unlikely to be due to inhibition of PADPRP. Lautier *et al* (1994) found ADP-ribose polymer synthesis was inhibited by ~90% in intact cells following treatment with the oxidising agent xanthine-xanthine oxidase, in the presence of 100μM BZ. Therefore, the high PADPRP inhibitor concentrations required to effect cell kill, e.g. 10mM BZ, would be highly unlikely to be because of PADPRP targetting.

The ability of the four compounds to potentiate TM induced growth inhibition and cytotoxicity was used as a measure of inhibitor potency. The effect TM alone had on the growth and survival of the L1210 cells was initially determined. Different periods of exposure were utilised in the two assays, i.e. 48 and 16 hour continuous exposures were used in the growth inhibition and cell survival assays respectively, but very similar IC_{50} and LD_{50} values were calculated, 361 ± 25μM and 251 ± 13μM respectively. The half life of TM *in vitro*, in human plasma (Stevens *et al*, 1987) and in murine lymphoma cells

(Tsang *et al*, 1991) was determined as ~40 minutes and ~50 minutes respectively. TM is therefore a relatively slow acting agent with damage induced over an extended period of time. It can be calculated using the murine $t_{1/2}$ of 50 minutes that a period of 6 hours is required for 99% of the TM to have degraded to MTIC. The MTIC subsequently decomposes to the methyldiazonium ion with a half life of ~4 minutes. The different incubation times of 48 hours and 16 hours used for the growth inhibition and survival protocols, respectively would both encompass the complete period of TM decomposition, and therefore the similarity between the IC_{50} and LD_{50} values is not surprising.

Both L1210 cell growth and survival was decreased in a concentration dependent manner in the presence of TM. DNA damage results in a transient cessation of DNA synthesis due to the cell cycle arrest at G_1/S and G_2/M boundaries, which consequently retards the growth of the cell (Little, 1968, Kastan *et al*, 1992). With minimal damage to the DNA, e.g. at low concentrations of TM, the efficient repair processes of the cell would rapidly repair the lesions with the subsequent continuation of cell growth. However, base methylation encompasses a broad spectrum of lesions with reportedly different $t_{1/2}$ repair rates. The efficient activity of 3meA glycosylase ensures the fast excision of this modified base (half-life ~3-8 hours), whereas 7meG although actively removed has a half life of ~40 hours. As 7meG basepairs normally with cytosine, it is not considered to be a cytotoxic lesion. However, 3meA is considered lethal to the cell as it blocks DNA replication. Hence, the persistence of such lesions could result in an increased growth delay with eventual cell death. The lesion initially considered as lethal was O^6meG. O^6meG is removed by MGMT but the levels of this enzyme vary between different cells. L1210 cells have little or no MGMT present (unpublished result - C.E. Arris, Cancer research Unit, The University of Newcastle-Upon-Tyne) and this is demonstrated by the increased sensitivity of L1210 cells to TM as compared to other cell lines (Hepburn & Tisdale, 1991). O^6meG also forms basepairs in the DNA, but lethality occurs due to its removal via the mismatch repair process (Karran & Bigami, 1992). The position of the cell in the cycle at the time of damage would also be of importance, as once a cell is

committed to divide it must complete the cycle. In such cases, a damaged template would be replicated during S phase. With the subsequent increase in the TM concentration, the number of modified bases would be extensively raised, saturating the repair mechanisms, resulting in extensive growth delays and intensifying the cytotoxic effects.

The effect of increasing concentrations of the PADPRP inhibitors when coincubated with a fixed concentration of TM (100μM) on the effect on L1210 cell growth was determined. Each inhibitor potentiated the growth inhibitory effects of the TM in a concentration dependent manner. However, the concentrations of NU1025 and PD 128763 required to potentiate growth inhibition to a similar level as 3AB were reduced ~60 and ~110 fold. This gave the same order of relative potency as was observed for the *in vitro* PADPRP inhibition study (see Section 3.3). The PADPRP inhibitors, 3AB and BZ have been shown to inhibit PADPRP competitively with k_i values of 2.6μM and 1.0μM respectively (Purnell *et al*, 1985). The novel compounds, NU1025 and PD 128763, were also shown to inhibit PADPRP in a competitive manner (unpublished results, J.K. Porteous, Cancer Research Unit, The University of Newcastle Upon Tyne). These experiments therefore provide an indirect measure of the potential of these compounds to inhibit PADPRP in intact cells. The induction of DNA damage resulting from an incubation of TM will mobilise cellular repair mechanisms. Excision of the damaged bases creates breaks in the DNA which is the stimulus for PADPRP activation. Inhibition of PADPRP is quantitated in these experiments by the potency of the inhibitors as potentiators of TM induced growth inhibition.

Growth inhibition experiments were again utilised to determine the effect fixed concentrations of the PADPRP inhibitors had on an increasing concentration of TM. Each inhibitor potentiated the growth inhibitory effects of TM in a concentration dependent manner. However, the relative abilities of BZ, NU1025 and PD 128763 to potentiate the growth inhibitory activity of TM in relation to 3AB were ~2, ~80 and ~130 respectively. Again, these data correlate extremely well with the ability of the compounds to inhibit

in vitro PADPRP activity (see Section 3.3), where the relative potencies of BZ, NU1025 and PD 128763 in relation to 3AB, were ~1, ~43, and ~53 respectively (see Table 3.1). Again, the process of repair induced by the TM related DNA damage will be impaired in the presence of the PADPRP inhibitors. A number of studies have shown that the inhibition of PADPRP in the presence of DNA damage results in a prolonged G_2 phase arrest (Das *et al* 1984, Boorstein & Pardee 1984, Jacobson *et al* 1985a). Thus, growth inhibition mediated by PADPRP inhibitors in the presence of TM presumably reflects inhibition of DNA repair and extended growth arrest.

Cytotoxicity of TM was also potentiated by fixed concentrations of the PADPRP inhibitors in a concentration-dependent manner. The relative potencies of the inhibitors in relation to 3AB were ~5, ~100 and ~140 for BZ, NU1025 and PD 128763 respectively. These again exhibit a reasonable correlation with the relative potencies calculated in the *in vitro* PADPRP inhibition assay (see Section 3.3). The results calculated for PD 128763 are also in excellent agreement with those obtained by Sebolt-Leopold and colleagues. Utilising the monofunctional alkylating agent, streptozotocin in conjunction with 500μM PD 128763 they observed a 7 fold increase in the cytotoxicity (Sebolt-Leopold & Scavone 1992). However, it appears that they vastly underestimated the inhibitory potential of their novel compound. The present study indicates 50-100μM PD 128763 in the presence of TM results in the maximum decline of cell survival, and that at a dose of 10μM, a significant level of potentiation is recorded. However, dissimilar concentration dependent effects of the PADPRP inhibitors may arise with the use of different damaging agents, and different cell lines (For example, cell lines with lower NAD^+ concentrations would be predicted to be sensitive to lower concentrations of PADPRP inhibitor, because of their competitive nature (see Chapter 6)).

For a compound to act as an effective resistance modifier it must potentiate the cytotoxicity of the damaging agent with little or no inherent toxicity of its own. 5mM 3AB gave a DEF_{10} of 4 whereas a maximum enhancement value of 7 was observed

using 100μM PD 128763. In comparison, toxicity due to a 24 hour exposure to 5mM 3AB alone reduced the cell survival by ~8% whereas at 10mM 3AB, survival was reduced by 34% (see Figure 3.2). Similarly, although maximum potentiation of TM cytotoxicity was apparent at 3mM BZ, alone this reduced cell survival by 34%. However, the concentrations of NU1025 and PD 128763 which resulted in a maximum enhancement of TM cytotoxicity, i.e. 50-100μM, (see Figure 3.7) showed a negligible effect, i.e. <5% reduction in cell survival after a 24 hour incubation (see Figure 3.2). Therefore, NU1025 and PD 128763, exhibited increased potency towards PADPRP, with less toxic effect *per se* compared to 3AB and BZ, and are thus potential candidates for resistance modifying agents.

The depletion of NAD^+ associated with PADPRP activation is utilised as an indirect measure of the enzyme activity. Therefore, the effect increasing concentrations of TM had on the intracellular NAD^+ concentration was determined. The NAD^+ was found to be depleted in a concentration and time dependent manner, with a 50% NAD^+ depletion observed at a concentration of 2mM TM following a 6 hour incubation. However, this was an ~10-fold greater TM concentration than that required to achieve significant growth inhibition and cytotoxic effects. Therefore, hyperlethal concentrations of TM must be utilised to obtain a measurable level of NAD^+ depletion. As the induction of TM related DNA damage occurs over a 6 hour period, so the break formation due to the action of constitutive repair enzymes, i.e. AP endonucleases, will be prolonged, rather than the immediate saturation of breaks characteristic of the more direct acting agents. A continuous, but steady depletion of the cellular NAD^+ might therefore be expected as opposed to the massive decline following administration of agents such as DMS and MNU. This theory was illustrated by a direct incubation with MTIC, the active metabolite of TM. An ~50% depletion of NAD^+ was achieved using 1mM MTIC following just a 1 hour incubation, and by 6 hours the NAD^+ levels had almost reattained those of control (see Figure 3.8A & B). Unfortunately the TM data only covers a 6 hour period, therefore

it would be of interest to follow the NAD^+ effects at increased times to determine whether the control levels are also reattained over a prolonged incubation. The ability of inhibitors of PADPRP to abrogate the NAD^+ depletion provides further evidence that PADPRP is responsible for the decrease in NAD^+. Both the classical and the novel PADPRP inhibitors, 3AB and PD 128763 prevented the NAD^+ depletion in a concentration dependent manner, as had been previously shown by Skidmore *et al* (1979) and James & Lehmann (1985). However, the concentration of PD 128763 required to completely prevent the NAD^+ depletion was ~10 fold less than that of 3AB. This again shows the increased potential of the novel inhibitor, PD 128763 to inhibit PADPRP, with the concentrations required to abolish NAD^+ depletion analogous to those required to potentiate growth inhibition and cytotoxicity in TM treated cells. It should be noted that there was apparently only an ~10 fold difference between the concentrations of 3AB and PD 128763 required to abrogate the NAD^+ depletion, whereas 50-100 fold differences in concentration were observed in the growth inhibition and cytotoxicity studies. However, these concentration dependent effects spanned only a 2 fold depletion in NAD^+ levels, and hence cannot be expected to show accurate concentration differences.

Overall, a comparable level of enhanced potency was recorded for both NU1025 and PD 128763 in each of the biological endpoints chosen, with the relative potencies of the four inhibitors correlating well with *in vitro* PADPRP inhibition. However, the concentration ranges required to exert significant biological effects for each of the four PADPRP inhibitors in the *in vivo* studies were found to differ ~25-200 fold, in comparison to the IC_{50} concentrations determined in the *in vitro* assays. In the intact cell, the influx and efflux rate of the inhibitor, its possible metabolism once inside the cell, and reactions with acceptors other than PADPRP must be accounted for. Differences in the intracellular NAD^+ concentration between the *in vitro* and the intact cell studies could also account for discrepencies in IC_{50} values. As the inhibitors act in a competitive manner, a higher NAD^+ concentration, e.g. 500-1000μM, would require increased concentrations of the

inhibitors to effect a similar level of PADPRP inhibition as *in vitro* (e.g. a concentration of 75μM NAD^+ was used in the poly(ADP-ribose) polymerase assay). Given these limitations, overall, there was an excellent correlation of potency as PADPRP inhibitors *in vitro*, and potency as potentiators of growth inhibition and cytotoxicity following DNA damage in intact cells, both of which have been ascribed to PADPRP inhibition.

The potency of NU1025 and PD 128763 when co-incubated with the DNA damaging agent TM in growth inhibition and cytotoxicity studies was observed to be increased ~100-fold as compared to 3AB. There was a slight increase in the potency of PD 128763 as compared to NU1025 in intact cells, although the difference in potency in the *in vitro* assay against PADPRP was not significant (PD 128763 IC_{50} = 0.36μM ± 0.01μM; NU1025 IC_{50} = 0.44μM ± 0.13μM). However, both of the novel compounds, NU1025 and PD 128763, exhibit an excellent potential to become candidates as resistance modifing agents for *in vivo* studies, prior to entrance into clinical trials.

CHAPTER 4 : EFFECT OF THE PADPRP INHIBITORS ON TM INDUCED DNA STRAND BREAK LEVELS.

4.1 INTRODUCTION

Prior to 1966, attempts to quantify DNA strand break levels were unsuccessful, as shearing forces which occurred during DNA extraction introduced large numbers of artefactual DNA strand breaks. However, in 1966, McGrath & Williams introduced the technique "alkaline sucrose gradient analysis". Cells were lysed onto the top of the alkaline gradient prior to centrifugation, with the alkali causing the denaturation of DNA degradative enzymes and also effecting the release of the DNA in single strand form. The molecular weight of the DNA was proportional to the rate of sedimentation during centrifugation. Although the gentle lysis technique and use of centrifugal force minimised the shearing effect on the DNA, the technique proved insensitive to low levels of DNA damage. Alkaline elution, developed by Kohn *et al* (1981), exhibited a greater level of sensitivity and was more amenable to quantitative analysis compared to alkaline sucrose density gradients. It utilises filters that act as mechanical sieves to discriminate between different sized fragments of DNA. DNA is eluted in single strand form using a highly alkaline solution (i.e. pH 12.2), and the presence of single strand breaks is recorded as an increase in the rate of elution (see Materials and Methods for further details). The "nucleoid technique" also allowed for an increased level of sensitivity, but due to neutral conditions, differentiated between breaks formed as a result of enzymic incision, and those resulting from alkali labile sites (Cook & Brazell, 1975). Nucleoids form in the presence of high salt concentrations and nonionic detergents. They consist of the cellular DNA, present as large numbers of independent supercoiled loops, but lack most of the nuclear proteins. Upon introduction of a DNA strand break, supercoiling is relieved resulting in a slower rate of sedimentation. Hence, the loss and recovery of DNA supercoiling is followed by changes in the sedimentation rate through a neutral sucrose

gradient. Birnboin & Jevcak (1981) developed a modified "alkali induced DNA unwinding assay" which circumvented the requirement for the physical separation of single and double stranded DNA on hydroxyapatite columns. The dye, ethidium bromide, was utilised as a direct probe of the DNA structure, as it binds selectively to duplex double stranded regions contained within single stranded DNA stabilised by alkali treatment. Most recently, a "comet assay" was developed that analyses the effect of DNA strand breaks in a single cell utilising gel electrophoresis for separation. Only direct DNA strand breaks or those introduced via enzymatic action are measured (Ostling & Johannson, 1984).

Studies to assess the DNA strand break levels in cells cotreated with DNA damaging agents and PADPRP inhibitors have been performed by numerous groups utilising most of the techniques described above (e.g. Durkacz *et al*, 1980; Durkacz *et al*, 1981b; James & Lehmann, 1982; Cleaver *et al*, 1983; Huet & Laval, 1985; Cleaver *et al*, 1985; Cantoni *et al*, 1986; Hunting & Gowans, 1988; Lunn & Harris, 1988; Moses *et al*, 1988). However, most of the investigations used only a single, high dose of the chosen PADPRP inhibitor nearly always 3AB, e.g. 3-5mM, and concentrated on the effect this had on the level of DNA single strand breaks when used in conjunction with an increasing concentration of the damaging agent (see Chapter 1, Table 1.1 for examples). A couple of reports touched briefly on the effect increasing concentrations of 3AB had on DNA single strand break levels, when in the presence of a damaging agent. Cantoni *et al* (1986), in an experiment to identify the optimum dose of 3AB to utilise in their investigation into the effects of PADPRP inhibition on DNA strand break rejoining in H_2O_2 treated cells, showed 3AB (0.5-5mM) to inhibit the rejoining of H_2O_2 induced DNA strand breaks in a concentration dependent manner. In the presence of 0.5mM and 5mM 3AB, the relative number of H_2O_2 induced breaks rejoined during a 15 minute post incubation was 95% and 70% respectively. A more comprehensive study was reported by Cleaver *et al* (1985). They used a single dose of the alkylating agent, MMS, together

with a range of 3AB concentrations. The single strand break frequency was observed to actually decrease at the low concentrations of 3AB (0.05-1mM), with an increased frequency seen only at 2mM and above. However, in general the literature is completely lacking both a comprehensive and quantitative dose dependent analysis of the PADPRP inhibitors, compared to their inhibitory potency, on the DNA single strand break level in DNA damaged cells. In the previous Chapter, co incubation of the alkylating agent, TM together with PADPRP inhibitors resulted in the potentiation of TM induced cytotoxicity. Durkacz *et al* (1980), determined the effect of coincubating the monofunctional alkylating agent, DMS with 3AB on the level of DNA damage. Utilising alkaline sucrose density gradients, low molecular weight species of DNA persisted for up to 40 minutes after the removal of the DMS, with only intermediate molecular weight DNA recovered by 80 minutes, indicative of an impaired repair process. This increased break frequency, in the presence of PADPRP inhibition was correlated with the cocytotoxic effect. Coincubation of DMS with 3-5mM 3AB resulted in a 4 fold increase in cytotoxicity, with a significant increase in the DNA strand break levels over a 90 minute period (Durkacz *et al*, 1980; James & Lehmann, 1982). This correlation has been reproduced utilising a number of different damaging agents and inhibitors of PADPRP (see Chapter 1, Table 1.1). However, a coexposure to UV radiation in the presence of 3AB showed no effect on either the enhancement of cytotoxicity or the level of DNA single strand breaks (James & Lehmann, 1982; Cleaver *et al*, 1983), confirming the subsequent proposed involvement of PADPRP in BER as opposed to NER (Satoh *et al*, 1993).

4.2 AIMS

The aim of this second results Chapter was to determine whether the two novel compounds, NU1025 and PD 128763, previously observed to inhibit PADPRP *in vitro* with a ~60 fold increased level of potency as compared to 3AB and BZ, also resulted in an increased DNA single strand break level following treatment with a

chemotherapeutically relevant alkylating agent that promotes BER. If this were the case, did the concentrations required correlate with their increased potency as PADPRP inhibitors ? This was achieved by performing a quantitative evaluation of a range of PADPRP inhibitor concentrations required to increase the DNA single strand break levels, when used to treat the cells in the presence of a DNA damaging agent.

To allow a comparison of these results with the cytotoxicity data, TM was again utilised as the damaging agent. TM is degraded in biological milieu to the direct methylating agent, MTIC with the subsequent formation of the modified bases, 3meA, 7meG and O^6meG (for further details see Chapter 1, Section 1.4.3b) (Tisdale, 1988; Hepburn *et al*, 1991). The action of specific glycosylase enzymes removes the damaged bases to leave AP sites which are subsequently cleaved to create DNA single strand breaks. The technique of alkaline elution was used to quantify the level of single strand breaks (Kohn *et al*, 1981). However, the AP sites generated are extremely alkali labile and breaks formed at the AP sites are identified by a convex curvature of the elution curves, as compared to the near linear curves observed with X irradiation, and have a distinct dependence on the pH range (Kohn *et al*, 1981). This was taken into account when examining the alkaline elution profiles obtained following TM treatment.

4.3 CHOICE AND JUSTIFICATION OF INTERNAL STANDARD CONCENTRATION

Coelution of internal standard cells with the treated sample cells defines a corrected time scale for the assay (Kohn *et al*, 1981). This increases the precision of elution by reducing intersample variations (see Chapter 2, Section 2.12).

The elution of internal standard cells requires the introduction of defined levels of breaks into the DNA. X irradiation is the most widely employed method, with breaks introduced directly into the DNA strands. A suitable dose of X rays for use on the internal standard cells, and which was utilised in subsequent experiments, was determined. [^{14}C] Thd

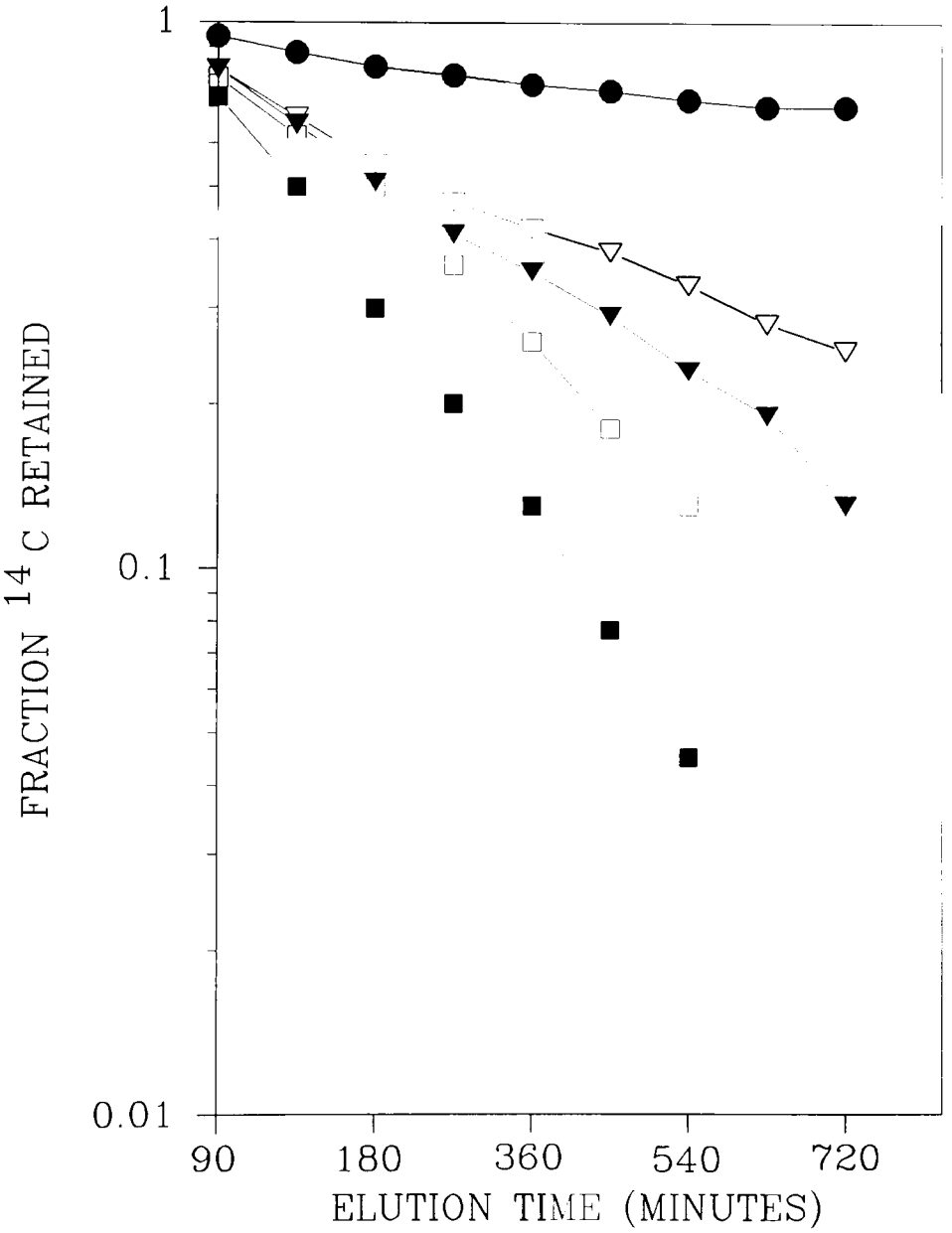


Figure 4.1: Representative alkaline elution profiles of X-irradiated cells.
● control, ▽ 100rads, ▼ 200rads,
□ 300rads, ■ 400rads.

labelled L1210 cells were exposed to increasing doses of X rays, prior to elution of DNA. The elution rates were plotted against time. Figure 4.1 shows the slopes of the elution profiles increased in a dose dependent manner. Each dose exhibited a linear elution profile. The X ray dose chosen to irradiate the internal sample cells was 300 rads, as the fraction of ^{14}C retained on the filter after completion of the elution was ~0.1.

4.4 THE EFFECT OF TM ON THE DNA STRAND BREAK FREQUENCY

Previously, TM was observed to increase the inhibition of L1210 cell growth as well as significantly reduce cell survival in a concentration dependent manner (see Chapter 3). The effect of the TM treatment on the level of single strand breaks was therefore determined, and the correlation with cytostatic and cytotoxic effects assessed.

Initially, the effect of two concentrations of TM on the DNA single strand break level, over a period of 24 hours was investigated. The two doses chosen, 200μM and 1000μM TM, reduced cell survival to approximately 50% and 1% respectively. The results presented in Figure 4.2 are in the "relative elution" (R.E.) format. R.E. is a ratio of, "the increase in the rate of DNA elution observed with the treated cells as compared to untreated controls, at the point when 50% of the internal standard DNA has eluted" (see Chapter 2, Section 2.12) (Fornace & Little, 1977). In the presence of both TM concentrations an increase in R.E. values was observed up to 4-6 hours, whereas over the subsequent incubation periods the R.E decreased, approaching control (undamaged) levels by 24 hours. This pattern was concentration dependent, with the effect produced by 1000μM TM ~3 fold greater than that observed for 200μM TM. The R.E. value obtained at any one time represents a balance between the level of single strand break formation and break religation. Over the initial 6 hours, the rate of single strand break formation would appear to be in excess over the rate of ligation. 1000μM TM treatment caused significantly more single strand breaks than was observed with 200μM TM, with the majority formed during the first two hours. The R.E., after an incubation of 1 hour was

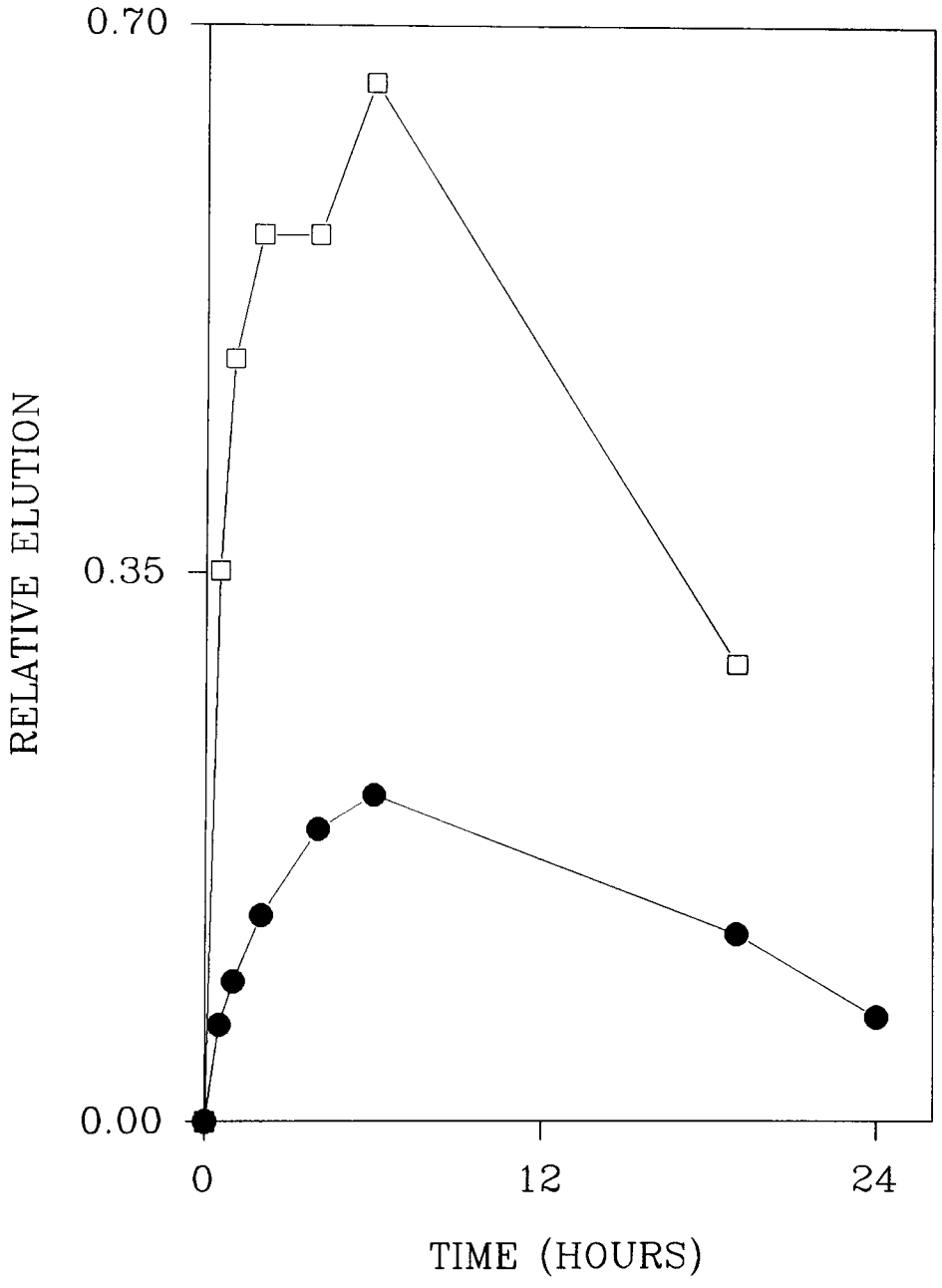


Figure 4.2: Relative elution plotted against time, of DNA from cells treated for increasing incubation periods with 200μM TM ● or 1000μM TM □ . The points were calculated from the data of a single experiment.

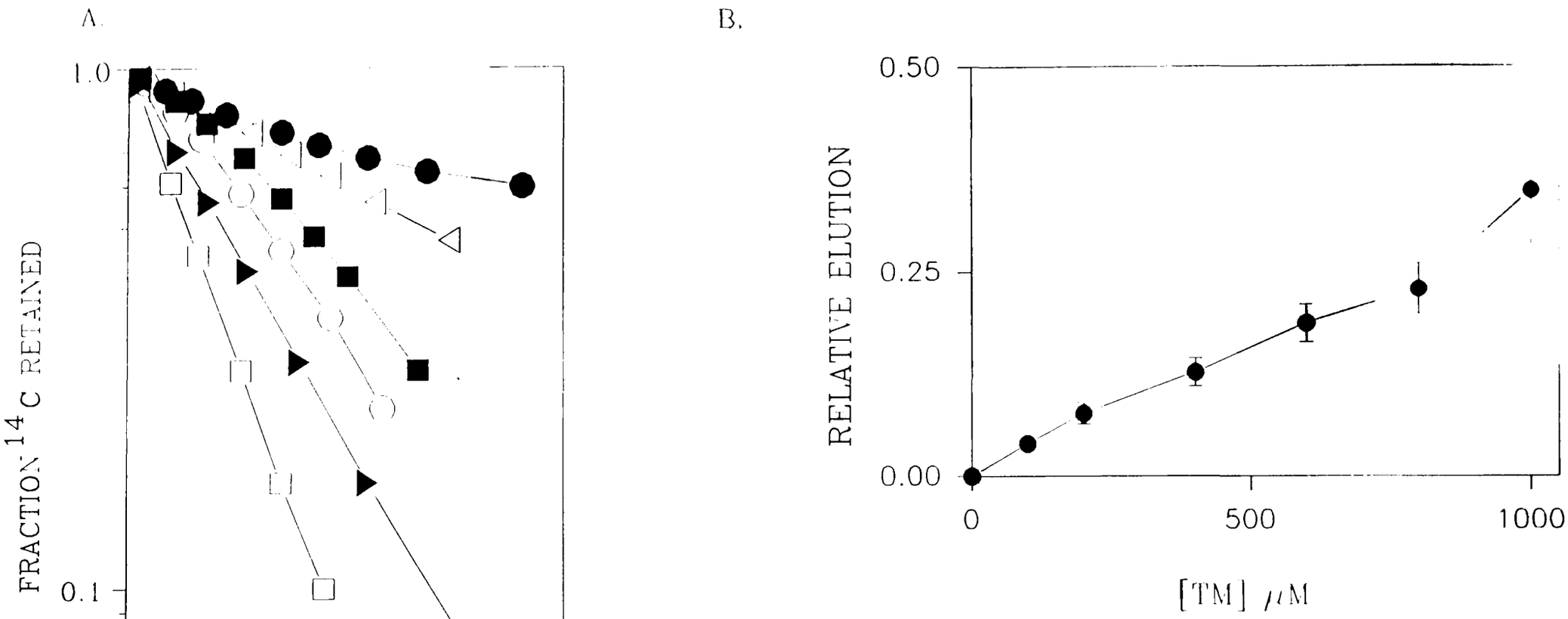


Figure 4.3: Effect of a 1 hour incubation with increasing concentrations of TM on the DNA single strand break levels in L1210 cells.
A. Alkaline elution profiles, ● control, ◁ 200μM TM, ■ 400μM TM, ○ 600μM TM, ▶ 800μM TM, □ 1000μM. B. Relative elution profile.

rapidly increasing in the presence of both 200μM and 1000μM TM (see Figure 4.2). Therefore, L1210 cells were incubated for 1 hour to determine the effect an increasing concentration of TM had on the frequency of single strand DNA breaks. Figure 4.3A shows the elution rates increased in a concentration dependent manner. At the lower TM concentrations, i.e. 200-400μM TM, the elution profiles appeared slightly convexed, suggestive of alkali labile sites produced by glycosylase action at sites of base methylation producing AP sites. However, as the dose increased, i.e. 800-1000μM TM, the profiles assumed linearity. The R.E. plot of the data is shown in Figure 4.3B, and demonstrates a near linear relationship between the dose of TM and the R.E. value. This suggests the damaging effect of the TM on the DNA is related to the formation of the single strand breaks.

4.5 THE EFFECT OF THE PADPRP INHIBITORS *PER SE* ON THE DNA STRAND BREAK FREQUENCY.

Previously, the four inhibitors of PADPRP, 3AB, BZ, NU1025 and PD 128763 were all reported to lack intrinsic toxicity at the doses utilised in the chemopotentiation experiments, e.g. 1-5mM 3AB and 10-100μM NU1025. Only as the concentrations were significantly raised did a cytotoxic effect become apparent, e.g. 10mM 3AB and 2mM NU1025 reduced survival by 34% and 55% respectively. Similarly, an important analysis prior to utilising TM in conjunction with the inhibitors, was to assess the effect of the inhibitors alone on DNA strand break frequency.

Following a 24 hour continuous exposure to high concentrations of the inhibitors, that had showed signs of cytostatic and cytotoxic effects, on the L1210 cells, i.e. 10mM 3AB, 10mM BZ, 1mM NU1025 and 1mM PD 128763, there was no observed increase in the frequency of single strand breaks in comparison to untreated control cells, as shown in Figure 4.4.

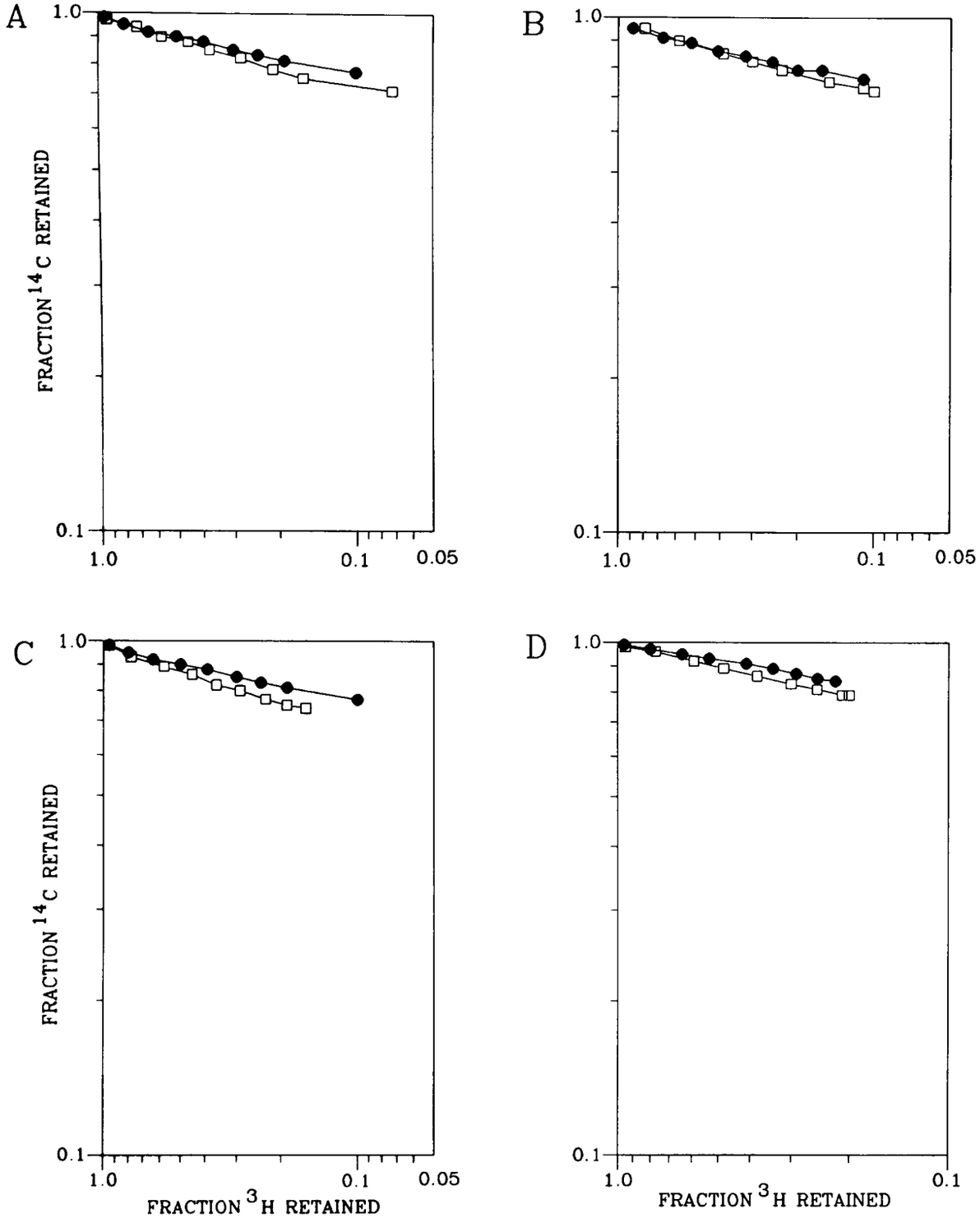


Figure 4.4: Alkaline elution profiles depicting the effect of a 24 hour continuous exposure of cells to PADPRP inhibitors *per se* on single strand break levels.
A. • control, ▫ 10mM 3AB. B. • control, ▫ 10mM BZ. C. • control, ▫ 1mM NU1025 D. • control, ▫ 1mM PD 128763.
The points were calculated from the data of a single experiment.

4.6 A COMPARISON OF THE ABILITY OF PADPRP INHIBITORS TO RETARD SINGLE STRAND BREAK REPAIR.

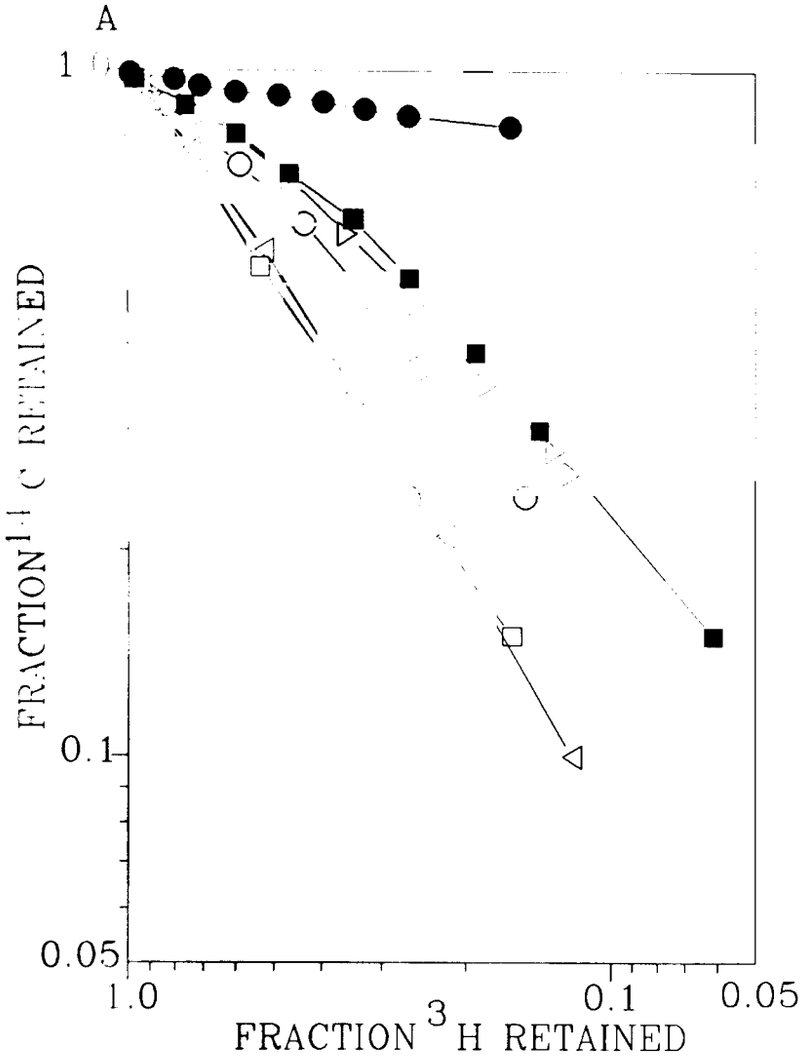
4.6.1 The effect of the PADPRP inhibitors on a fixed level of TM induced DNA damage.

The effect of the PADPRP inhibitors, 3AB, BZ, NU1025 and PD 128763 on the level of DNA single strand breaks following TM induced DNA damage was determined, and the results compared to their potency as PADPRP inhibitors. A quantitative evaluation of the DNA single strand break levels was performed over increasing concentrations of the inhibitors.

To allow an accurate comparison, the same starting level of TM induced DNA damage was required. Utilising the alkaline elution profile shown in Figure 4.3 a concentration of 150μM TM was chosen, as this itself resulted in a significant measurable level of single strand breaks, but allowed the possible potentiation effects of the PADPRP inhibitors on DNA single strand break levels to be observed.

L1210 cells were incubated for 1 hour with 150μM TM alone, or in the presence of increasing concentrations of the PADPRP inhibitors, prior to alkaline elution of the DNA. Figures 4.5 & 4.6 demonstrate a dose dependent increase in the rate of elution as the concentration of each of the PADPRP inhibitors was increased. Cantoni *et al* (1986) had previously observed a similar concentration dependent increase in single strand break frequency by 3AB in the presence of a fixed concentration of H_2O_2, as had Cleaver *et al* (1985) using the monofunctional alkylating agent, MMS.

The effect of the 1 hour incubation with TM alone gave rise to profiles with a slight convex nature, suggestive of the presence of alkali labile sites. In the presence of low doses of the inhibitors the convex shaped profiles were retained, whereas on attaining the higher inhibitor concentrations, the profiles became linear, e.g. 1mM and 3mM BZ produced convex curves, but at 10mM and 20mM BZ the profiles were linear (see Figure 4.5B). A comparison of the inhibitor concentrations that resulted in the production of



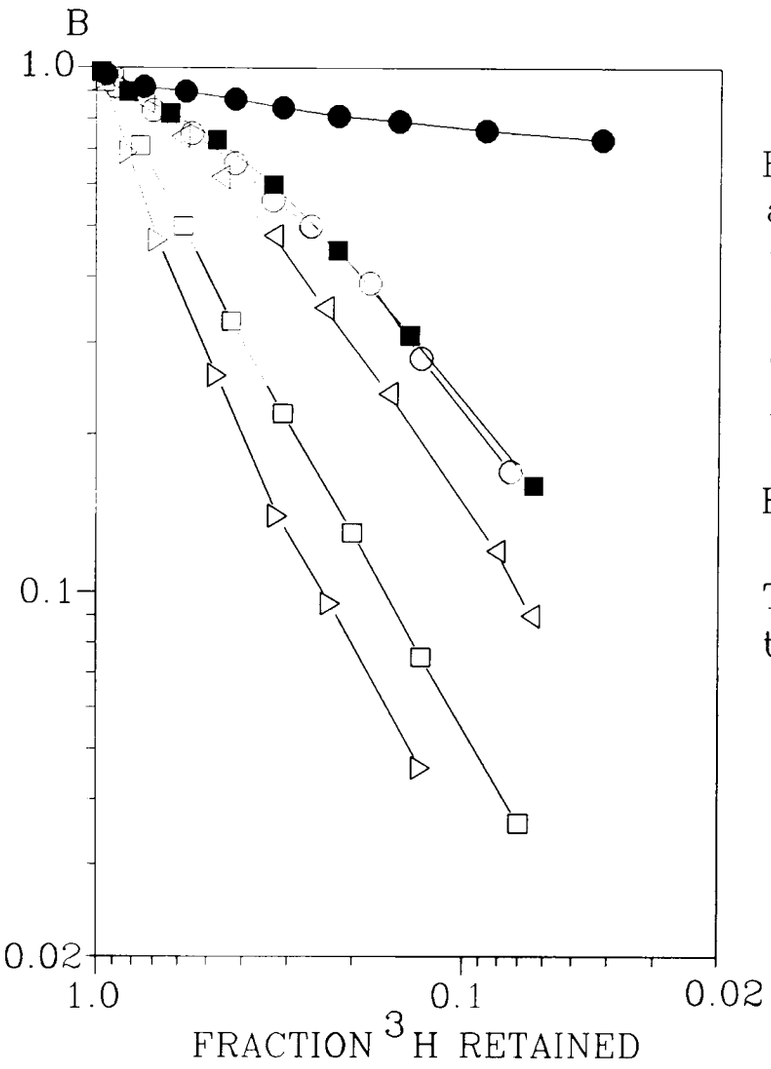


Figure 4.5: Representative alkaline elution profiles showing the effect of a 1 hour incubation of L1210 cells with 150μM TM alone ■ or in the presence of
A. ▷ 1mM 3AB, ○ 5mM 3AB, □ 10mM 3AB, ◁ 20mM 3AB.
B. ○ 1mM BZ, ◁ 3mM BZ, □ 10mM BZ, ▷ 20mM BZ.
The points were calculated from the data of a single experiment.

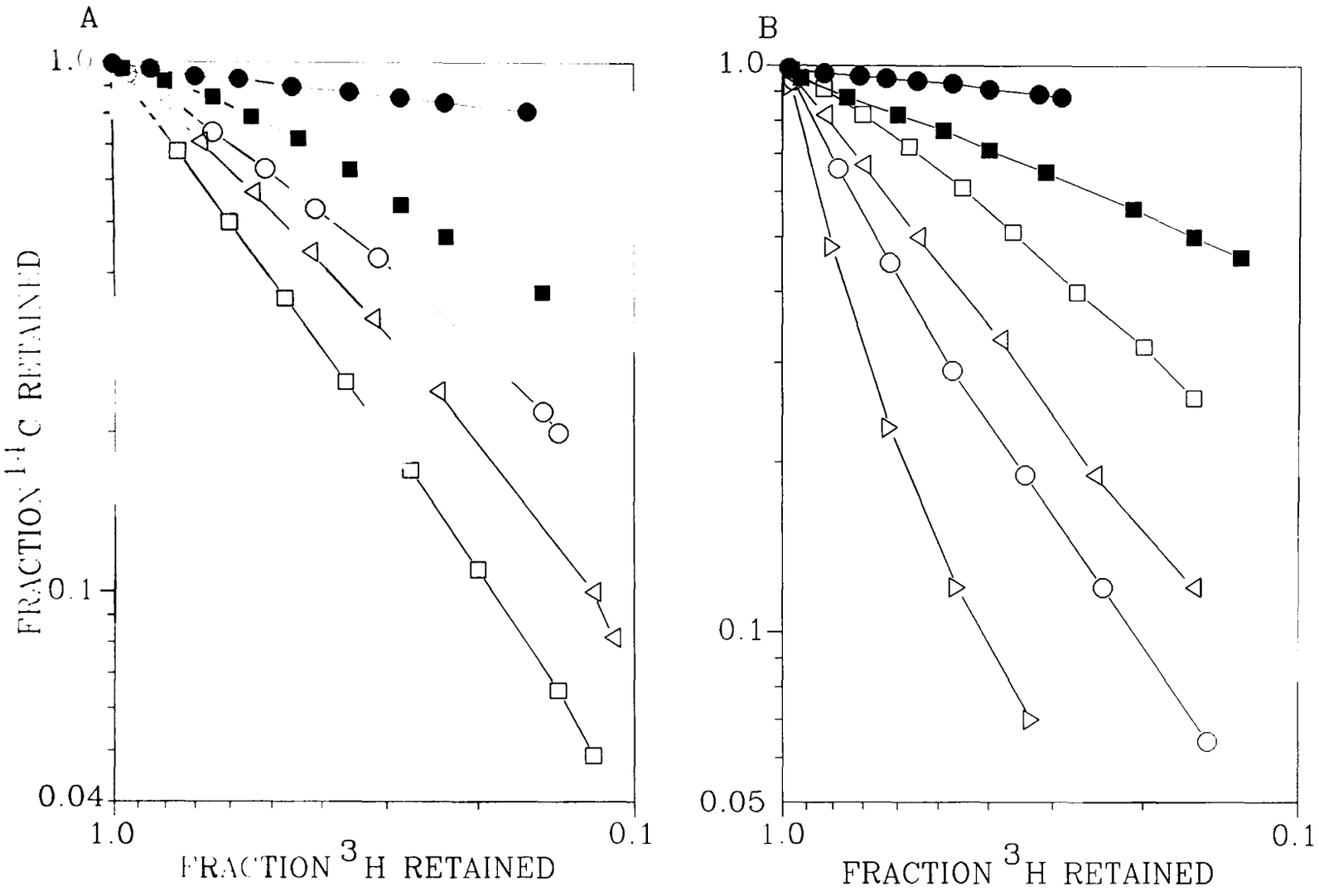


Figure 4.6: Representative alkaline elution profiles showing the effect of a 1 hour incubation of L1210 cells with 150μM TM alone ■ or in the presence of
A. ○ 0.3mM NU1025, ◁ 0.5mM NU1025, □ 1.0mM NU1025.
B. □ 0.1mM PD 128763, ◁ 0.3mM PD 128763, ○ 0.5mM PD 128763, ▷ 1.0mM PD 128763.
The points were calculated from the data of a single experiment.

linear profiles showed both 3AB and BZ required concentrations of 3-10mM whereas NU1025 and PD 128763 only needed 0.3-1mM.

The translation of the data presented in the profiles in Figures 4.5 & 4.6 to the R.E. values (see Figure 4.7) represents more clearly the effect the PADPRP inhibitor concentration had on DNA single strand break frequency. The R.E. data was calculated utilising the TM alone as the control, and therefore can be extrapolated back to zero, representing a zero inhibitor concentration. A large difference was observed between the concentrations of the two novel inhibitors and the "classical" PADPRP inhibitors, 3AB and BZ, required to enhance the single strand break level. An arbitrary R.E. value was selected of 0.125 as this gave readings on all four of the inhibitor curves, and the concentration of each inhibitor required to raise the level of single strand breaks to this degree was determined. The concentrations thus calculated are presented in Table 4.1:-

TABLE 4.1

INHIBITOR	$[INHIBITOR]_{0.125}$ (DNA strand breaks)	POTENCY AS COMPARED TO 3AB (DNA strand breaks)	POTENCY AS *IN VITRO* PADPRP INHIBITORS
3AB	12.0mM	1.0	1.0
BZ	5.3mM	~2	~2
NU1025	0.18mM	~70	~43
PD 128763	0.12mM	~100	~53

Table 4.1: An assessment of the PADPRP inhibitor concentrations required to increase the level of DNA single strand breaks, resulting from treatment with 150μM TM, by a similar magnitude. An arbitary R.E. value of 0.125 was utilised. The values were calculated from the averaged data presented in Figure 4.7.

As shown, ~70 & ~100 fold reductions in the concentrations of NU1025 and PD 128763 respectively were required to exhibit the equivalent single strand break frequency as 3AB. Only a ~2 fold difference was observed between the potencies of 3AB and BZ. These figures correlate well with the relative potencies obtained from the IC_{50} values calculated for PADPRP inhibition *in vitro,* also presented in Table 4.1 (see Chapter 3, Table 3.1). However, the inhibitor concentrations required to inhibit PADPRP *in vitro*, compared to those required to raise the DNA single strand break level to the R.E of 0.125 differ by several hundred fold. A concentration of 0.44μM NU1025 was sufficient to inhibit PADPRP activity *in vitro* by 50%, but a concentration of 200μM NU1025 was required before an effect on DNA single strand break levels was observed. However, a direct comparison between the intact cells and the permeabilised cell system is not possible, and the reasons for this will be discussed in Section 4.8.

4.6.2 The schedule dependency of the TM and PADPRP inhibitors.

The experiments described in the previous Subsection involved a 1 hour coincubation of the cells with a fixed dose of 150μM TM with increasing concentrations of PADPRP inhibitors. The results obtained demonstrated an increase in the level of single strand breaks. However, as TM and inhibitors were present simultaneously, an interaction of the two compounds, non-specific to PADPRP could be responsible for the observed effects. Therefore, an experiment was performed utilising NU1025 as the PADPRP inhibitor. One set of cells was treated simultaneously with TM and NU1025 for the 1 hour incubation as described previously. However, a second set of cells was incubated for 1 hour in the presence of 150μM TM, the TM was then removed, and the cells incubated for a further hour in fresh medium containing the increasing concentrations of NU1025. Figure 4.8 shows the results of the two dosing schedules on R.E. values. The R.E. profiles obtained for both the coincubation and pretreatment studies increased in a concentration dependent manner, confirming there to be no interaction between the TM and the PADPRP

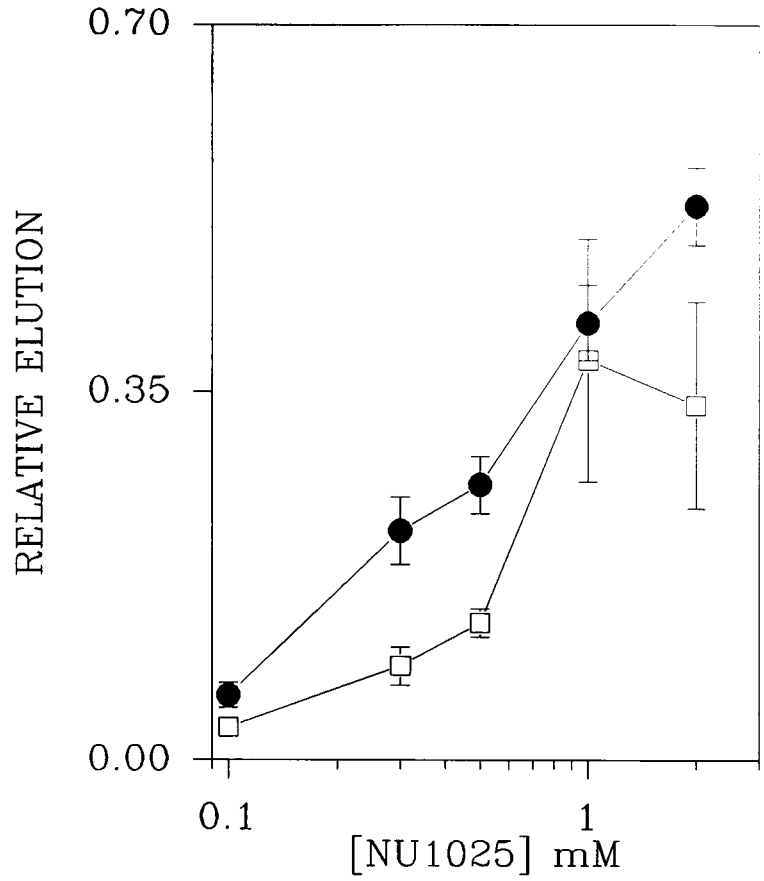


Figure 4.8: :The effect of schedule dependency of TM and NU1025 on DNA strand break levels. Relative elution values were compared from L1210 cells treated with ● a 1 hour coincubation with increasing concentrations of NU1025 + 150μM TM or □ a 1 hour incubation with 150μM TM alone, prior to a 1 hour incubation in fresh medium containing increasing concentrations of NU1025. Points were calculated from the data of a single experiment.

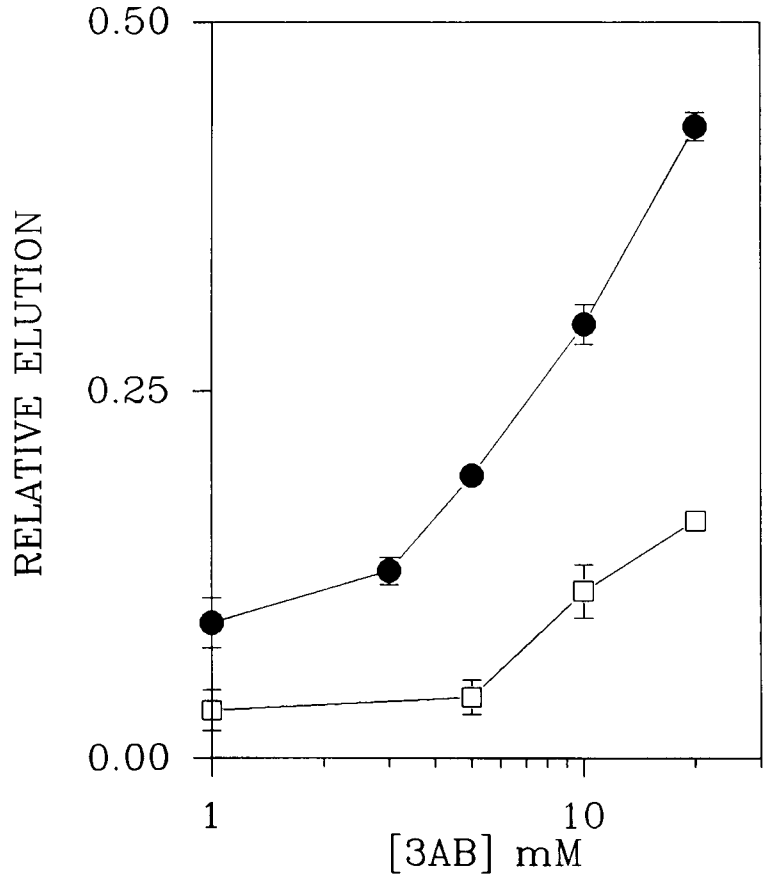


Figure 4.9: Relative elution depicting a 1 hour exposure of L1210 cells to increasing concentrations of 3AB in the presence of □ 150μM TM, or ● 250μM TM. Points were calculated from the data of a single experiment.

inhibitor. However, it should be pointed out that these results were derived from a single experiment, and the large errors in the high dose values would suggest further studies would be required to substantiate these observations.

4.6.3 The effect of PADPRP inhibition on the DNA single strand break levels resulting from fixed doses of TM.

3AB, in relation to the two novel PADPRP inhibitors, exhibited a much reduced level of potency as a PADPRP inhibitor as exemplified by the very high concentrations required to raise the single strand break frequency, i.e. ~70 fold increase in the concentration of 3AB was required as compared to NU1025, to produce a similar effect on DNA strand break frequency (see Figure 4.7). The potential of 3AB to increase the level of DNA single strand breaks was assessed utilising an increased concentration of TM which raised the initial break level. The TM concentration used was 250μM, and this was co-incubated with increasing concentrations of 3AB. Figure 4.9 indicates a raised frequency of single strand breaks when 250μM TM was used as opposed to 150μM TM. At both TM concentrations the DNA single strand break levels were increased when coincubated with increasing concentrations of 3AB. A slightly reduced 3AB concentration was apparently required to initiate the increased strand break frequency. 5mM 3AB was required in the presence of 150μM TM whereas only 3mM 3AB was necessary if 250μM was utilised. However, as the data presented in Figure 4.9 was derived from a single experiment, further studies would be required for validation. A comparison of the effect of an increased TM concentration in the presence of one of the more potent PADPRP inhibitors to observe the effect on DNA strand break frequency would be of interest.

4.7 THE EFFECT OF TIME ON THE ABILITY OF THE PADPRP INHIBITORS TO INCREASE THE SINGLE STRAND BREAK FREQUENCY FOLLOWING TM TREATMENT.

The effect of the PADPRP inhibitors on TM induced single strand breaks over a 24 hour period was investigated. A fixed dose of TM was coincubated for increasing periods of time in the presence of fixed concentration of PADPRP inhibitors. The effect of a fixed TM dose alone over time was presented in Figure 4.2 It was important that the dose of TM chosen for these experiments induced a significant level of damage into the DNA, but allowed for the observation of possible potentiation effects of the PADPRP inhibitors. A TM concentration of 200μM was chosen, as this produced a measurable level of single strand breaks as recorded by an increased rate of elution but was approximately 3 fold lower than that observed with a high TM concentration, e.g. 1000μM (see Figure 4.2).

For the study, just two of the four PADPRP inhibitors were assessed. 3AB and NU1025 represented a classical and a novel PADPRP inhibitor respectively, thereby allowing the potency of the inhibitors over time to be compared. The concentrations of the two inhibitors used were 5mM 3AB and 300μM NU1025. Both resulted in the increased frequency of single strand breaks after just a 1 hour incubation with 150μM (see Figure 4.3). The cytotoxicity studies (see Chapter 3, Figure 3.6) gave ~6% survival following 200μM TM + 5mM 3AB. However, as shown, maximal potentiation of TM cytotoxicity was observed with 50-100μM NU1025, giving ~3% survival at 200μM TM. The concentration of NU1025 utilised in this time course study was 300μM, as this gave a significant effect on the single strand break levels over a 1 hour incubation.

Both of the PADPRP inhibitors increased the R.E. values of the TM treated DNA over the whole 24 hour period, as observed in the R.E. plots of Figure 4.10, indicating an increased frequency of single strand breaks. There was a ~3 fold increase in this level when the rate obtained in the presence of 300μM NU1025 was compared to that of 5mM 3AB. However, with both inhibitors, the DNA single strand break levels after an

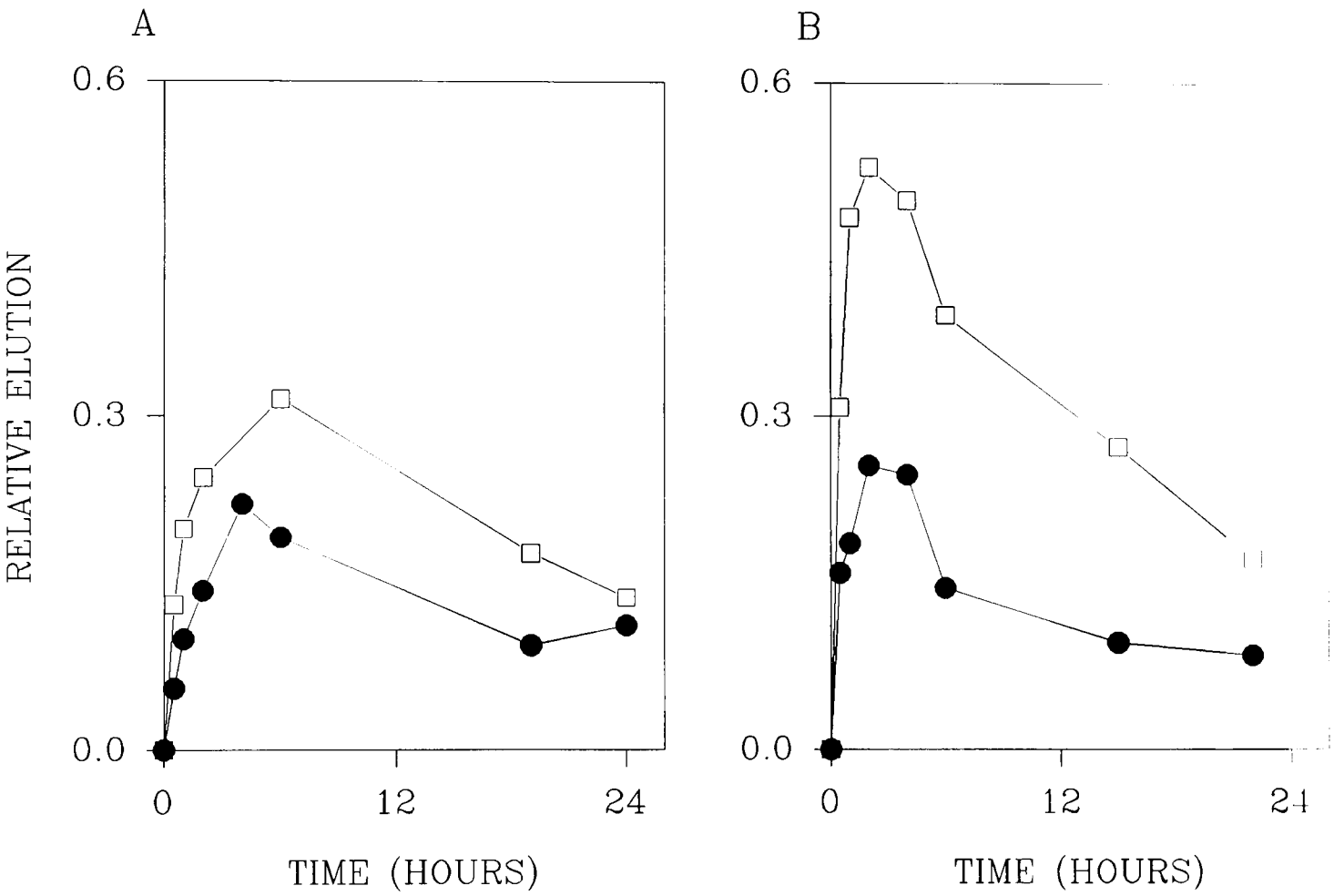


Figure 4.10: Relative elution of DNA from cells treated for increasing incubation periods with 200μM TM alone ● ,or in the presence of A. □ 5mM 3AB;. B. □ 300μM NU1025. These graphs were produced from the data of a single experiment, and are representative profiles.

incubation of 24 hours had fallen to nearly that of TM alone.

4.8 DISCUSSION

This Chapter investigated the effect that the clinically relevant alkylating agent, TM, had on DNA single strand break levels in L1210 cells, in the presence and absence of the four PADPRP inhibitors 3AB, BZ, NU1025, and PD 128763. The PADPRP inhibitor concentration range over which the level of TM induced DNA single strand breaks was raised was determined.

TM, through its active metabolite, MTIC, methylates DNA bases, e.g. 3meA, 7meG, O^6meG. The BER of these lesions results either in the formation of DNA single strand breaks due to the action of AP endonucleases (see Chapter 1, Section 1.4.5c), or, as in the case of O^6meG, breaks form due to the mismatch repair of incorrect bases paired opposite the O^6meG. As the concentration of TM was increased the level of DNA single strand breaks was also observed to increase in a concentration dependent manner (see Figure 4.3A), with the peak break level attained after 4-6 hours (see Figure 4.2). By 24 hours, the level of DNA single strand breaks was similar for untreated controls demonstrating that repair had occurred. TM degrades to MTIC with a half-life of ~50 minutes in murine lymphoma cells (Tsang *et al*, 1991) with the MTIC reaching a peak after 20-40 minutes. This correlates favourably with the peak elution rate observed between 1-4 hours for the TM treated L1210 cells. The subsequent reduction in R.E. values (see Figure 4.2) would indicate that the balance between break formation and break ligation alters. As the TM degrades, the level of base modification and hence the substrate for the AP endonuclease would decrease, thereby resulting in a reduced level of enzyme activity. Ligase action would therefore predominate.

The elution profiles of Figure 4.3A, especially at the lower TM concentrations, e.g. 100-400μM, were found to be of a convex nature. However, as the TM concentration was increased the profiles were observed to be more linear. To explain this occurrence, the

kinetics for the generation of DNA single strand breaks resulting from AP sites because of alkaline lability, and the kinetics of AP-endonuclease induce break formation must alter at increased levels of damage, with the latter increasing and/or the former becoming rate limiting. However as the peak elution rate correlates well with the degradation profile of TM, an increase in the activity of the AP endonuclease would be anticipated.

An assessment of the effect the four PADPRP inhibitors alone had on the DNA single strand break levels showed there to be no difference as compared to untreated controls after a 24 hour continuous exposure to high concentrations of inhibitor, e.g. 10mM 3AB, 1mM NU1025. The cellular DNA is continuously subject to "spontaneous damage" resulting from deamination, depurination, methylation and oxidation reactions within the cell (see Chapter 1, Section 1.4.2). The BER process is mainly responsible for the repair of these types of damage. Because of the known requirement for PADPRP function in BER, an incubation of cells with PADPRP inhibitors might be expected to slow this repair rate, resulting in the increased levels of single strand breaks. However, no effects on the single strand break frequency were observed, indicating the ultimate toxicities of the inhibitors were not due to an inhibition of the repair of spontaneous damage. The levels of spontaneous damage appear relatively high, (e.g. 2000-10000 depurinations/day), but when the number of base pairs in the DNA of a single chromosome is taken into consideration e.g. *Homo sapiens* contains $\sim 10^8$ nucleotides, the level of spontaneous damage appears rather insignificant (e.g. 1 break per $\sim 10^4$ nucleotides). This level of damage would be measurable using the alkaline elution technique, whose sensitivity is of the order of 1 break/10^7 nucleotides (Kohn *et al*, 1981). However, the number and class of DNA strand breaks introduced by DNA damaging agents, e.g. TM, could be an important factor in the contribution of PADPRP inhibition to increased cytotoxicity and increases in DNA strand break levels.

When increasing concentrations of the PADPRP inhibitors were coincubated with a fixed concentration of TM, which itself resulted in a significant, measurable level of DNA

damage, the DNA single strand break frequency was increased in a concentration dependent manner. However, there was a ~70-100 fold reduction in the concentrations of NU1025 and PD 128763 required to increase the break level to a similar degree as 3AB.

To the best of my knowledge this represents the first comprehensive study to determine the concentration dependent effect of the PADPRP inhibitors on single strand break levels in DNA damaged cells. The data presented herein does corroborate the few publications that have briefly reported the concentration dependency of the PADPRP inhibitor, 3AB, on the level of DNA single strand breaks (Cleaver *et al*, 1985; Cantoni *et al*, 1986). However, in this study, I found the single strand break frequency to increase, albeit very slightly from 0.3-5mM 3AB, in the presence of a fixed dose of TM (150μM) (see Figures 4.5A and 4.6). Only at 10mM 3AB was there an observable increase in the DNA single strand break level (see Figure 4.7). Cleaver *et al* (1985) had reported a decreased frequency of DNA single strand breaks between the concentrations of 0.5-1mM 3AB, with an increase observed only at 2mM 3AB and above. As well as demonstrating the concentration dependent effect of 3AB, the PADPRP inhibitors, BZ, NU1025 and PD 128763, were also found to exhibit similar concentration dependent increases in DNA single strand break levels. The ability of the inhibitors to increase DNA single strand break levels was shown to be in excellent agreement with their potencies as PADPRP inhibitors. The order of relative potency of the four inhibitors towards PADPRP was as observed for the *in vitro* PADPRP inhibition study (see table 4.1).

Interestingly, the concentrations of inhibitor required to inhibit PADPRP *in vitro* were ~1000 fold less than those required to increase the DNA single strand break levels, e.g. 3AB, gave an IC_{50} value for *in vitro* PADPRP inhibition of 19.1μM, but a significant increase in DNA strand break levels was not observed until a concentration of at least 5mM 3AB was achieved. However, these values can not be compared in a quantitative manner, as the R.E. values are only arbitrary figures. Nevertheless, the dissimilarity between the concentrations required to generate the effects could be due to the influence

of a number of parameters. Perhaps most importantly. to effect the level of DNA strand breaks a greater than 50% inhibition of the PADPRP activity could be required. This would result in a requirement for much higher PADPRP inhibitor concentrations. Also. the inhibitors rather than entering the cell by simple passive diffusion could require active transport into the intact cells (Olsson *et al*, 1993), or simply not permeate cell membranes sufficiently well. In this respect, efflux of the inhibitors may also contribute to concentration variations. Once inside the cell, the inhibitors, due to the amide grouping shared with nicotinamide, may bind to other NAD^+ dependent proteins, e.g. alcohol dehydrogenase, glyceraldehyde dehydrogenase. Also. other nonspecific binding to intracellular proteins could occur all of which would reduce the inhibitor concentration available to inhibit PADPRP. The intracellular NAD^+ concentration in intact cells varies between 100-1000μM whereas in the *in vitro* PADPRP assay the NAD^+ was maintained at a constant concentration of 75μM. Therefore, due to the competitive nature of the PADPRP inhibitors, cells with high intracellular NAD^+ levels would require increased concentrations of the inhibitors to effect a similar level of PADPRP inhibition. Metabolic degradation of the inhibitors could also provide an explanation for a reduced intracellular inhibitor concentration.

Satoh & Lindahl (1992) proposed a model for the involvement of PADPRP in the repair process. In DNA damaged cells, PADPRP binds to DNA single strand breaks, which activates the synthesis of poly(ADP-ribose) and results in auto- and hetero-modification. Automodified PADPRP exhibits a reduced affinity for DNA, and its release from the strand break allows the access of the repair enzymes to the damaged site. Utilising MNNG treated cells transfected with the DBD polypeptide, Molinete *et al* (1993) found the DBD competed with the endogenous PADPRP for the DNA single strand breaks. However, once the DBD was bound, the lack of automodification prevented its release with a consequential decrease in repair capacity. The transient inhibition of DNA repair by inhibitors of PADPRP can be explained utilising this model. The inhibition of

PADPRP would significantly inhibit the automodification reaction, therefore PADPRP would remain bound to the damaged site for an increased period, thus preventing the access of the repair enzymes. Inhibitors with increased potency towards PADPRP would retard the release from the DNA single strand break to an even greater extent, with this manifested as an increased delay of DNA repair.

The effect two different TM and NU1025 dosing schedules had on the R.E. profiles was considered (see Figure 4.8). Both in the coincubation study, (i.e. 150μM TM coincubated with increasing concentrations of NU1025), and the preincubation study, (i.e. L1210 cells were pre-incubated with 150μM TM, prior to incubation with the NU1025), the R.E. values were observed to increase as the concentration of NU1025 was increased. Therefore, the concentration dependent increase in DNA single strand break levels was not due to an interaction between the two drugs. Interestingly, the initial DNA single strand break levels due to TM alone are extremely comparable in both of the dosing schedules, which would suggest an efficient "wash out" of TM. In the preincubation study, TM was removed prior to treatment with the NU1025 concentrations. Therefore, if the TM was not effectively removed, or became trapped in the cell, e.g. TM efflux was transporter dependent or totally prevented due to complexing with intracellular proteins, TM would have been present for a 2 hour period, as opposed to 1 hour in the coincubation study. Therefore, a higher initial DNA strand break level due to TM alone would have been anticipated.

Several studies have been published which indicate PADPRP function resulted in the reduction of the steady state level of single strand breaks present in the DNA following damage. Lehmann & Broughton (1984) utilised alkaline sucrose gradients to analyse the effect of a DMS and 3AB cotreatment on the DNA single strand break frequency in serum starved cells. These G_0 arrested cells showed an equivalent DNA single strand break frequency to a growing cell population. In the absence of 3AB, high molecular weight species were regained as most of the DNA breaks were rejoined. However, upon

addition of 3AB the break level rose, with the level of DNA single strand breaks returning to the initial frequency. Cleaver & Morgan (1983) found that the addition of 3AB at any time between 6-24 hours after alkylation damage resulted in a similar level of breaks, as if the coincubation with 3AB had been continuous. With the subsequent removal of the inhibitor, the DNA single strand break frequency returned to that observed in the absence of PADPRP inhibition (Cleaver & Morgan, 1983; Cantoni *et al*, 1986).

As the concentration of 3AB required to exhibit a similar increased level of DNA single strand breaks was ~70 fold greater than that of NU1025, the effect of increasing the level of damage was assessed. At both 150μM TM and 250μM TM the DNA single strand break levels were increased in a concentration dependent manner. However, the increase observed at 250μM TM was greater than that at 150μM TM and the 3AB concentration at which the break level initially rose was slightly reduced. Therefore, the effectiveness of the PADPRP inhibitors may be dependent on the level of activation of PADPRP, which is itself activated by the level damage to the DNA. For example, the amount of PADPRP required for binding and activation may become rate limiting at higher levels of strand breaks. Panzeter *et al* (1992) proposed the homomodification of PADPRP, with one PADPRP molecule bound to DNA strand break ends, which actively produced poly(ADP-ribose) to modify a second acceptor PADPRP molecule. Therefore, less inhibition would be required to affect strand break frequencies if PADPRP itself became rate limiting for strand breaks. However, this result may be at variance with the results presented in Chapter 5, where L1210 cells were treated for increasing incubation periods with a fixed dose of 150μM TM, in the presence of increasing PD 128763 concentrations. There was no significant difference in the concentration of PD 128763 that resulted in an increased level of DNA single strand breaks, although the initial break levels due to TM alone was increased.

The increased single strand DNA break level resulting from a coincubation of TM with the PADPRP inhibitors was followed over a 24 hour period. Both 5mM 3AB and 300μM

NU1025, when coincubated with 200μM TM, led to increased R.E. values over the entire 24 hour period. However, this DNA strand break increase was 3 fold greater when 300μM NU1025 was utilised as opposed to 5mM 3AB. By 24 hours control levels were nearly reattained. The opinion generally upheld, is that the rate of religation of the single strand breaks is the factor modified by PADPRP in the dynamic process of break formation and repair. An increase in the activity of DNA ligase, due to direct or indirect modification by PADPRP is a proposed method by which PADPRP alters the steady state level of the single strand breaks (Creissen & Shall, 1982; Ohashi *et al*, 1983) (see Chapter 1, Section 1.5.2 for detailed discussion). The inhibition of PADPRP results in a shift in the balance of this equilibrium. The increased rate of elution observed with NU1025 as opposed to 3AB can be interpreted as an increased ability of this compound to inhibit the action of PADPRP. The increased level of PADPRP inhibition results in the increased upset to the dynamic relationship between break formation and subsequent religation. The shape of the elution curves as well as the maximal R.E. value obtained (i.e. at 6 hours) in the presence of 3AB and NU1025 was observed to be similar to that of TM alone. By 24 hours, the control levels were almost reattained, although there was still a slightly raised level of DNA single strand breaks observed with NU1025.

The results presented in this chapter have clearly demonstrated that all four of the PADPRP inhibitors, 3AB, BZ, NU1025 and PD 128763, when incubated in the presence of TM, result in an increased frequency of DNA single strand breaks. The concentrations of the inhibitors required to increase the DNA single strand break levels in the presence of a fixed dose of TM showed an excellent correlation with the level of *in vitro* PADPRP inhibition that was demonstrated in Chapter 3. A 70-100 fold decrease in the concentration of the novel compounds was required to increase the single strand break frequency to that observed with 3AB. This also gave an excellent correlation with the relative potencies observed in the growth inhibition and cytotoxicity studies.

The inhibitors themselves lacked effect on DNA strand break production, thus indicating,

that the cytotoxicity of the PADPRP inhibitors observed at high concentrations was not explainable by the inhibition of repair of spontaneous damage. The cytotoxicity of DNA damaging agents in the presence of inhibitors of PADPRP was reportedly correlated with an increase in the level of DNA single strand breaks resulting from the repair of lesions in the DNA bases. PADPRP function was associated with reducing the steady state break level, by reducing their half life, and thereby increasing survival. The inhibition of PADPRP altered this dynamic balance, and correspondingly decreased the cell survival. However, although the inhibitors were shown to potentiate the cytotoxicity of TM, together with a significant increase in the frequency of single strand breaks there was an observed lack of correlation between the concentrations over which these two biological endpoints occurred:- e.g. at 50μM PD 128763, maximum potentiation of TM cytotoxicity was observed (Chapter 3, Figure 3.6) but at this concentration there was little or no apparent increase in the DNA strand break level (Figure 4.7). The following Chapter will aim to investigate this observation more thoroughly with the presentation of comparative data.

CHAPTER 5 : EVALUATION OF PADPRP INHIBITOR CONCENTRATIONS REQUIRED TO POTENTIATE TM INDUCED CYTOTOXICITY AND DNA STRAND BREAK LEVELS.

5.1 INTRODUCTION

When the results of Chapters 3 and 4 were analysed together, as briefly described in the summary of Chapter 4, a discrepancy was observed between the PADPRP inhibitor concentration that resulted in the potentiation of TM cytotoxicity, and the concentrations required to increase the single strand break frequency, following TM treatment. As described in the previous Chapter, the literature lacks a quantitative and comprehensive dose dependent correlation between the effects of the PADPRP inhibitors on the DNA strand breaks, and effects on cytotoxicity. Most of the studies utilised just a single, high dose of inhibitor in conjunction with increasing concentrations of the damaging agent. Cleaver *et al* (1985) and Cantoni *et al* (1986), found that DNA single strand break levels resulting from fixed concentrations of MMS and H_2O_2 respectively, were increased in a concentration dependent manner by a co-incubation with 3AB, but this was not correlated with the effects on cytotoxicity. The potentiation of cytotoxicity of DNA damaging agents, especially those resulting in initiation of BER, by inhibitors of PADPRP, was suggested to correlate with an increase in the DNA single strand break frequency (e.g. Durkacz *et al*, 1980; James & Lehmann, 1982; Huet & Laval, 1985; Cantoni *et al*, 1986; Lunn & Harris, 1988). However, a number of studies reported a lack of correlation between cytotoxicity and DNA single strand break levels. In 1985, Cleaver *et al* published evidence to suggest that the increase in single strand break levels following damage to the DNA, were observed only at concentrations of 3AB of 2-5mM or over, and that below this there was an apparent decrease in DNA strand break level. However, the repair replication levels increased sharply at the lower inhibitor concentrations, so

negating the affiliation between repair replication and DNA strand break frequencies (Cleaver, 1985). However, different MMS concentrations used in the dosing schedules of these experiments could account for the discrepencies. Recent data, utilising an *in vitro* cell extract assay which assessed the rejoining ability of a nicked plasmid, found that break rejoining was inhibited in a concentration dependent manner in the presence of 3AB. However, 1mM 3AB which only reduced the repair capacity by ~70% of control levels resulted in an 80-90% inhibition of poly(ADP-ribose) polymer formation (Satoh *et al*, 1994). In fact even in the presence of just 200μM 3AB, a concentration which reduced strand rejoining by 20% of control levels, polymer formation was depleted to 80-90% of that found in control extracts. The 10-20% (ADP-ribose) polymer that was formed under such conditions was of a much reduced length, i.e. oligomers of less than 20 residues (Satoh *et al*, 1994). The proposed theory suggested PADPRP acted in a negative manner in respect to DNA repair. PADPRP bound to the DNA single strand break ends, required automodification to effect release, thereby allowing the repair enzymes entrance to the damaged site. The results indicated that modification by short ADP-ribose polymers of less than 20 residues was sufficient to allow chromatin decondensation, and therefore repair was inhibited only following complete inactivation of PADPRP.

Jacobson *et al* (1985) confirmed the results of Boorstein & Pardee (1984), who had shown that the PADPRP inhibitors caused an increase in the cytotoxicity of DNA damaging agents only if the cells traversed the S phase of the cell cycle. When quiescent and dividing cells were treated with MNNG in the presence of the PADPRP inhibitor, 3-MBZ similar increased levels of breaks were observed, and this increased break frequency was maintained throughout a 48 hour incubation period (Jacobson *et al*, 1985). However, the quiescent cells failed to exhibit an enhancement of cytotoxicity. Such cells are in G_0, and so have an indefinite period of time in which to repair the DNA strand breaks. Therefore, the slowing of repair by inhibition of PADPRP activity does not increase

cytotoxicity. Boorstein & Pardee (1984) also recorded a lack of correlation between the level of single strand breaks and the enhanced lethality in quiescent cells, due to S-phase dependency of the PADPRP inhibitors. Such data suggests that the processing of the DNA strand breaks by replication renders them lethal, and so results in cytotoxicity. The technique of alkaline elution was suggested to measure two subpopulations of DNA single strand breaks:- (1) benign repairable, and (2) lethal unrepairable (Boorstein & Pardee, 1984). The second class was proposed to originate from breaks in the initial class that had interfered in replication processes. Inhibition of PADPRP impedes repair. Jacobson *et al* (1985) demonstrated that if the PADPRP inhibitor was removed prior to the end of the 48 hour incubation period, then the break level was reduced to that of MNNG alone, but if inhibitor was added only after a ~36 hours incubation, the break frequency was increased. These data would support the theory proposed by Cleaver & Morgan (1983) and Lehmann & Broughton (1984), that PADPRP was involved in the regulation of single strand, steady state break levels. A further explanation for the discrepancies between enhanced lethality and DNA strand break levels would be that PADPRP was not directly involved in the repair process. Due to the raised poly(ADP-ribose) polymer levels at the G_1 and S phases of the cell cycle (Kidwell & Mage, 1976; Leduc *et al*, 1988), Jacobson *et al* (1985) postulated PADPRP was required by DNA damaged cells for the successful progression through the cell cycle, either by modulation or stabilisation of the chromatin structure. Realini & Althaus (1992) proposed the elegant histone shuttle model, describing such a mechanism for the decondensation of the chromatin structure.

5.2 AIMS

The aim of this third results Chapter was to further investigate the apparent discrepencies between the range of PADPRP inhibitor concentrations required to potentiate TM cytotoxicity (Chapter 3) and the concentrations that resulted in an increased single strand break frequency in the DNA (Chapter 4). To allow such comparisons, the concentration

of TM was maintained at a constant 150μM. and the concentration range of PD 128763 and BZ were varied in cytotoxicity studies and DNA strand break analyses for the purposes of direct comparison.

A comparison of TM cytotoxicity and the concentrations of TM that resulted in DNA single strand breaks was also assessed, and the results compared with those from the combination studies.

5.3 CONVERSION OF R.E. TO RAD EQUIVALENTS.

The R.E. value represents, "the increase in DNA elution observed with treated cells as compared to untreated controls, at the point when 50% of the internal standard DNA has eluted" (see Figure 2.1, for method of calculation).

Alkaline elution profiles, constructed from exposure to X rays, are almost first order with respect to dose, and the linearity of the elution curves signifies a near random distribution of the single strand breaks through the DNA (Kohn *et al*, 1981). This feature of X-rays makes them an ideal candidate for use as a standard of break frequency. The calculated R.E. values were converted to rad equivalents, and a linear relationship was shown between the R.E. and the X-ray dose ($r^2 = 0.92$) (see Figure 5.1), therefore the R.E. values directly reflect the DNA single strand break levels.

The R.E. values for cells (the cells utilised must be the same as those used in X-ray exposure) treated with other DNA damaging agents, e.g. alkylating & oxidising agents, ionising radiation can then be expressed in rad equivalents, i.e. that is the X-ray dose which would produce an equivalent frequency of breaks.

5.4 COMPARISON OF CYTOTOXICITY AND DNA STRAND BREAK LEVELS IN TM TREATED L1210 CELLS.

Figure 5.2 shows that the cytotoxicity (Section 3.5), and the frequency of DNA strand breaks (Section 4.4) in TM treated L1210 cells are both concentration dependent over a

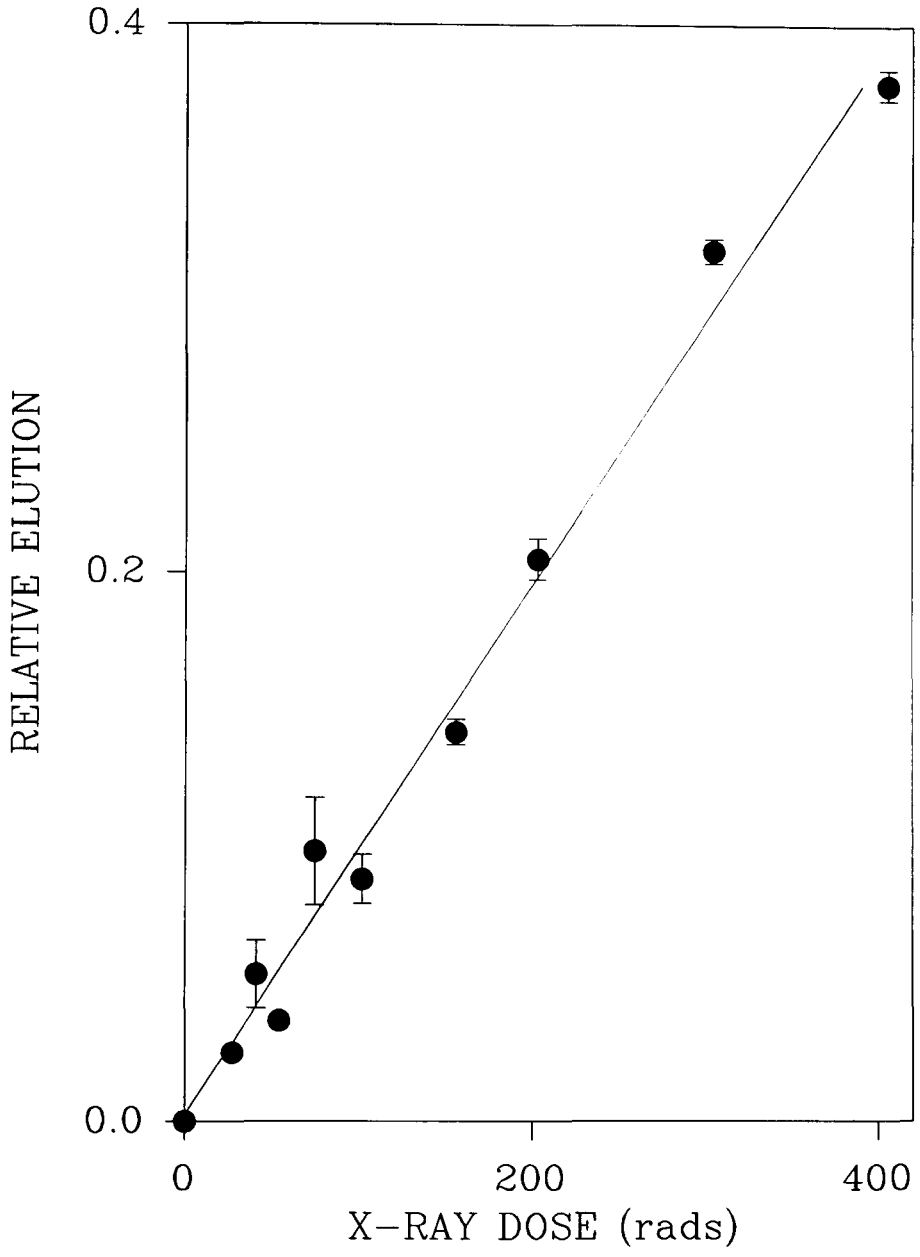


Figure 5.1: Relative elution values for L1210 cells exposed to increasing concentrations of X-rays. Points represent the mean of eight samples, taken from four independent experiments.

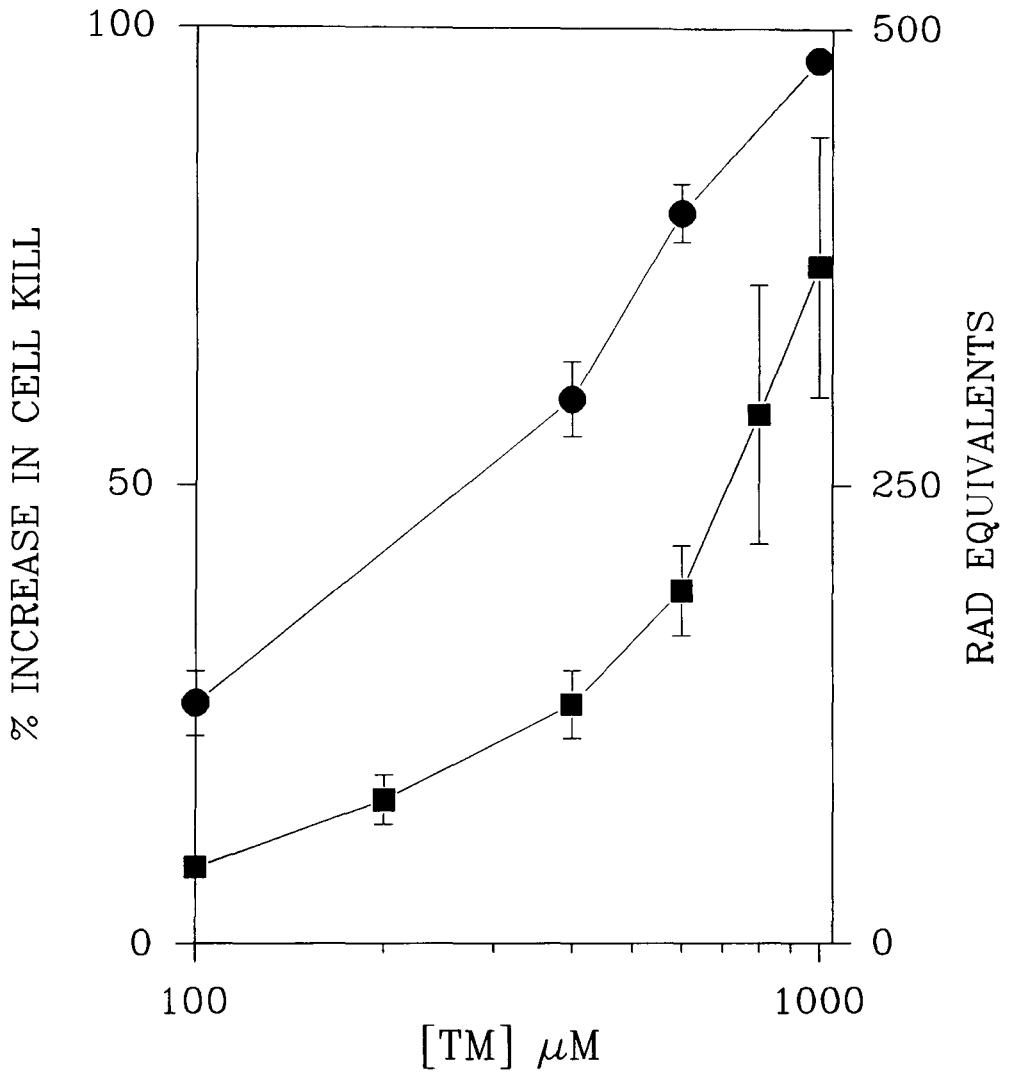


Figure 5.2: Concentration dependent effects of ● TM induced cytotoxicity and ■ DNA strand break levels in L1210 cells continuously exposed for 16 hours and 1 hour respectively. The cytotoxicity data points represent the mean of at least six independent experiments, whereas the DNA strand data was averaged from at least six samples, taken from two independently dosed experiments.

similar range of TM doses, i.e. 100-1000μM. A 25% increase in cell kill correlates with an increase of ~125 rad equivalents, and this was raised to ~250 rad equivalents when a 50% increase in cell kill was observed.

5.5 CONCENTRATION DEPENDENT EFFECTS OF THE PADPRP INHIBITORS ON TM CYTOTOXICITY AND DNA SINGLE STRAND BREAK LEVELS.

In Section 3.6.1, the effect of 100μM TM in the presence of increasing concentrations of the PADPRP inhibitors on the growth of L1210 cells was determined. For the current study, this experimental design was repeated, but in the form of a clonogenic survival assay (see Section 2.11) which allowed the percentage increase in cell kill due to PADPRP inhibitor treatment to be determined. To permit comparisons with the DNA single strand break data, a fixed concentration of 150μM TM was used. The DNA strand break study was itself extended from that in Section 4.6.1 to include doses of BZ and PD 128763 over a lower range of concentrations.

Figure 5.3 clearly demonstrates that, in marked comparison with the effects of TM on cell kill and DNA single strand breaks, both BZ and PD 128763 lacked a concentration dependent correlation between the potentiation of TM cytotoxicity and the increased DNA single strand break frequency. It is important to mention at this point that the cytotoxicity data was calculated using TM alone as the control representing 0% cell kill, whereas the rad equivalent values were plotted inclusive of the effect TM alone had on the DNA single strand break level (i.e. using an untreated control for calculating R.E. values). Unlike for TM alone (Figure 5.2), the % increase in cell kill and the increase in DNA single strand break levels resulting from a coincubation of either BZ or PD 128763 with TM, do not increase over obviously analogous concentration ranges. For each inhibitor, the curves representing the increase in cytotoxicity and the increase in DNA single strand break level were found to be sigmoidal in the semi log plots used. A sigmoid curve is divided into three phases:- (1) lag phase, (2) exponential phase and (3) plateau phase. As

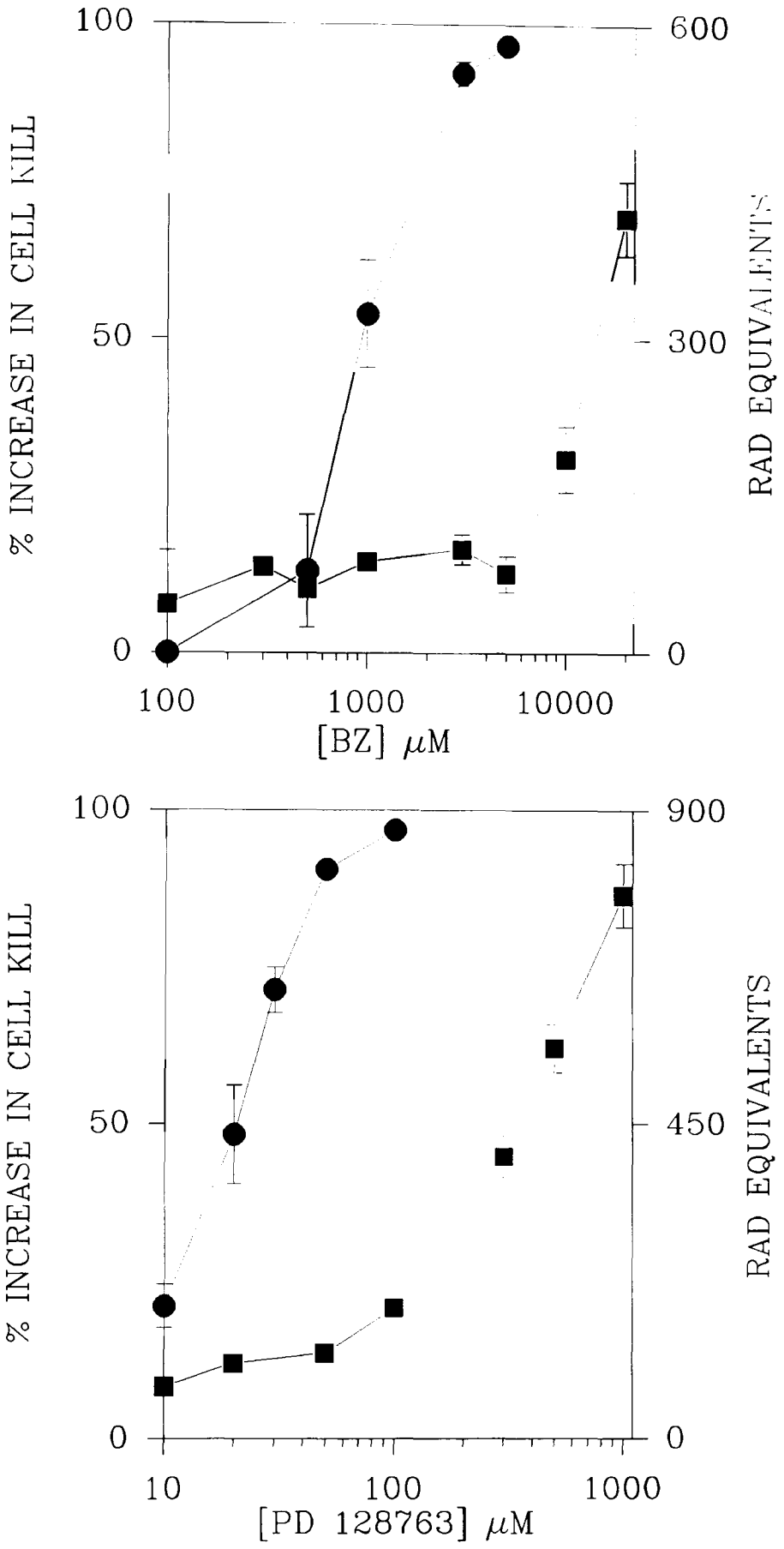


Figure 5.3: Concentration dependent effects of PADPRP inhibitors on ● TM induced cytotoxicity and ■ DNA strand break levels, following 16 and 1 hour continuous exposures respectively. The cytotoxicity data points represent the mean of four samples from two independent experiments, whereas, the DNA strand break data was averaged from at least six samples, taken from three independent experiments.

depicted in Figure 5.3, ~92% and ~91% increases in cell kill were observed on the exponential part of the curves for 3mM BZ and 50μM PD 128763 respectively. However, at these concentrations there was little evidence of any effect on DNA strand break levels (i.e. the data points remain on the lag phase of the curve).

In order to numerically portray the concentration dependency of the PADPRP inhibitors on DNA single strand break levels in relation to the % increase in cell kill, Tables 5.1 and 5.2 were constructed. The DNA single strand break levels were increased as the concentration of both BZ and PD 128763 were increased. At concentrations of BZ and PD 128763 which gave corresponding ~90% increases in cell kill, i.e. 3mM BZ and 50μM PD 128763, the DNA single strand break levels in rad equivalents were 107 ± 12 and 129 ± 8 respectively. The rad equivalents related to 150μM TM alone were 67 ± 7 and 81 ± 9 respectively (which are not significantly different). Although the DNA single strand break increase was calculated as being statistically significant (P values were 0.0145 and 0.0006 respectively), in relative terms this represents only a small increase in break levels, i.e. ~60% in both cases. This was clearly demonstrated in Figure 5.3, as 3mM BZ and 50μM PD 128763 are positioned on the lag phase of the rad equivalent curve whereas the % increase in cell kill at equivalent concentrations are positioned on the exponential part of the curve. A 50% cell kill was observed with 20μM PD 128763, but this only increased DNA single strand break levels by ~30% (although due to relatively high errors at low levels of strand breaks, this was not computed as significantly different from the TM alone levels by the Student's T test). When the inhibitor concentrations were raised further, a concentration dependent increase in the DNA strand break frequency was found, giving ~4 fold increases by 20000μM BZ and 500μM PD 128763.

For the two inhibitors, the rad equivalent data was plotted against the % cell kill to allow the identification of possible correlations and this was compared with the effect of TM alone (see Figure 5.4). Both BZ and PD 128763 were found to produce an almost total

enhancement of cytotoxicity with only a slight corresponding increases in DNA strand breaks. These plots are in contrast to that for TM alone (also Figure 5.4) where the cytotoxicity was observed to increase in relation to increases in the DNA single strand break levels. However, once the % cell kill reached ~80%, there was decrease in the gradient of the slope. This increase must be considered in a relative manner, thus, larger increases in DNA strand break levels have a lesser effect on cytotoxicity.

TABLE 5.1

DRUG TREATMENT	% CELL KILL	RAD EQUIVALENTS	SIGNIFICANCE (P VALUES)
TM alone	0	81 ± 9	
TM + 10μM PD 128763	21 ± 3	81 ± 14	0.9979
TM + 20μM PD 128763	48 ± 7	107 ± 11	0.1236
TM + 50μM PD 128763	91 ± 1	129 ± 8	0.0006
TM + 100μM PD 128763	97 ± 1	186 ± 10	<0.0001
TM + 300μM PD 128763	>99	403 ± 27	<0.0001
TM + 500μM PD 128763	>99	558 ± 31	<0.0001

Table 5.1: The effect of a 16 hour, or 1 hour continuous incubation with a fixed concentration of TM, in the absence or presence of increasing concentrations of PD 128763 on the % cell kill, and DNA single strand break levels respectively. A two-tailed, unpaired students t test was utilised to determine whether, the difference between the rad equivalents of TM alone and TM + increasing concentrations of PD 128763 were significant. The "GraphPad InStat" computer programme was used to aid computation. Figures were calculated from at least 3 individually dosed samples.

TABLE 5.2

DRUG TREATMENT	% CELL KILL	RAD EQUIVALENTS	SIGNIFICANCE (P VALUES)
TM alone	0	67 ± 7	
TM + 100μM BZ	0	46 ± 36	0.2185
TM + 500μM BZ	13 ± 8	61 ± 6	0.6284
TM + 1000μM BZ	54 ± 7	87 ± 10	0.1216
TM + 3000μM BZ	92 ± 2	107 ± 12	0.0145
TM + 5000μM BZ	97 ± 1	76 ± 16	0.5707
TM + 10000μM BZ	>99	199 ± 29	0.0008
TM + 20000μM BZ	>99	417 ± 32	0.0001

Table 5.2: The effect of a 16 hour, or 1 hour continuous exposure to a fixed concentration of TM, in the absence or presence of increasing concentrations of BZ on the % cell kill, and DNA single strand break levels respectively. The statistical analysis was as described for Table 5.1.

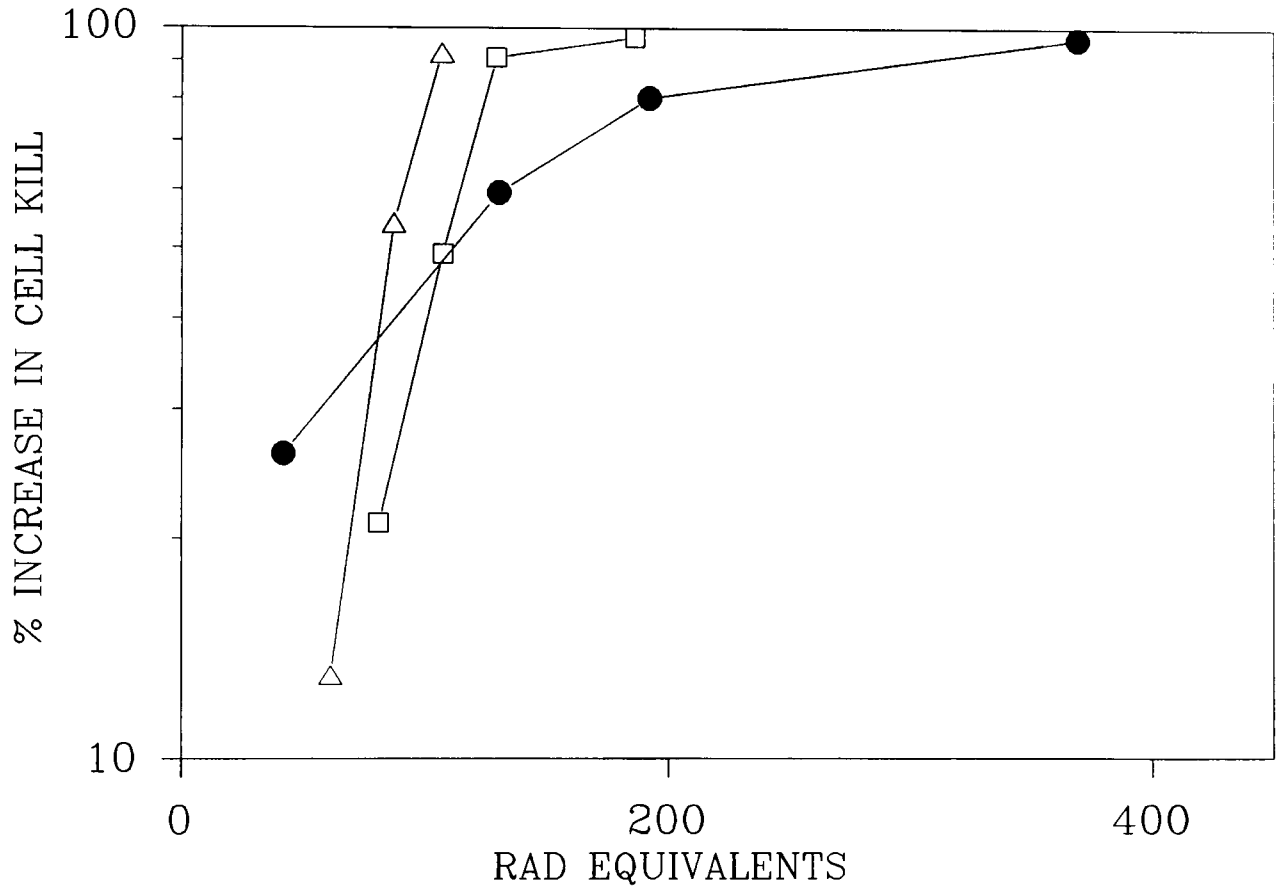


Figure 5.4: Correlation between the DNA strand break level and cytotoxicity ● increasing TM concentrations, □ 150μM TM + increasing concentrations of PD 128763, △ 150μM TM + increasing concentrations of BZ.

The similar position and the parallel slopes of the curves for the two inhibitors would suggest a parallel mechanism of action. This is an important observation. as once again an ~60 fold increase in potency towards PADPRP was observed with PD 128763 as compared to BZ (see above: 50μM PD 128763 as compared to 3mM BZ).

Tables 5.1 and 5.2 clearly demonstrate an increase in rad equivalents observed as the concentration of inhibitor increased, e.g. PD 128763: 10μM = 81 ± 14 rads, 20μM = 107 ± 11 rads, 50μM = 129 ± 8 rads. This may imply, that rather than there being a total dissociation between the % increase in cell kill and DNA single strand strand break frequency, a modest increase in DNA single strand breaks, e.g. perhaps originating from a distinct base modification, is sufficient to account for the increased cytotoxicity (see Section 5.6 for further Discussion of this point).

At present a detailed analysis for the effect of time on the relationship between cell kill and DNA strand break levels is lacking. The cytotoxicity data presented above was calculated from a 16 hour continuous exposure to the TM and PADPRP inhibitors, whereas just a 1 hour co-incubation was given in the DNA strand break assessment. However, preliminary strand break data utilising increased incubation periods of 4 and 6 hours would indicate the PD 128763 concentration dependent increase in DNA single strand break frequency to show similar responses at different time points (see Figure 5.5). The only apparent difference between each time point is the initial level of DNA single strand breaks. TM is a slow acting agent, with a $t_{1/2}$ of ~50 minute in murine lymphoma cells (Tsang *et al*, 1991), and Figure 4.2 showed the peak of DNA single strand breaks to occur after a 2-4 hour incubation period. Figure 5.5 shows linear plots of the increase in single strand break frequency for increasing concentrations of PD 128763 in the presence of 150μM TM at 1, 4, and 6 hour incubation periods. The initial number of breaks due to the 150μM TM alone increases with time, although the difference between the 4 and 6 hour incubations is only ~50 rad equivalents as opposed to ~320 rad equivalents between the 1 and 4 hour incubations. This data would again indicate the

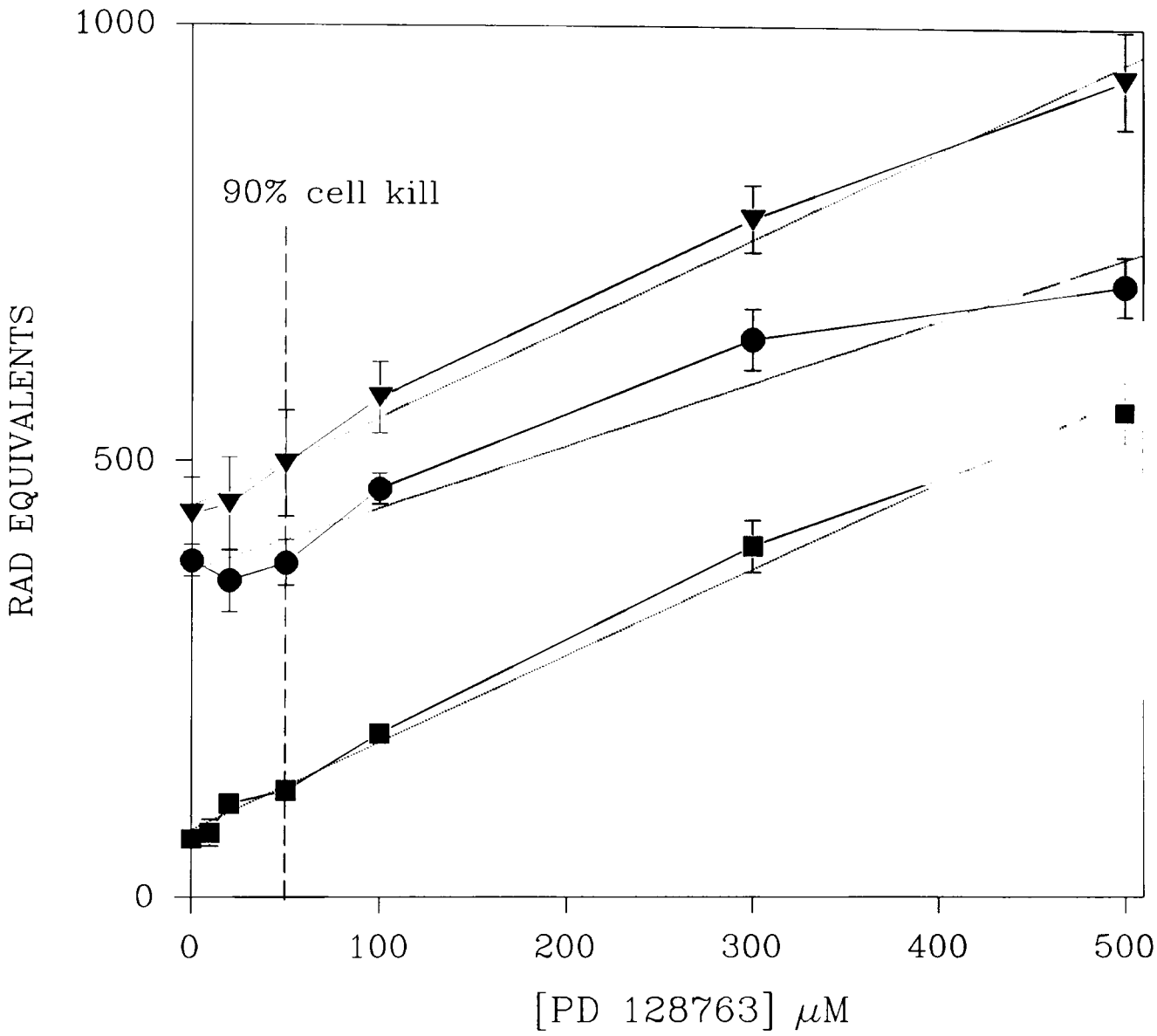


Figure 5.5: Effect of an increasing incubation on the PD 128763 dependent increased single strand break level in L1210 cells treated with 150μM TM ■ 1 hour, ● 4 hours, ▼ 6 hours. For each set of data, a linear regression analysis was performed. Points represent the mean of at least, six samples taken from three independently dosed experiments.

DNA single strand breaks to plateau after a 2-4 hour incubation as observed in Figure 4.2.

Initially, inspection of the data suggested a "null" effect of the low PD 128763 concentrations on DNA single strand break levels, but due to large errors. (i.e. between 10-20% at 10μM and 20μM PD 128763), a linear relationship was assumed. Interestingly, linear regression analysis for each of the three curves gave quite similar rates of increase in break level (see Figure 5.5), with the calculated slopes being, 0.38, 0.31 and 0.5 for 1, 4 and 6 hours respectively. Utilising the linear regression slopes, figures were derived for the effect of specific concentrations of inhibitor on rad equivalent values. The data is presented in Table 5.3, along with the % increase in DNA single strand break levels that these figures yield. Upon analysis of, for example, the 1 hour incubation data only slight % increases in the DNA single strand break levels were observed as the concentration of PD 128763 was raised, e.g. at 20μM PD 128763 there was a ~10% increase in the DNA strand break levels, whereas by 50μM PD 128763 the % increase in DNA strand break levels was just ~25%. A notable increase in the DNA single strand break level only became apparent at a concentration of 300μM PD 128763. Due to the considerable errors at the lower concentrations, the linear regression analysis was repeated ommiting these values (e.g. 0-50μM PD 128763) However, there was no significant differences observed, with similar values calculated for the gradients of the curves.

Table 5.3 also shows the data for the 4 and 6 hour incubations. Significant increases in DNA single strand break levels were only observed when 300μM PD 128763 was utilised. Interestingly, the % increases in DNA strand breaks observed at these later times were much smaller than those calculated for the 1 hour incubation, e.g. at 50μM PD 128763, a 1 hour incubation gave a ~25% increase whereas just ~4% and ~6% increases were observed at the 4 and 6 hour incubations respectively. At 100μM PD 128763 a % cell kill of > 95% was observed, but at the 4 and 6 hour incubations the increase in the level of DNA single strand breaks was just ~8-12% as opposed to ~50% at 1 hour.

Therefore, the % increases in strand breaks when % cell kill is greater than 90% become even more "modest" at later incubation time points. Note however, that in "absolute" terms, these % increases represent larger numbers of breaks because the starting levels are higher at these later times (e.g. 50μM PD 128763 at 1 hour: 25% increase ≡ ~20 rad equivalents, 50μM PD 128763 at 4 hour: 4% increase ≡ ~16 rad equivalents).

TABLE 5.3

PD 128763 (μM)	1 HOUR INCUBATION		4 HOUR INCUBATION		6 HOUR INCUBATION	
	RAD EQ.	% INC.	RAD EQ.	% INC.	RAD EQ.	% INC.
0	76	-	370	-	425	-
20	84	~10	377	~2	435	~2
50	95	~25	386	~4	450	~6
100	114	~50	401	~8	475	~12
300	189	~150	463	~25	575	~35

Table 5.3: Effect of time on the PD 128763 related % increase in DNA single strand breaks in L1210 cells treated with 150μM TM. The data presented is derived from linear regression analysis of the data using the "Graphpad Inplot tm." software programme, and values represent, at least, five samples taken from three independently dosed experiments. The percentage increases in DNA single strand break levels were calculated utilising the following equation:- PD 128763_X - control/control x 100%. Where X = the concentration of PD 128763.

5.6 DISCUSSION

This results Chapter, due to the preliminary stage of the research, and experimental limitations because of the accuracy of the data, especially at low levels of DNA strand breaks, includes a number of perhaps highly contentious theories and interpretations. However, it was considered important to include this comparison to allow a full interpretation of the results in Chapters 3 and 4.

Initially, the correlation between the TM concentrations that effected cytotoxicity and caused an increase in the DNA single strand break frequency was assessed. Both endpoints were observed to increase over a similar range of TM concentrations, suggestive that the increased break frequency was related to the increased cytotoxicity.

TM, degrades to the methylating agent, MTIC which results in base modification (see Figure 1.13). To date three such bases have been described, O^6meG, 3meA and 7meG. However, the structurally related compound DTIC, has been identified as modifying 12 different sites on the bases within the DNA (Mitchell & Dolan, 1993), and as both DTIC and TM degrade to the active metabolite MTIC, it is likely that TM also exhibits a more varied range of base modifications, which as yet remain unidentified. The mechanism by which TM exerts its cytotoxic effect also has yet to be fully defined. Reports have suggested O^6meG to be implicated in the cytotoxic event, especially as cells with low levels of the O^6meG repair enzyme, MGMT show an increased level of sensitivity to TM treatment (Tisdale, 1988; Hepburn & Tisdale, 1991; Baer, 1993). The persistence of O^6meG fails to impede the replication fork but allows the continuation of DNA replication. However, the base placed opposite O^6meG during replication is T, so giving a GC $\rightarrow$ AT transition (Coulondre & Miller, 1977). O^6meG levels have been shown to correlate well with the induction of mutations, but correlations with cytotoxicity have proved contentious. Whilst Kalamegham *et al* (1988) found that unrepaired O^6meG was correlated with cytotoxicity, probably due to futile cycles of mismatch correction, others failed to do so (Newbold *et al*, 1980). The recent identification of the "tolerance"

phenotype could possibly provide an explanation for this discrepency (see Chapter 1, Section 1.4.5c).

The results presented herein demonstrate TM related cytotoxicity in L1210 cells to be correlated with a concentration dependent increase in the DNA single strand break frequency, e.g. at a 50% increase in cell kill the increase in rad equivalents was ~250, and when the cell kill was increased to 75%, the number of rad equivalents had increased to ~375. L1210 cells are almost completely lacking MGMT (unpublished data, C.E. Arris, Cancer Research Unit, The Universisty of Newcastle upon Tyne), and hence mismatch correction would be initiated due to the persistence of O^6meG lesions in the DNA. O^6meG repair is a slow repair process, with a complex of enzymes and proteins involved formed prior to the strand break formation. However, Hepburn & Tisdale (1991) reported that TM treatment also resulted in the formation of the repairable bases, 3meA and 7meG. 3meA is removed from the DNA by a specific 3meA glycosylase. The rate of removal of 3meA (Medcalf & Lawley, 1981) was found to correlate with growth inhibitory effects of TM, up to 24 hours after treatment, in cells treated directly with TM or in cells transfected with TM damaged DNA (Hepburn & Tisdale, 1991). 3meA is considered a lethal lesion as it blocks DNA replication (Lindahl *et al*, 1988). Although this base modification has yet to be implicated as a cytotoxic effector in eukaryotes, Karren *et al*, (1980) found 3meA was involved in the cytotoxicity of methylating agents in bacteria. Hepburn & Tisdale (1991) found that of the total modified bases in TM treated cells, ~70% were 7meG and just ~10% 3meA. As 7meG fails to disrupt the genetic sequence, a significant role in TM cytotoxicity would be highly improbable (Lehmann & Karren, 1981). In summary, TM treatment leads to the formation of subsets of lesions, which either fail to produce DNA strand breaks, e.g. O^6meG, or which represent just a small percentage of the total lesions present, but may have a greater effect on the cytotoxicity than the overall break levels, e.g. 3meA. Thus, although a correlation was observed between the TM concentrations that effected cytotoxicity, and the DNA

single strand break levels, it may be too simplistic to interpret the increased break levels as being responsible for the increase in cytotoxicity. Overall TM cytotoxicity could be a combination of events, only some of which are dependent on strand breaks, all interacting to result in the death of the cell.

The time difference between the incubation periods of the two biological endpoints could lead to complications in the interpretation. The cytotoxicity studies were performed following a 16 hour continuous exposure to TM whereas the DNA strand break analysis utilised just a 1 hour exposure. Due to possible variations in repair kinetics of individual lesions, the correlation observed between TM cytotoxicity and DNA single strand break levels may be unrepresentative due to the dissimilar incubation periods. Figure 4.2 showed that the level of DNA single strand breaks in TM treated cells plateaued between 4-6 hours. However, after only a 1 hour exposure to TM, even a TM concentration of 200μM gave a significant level of DNA single strand breaks. The effect of increasing concentrations of PADPRP inhibitor in the presence of a fixed concentration of TM was then considered (see Figure 5.3). Unlike TM alone, where the increase in strand breaks paralleled the increase in cytotoxicity, a discrepency was observed between the concentrations of inhibitor required to increase cytotoxicity, and those required to increase the DNA single strand break levels (see Figure 5.3). At 3mM BZ and 50μM PD 128763 in the presence of 150μM TM, a >90% increase in cell kill was observed, but the increase in DNA single strand break frequency although statistically significant remained on the lag phase of the curve. Nevertheless, it could still be a possiblity that the increases in DNA single strand breaks do account for the increased level of cytotoxcity, e.g. a 50% cell kill was observed with 20μM PD 128763, and at this concentration an ~30% increase in DNA single strand breaks was observed. Figures 5.5 and 5.6, could support this proposal, as on linear plots, DNA single strand break levels could be interpreted as increasing in a concentration dependent manner, rather than there being a "null" effect at the lower PADPRP inhibitor concentrations which cause high degrees of cytotoxicity

e.g. 90% cell kill. This takes account of the high errors observed at the lower inhibitor concentrations (e.g.10-20%). However, the data would suggest that the theory postulated ~15 years ago, that potentiation of cytotoxicity by PADPRP inhibitors was directly related to the increase in the DNA single strand break level, is at best too simplistic, especially when compared to the TM alone data where a good correlation was shown.

How has this discrepancy previously failed to be recognised ? Cytotoxic mechanisms of DNA damaging agents are extremely complex, as previously described, e.g. O^6meG and 3meA. For the PADPRP inhibitors to cause cytotoxicity due to an altered rate of repair of these lesions, then the involvement of PADPRP in their repair must be considered. The direct repair of O^6meG via MGMT does not introduce breaks into the DNA strands, as previously described. However, persistence of the lesion resulting from a lack of the MGMT would lead to mismatch repair. This form of repair does result in DNA strand breaks with which PADPRP could interact, but requires the interaction of several enzymes/proteins in the form of a complex to incise the DNA strands (see Section 1.4.5c). The complex could therefore mask the DNA strand breaks from PADPRP, thereby preventing its attachment to the strand ends. 3meA is repaired via the BER process. A specific 3meA glycosylase removes the modified base from the DNA, resulting in the formation of AP sites that are subsequently processed by AP endonucleases to form DNA single strand breaks. This reaction sequence activates PADPRP only after the generation of the DNA strand breaks and therefore is unlikely to modulate the rate of removal of 3meA. Furthermore, the PADPRP inhibitor 5meNic had no effect on the removal of 7meG from the DNA following treatment with MNU (Durrant *et al*, 1981). However, a 2 fold enhancement of O^6meG was observed (Durrant *et al*, 1981). The classical inhibitors, e.g. BZ and 3AB were known to be of low potency and specificity towards PADPRP, therefore the concentrations of 1-5mM, which gave moderate potentiation of cytotoxicity, but had little or no effect on other metabolic reactions were generally used (Durkacz *et al*, 1980; James & Lehmann, 1982; Huet & Laval, 1985; Cantoni *et al*, 1986; Boorstein, 1987; Moses *et al*, 1988; Lunn & Harris,

1988) (see Section 4.1, Chapter 4 for a more detailed review of the literature). However, with the availability of novel PADPRP inhibitors, which exhibit increased levels of potency towards PADPRP, it was possible to carry out a comparative analysis with the "classical" inhibitors to compare the range of inhibitor concentrations that resulted in increased cytotoxicity and a raised level of DNA single strand breaks in DNA damaged cells.

PADPRP has previously been proposed as a possible signal which would alert the cell to DNA damage. As presented in the introduction to this Chapter, Jacobson *et al* (1985) postulated that raised polymer levels during the G1 and S phases of the cell cycle were required for correct progression through the cell cycle. However, from the observed effects of the PADPRP inhibitors, PADPRP may have control over two different endpoints. A report by Satoh *et al* (1994) suggested that the poly(ADP-ribose) polymer formed had a dual function, as almost all PADPRP activity could be suppressed *in vitro* by either 200μM 3AB or 50μM 2-nitro-6(5*H*)-phenanthridinone (a novel PADPRP inhibitor developed by Banasik *et al*, 1992), with little effect on the repair capacity. Short polymer chains were all that were required for the repair of damage to the DNA and they proposed that the synthesis of long polymer chains was required to prevent homologous recombinational events. However, another interpretation for these results, which would also support the results presented in this Chapter would suggest that two different biological endpoints, cytotoxicity and DNA repair are controlled by modifications to the long and short polymer chains respectively. The synthesis of long polymers (up to 200 residues (Alvarez-Gonzalez & Jacobson, 1987; Kiehlbauch *et al*, 1993)) could be the means by which DNA damage is signalled to the cell. The PADPRP inhibitors, due to their competitive nature, transiently inhibit PADPRP activity in a concentration dependent manner, i.e. at the lower inhibitor concentrations PADPRP remains active but at a reduced level (Sims *et al*, 1983). (ADP-ribose) polymers form in a processive manner (Naegelli *et al*, 1989) which would account for the concentration-dependent effect

observed on the potentiation of cytotoxicity. The poly(ADP-ribose) glycohydrolase is responsible for the intracellular breakdown of the polymer. Research has shown that the (ADP-ribose) polymers are degraded in a biphasic manner (Wielckens *et al*, 1982; Hatakeyama *et al*, 1986) with the long polymers processively degraded in either a endoglycosidic or exoglycosidic manner, until short polymers remain (Miwa *et al*, 1974; Braun *et al*, 1994; Brochuet *et al*, 1994). The short polymers are subsequently degraded in a distributive manner. Therefore, at the lower PADPRP inhibitor concentrations, preferential inhibition of long polymer synthesis could occur. A further possibility could be that, different concentrations of PADPRP inhibitor could inhibit the initiation and elongation reactions of poly(ADP-ribose) chain formation. The K_m for each of these reactions would be an important factor. The overall K_{mNAD+} for PADPRP was calculated to be ~50μM (Ueda *et al*, 1982), with the cellular NAD^+ levels at 300-1000μM (Shall, 1984). Due to the low K_m, the NAD^+ levels must be significantly reduced before any effects on the velocity of the reaction are observed, or in the case of the PADPRP inhibitors, their concentration must be sufficiently high to compete with the NAD^+ substrate. To sum up, the presence of low inhibitor concentrations may result only in the formation of short polymers. The longer polymers would be required to signal cellular processes leading to cell survival. The short polymers suffice for chromatin decondensation and repair, therefore much higher inhibitor concentrations would be required before an observed inhibition of repair.

The target of the long poly(ADP-ribose) polymers could be a cellular component(s) involved in cell cycle arrest and cell survival. A speculative idea for such a target, emanating from the cell cycle literature could be, for example, the nuclear protein, p53. Following damage to the DNA e.g. by UV, ionising radiation and chemotherapeutic agents, the level of p53 in the cell rises due to a post-translational, stabilisation mechanism, which subsequently causes the induction of "damage inducible" gene transcription, e.g. p21, which result in a G_1 cell cycle arrest (see Figure 1.18) (Xiong *et*

al, 1993). The stimulus for p53 stabilisation has been reported as DNA single and double strand breaks (Nelson & Kastan, 1994). With the cell cycle arrested, DNA replication is prevented and the polymers bound to PADPRP and histones etc. would remain capable of chromatin decondensation thereby facilitating the repair process. p53 was also shown to induce expression of the damage inducible Gadd45 gene (Smith *et al*, 1994). This was found bound to PCNA, with an enhancement of DNA repair observed.

Again, as for the TM alone study, an alternative explanation could be that the different incubation periods utilised (16 hour: cytotoxicity studies, 1 hour: DNA strand break assay) could possibly explain the observed discrepancy between the concentrations of PADPRP inhibitor required. The initial studies to further investigate the effect of time on the range of PADPRP inhibitor concentrations required, would indicate that over a period of 1-6 hours the rate of inhibitor dependent increase in break levels was similar (see Figure 5.6). However, a more detailed analysis would be required before any serious conclusions could be drawn, (although again, this may be limited by the accuracy of the techniques employed).

A completely distinct theory for the difference in concentrations required to inhibit repair and enhance cytotoxicity would be that the PADPRP inhibitors themselves were affecting two different cellular targets. Rankin *et al* (1989) showed that the PADPRP inhibitors over a low range of concentrations, e.g. 1-100μM BZ, inhibited PADPRP, whereas when the concentrations were raised, e.g. 100μM-10000μM BZ, the mono(ADP-ribose) transferases were inhibited. Lautier *et al*, (1994) also found poly(ADP-ribose) polymer synthesis to be inhibited by ~90% in intact cells following treatment of cells with the oxidising agent, xanthine-xanthine oxidase in the presence of 100μM BZ. However, the data presented herein shows only a 50% increase in cell kill at a concentration of 1mM BZ. Once again, this may represent either cell line specific effects, or more importantly could show dependence on the DNA damaging agent utilised, due to the subsets of lesions formed and the period of time over which damage occurs. However, the inhibition

of mono-(ADP-ribosyl)transferases can not be ruled out, and the use of a knockout$_{PADPRP}$ mouse cell system would be an elegant method for investigating such a possible involvement.

A generally accepted concept is that the progression of DNA single strand breaks through the replication fork accounts for a proportion of the cytotoxic effect of DNA damaging agents. However, this proposal suffers from a lack of mechanistic explanation. DNA single strand breaks entering onto daughter strands result in the presence of a non-coding lesion. In *E.coli* two mechanisms were predicted for overcoming replication blocks due to UV dimers, (1) DNA synthesis was reinitiated downstream of the template block, so creating discontinuities in the daughter strands which were infilled by a recombinational event, and (2) continuance of the replisome past the template lesion subsequent to an initial arrest (Friedberg, 1985). Cytotoxicity due to the presence of DNA strand breaks in daughter strands of mammalian cells could also be explained using the above two theories. Replication could halt at the break, but unlike the situation with the UV dimer, the replisome would totally lack a template base from which to code the leading/lagging strand, therefore further progression would not be possible. However, if the replisome could bypass the DNA break, replication would procede, but the single strand DNA break would convert to a double strand DNA break. A recombinational repair process would be subsequently required to replace the template base(s).

Interestingly, Boorstein *et al* (1992) found that a Chinese hamster cell line V79, resistant to HmdUrd, failed to express the HmUra DNA glycosylase. Cytotoxicity to HmUrd was reduced in the resistant cells as compared to the parental cell line, with the persistence of high levels of HmUra suggesting that the substitution of HmUra for thymidine in the DNA was not the cytotoxic lesion, but that their subsequent repair was. Further evidence indicative of the cytotoxic event being the presence of DNA strand breaks would be the observed increase in the number of SCEs found in the parental V79. Conversely, Chinese hamster ovary cells which overexpress the N-methylpurineDNA glycosylase show an

increased rate of removal of N-methylpurines from the DNA, but this was not associated with an increased level of resistance towards cell kill by the DNA damaging agents, MMS and MNNG (Coquerelle *et al*, 1995). The level of repair in this cell line would be enhanced, so increasing the numbers of DNA strand breaks. However, levels of SCEs were increased, and the DNA replication delay was prolonged. In the presence of inhibitors of PADPRP, e.g. BZ, 3AB, SCEs were observed to increase (Oikawa *et al*, 1980; Hori, 1981; Morgan & Cleaver, 1982; Schwartz *et al*, 1983). The inhibition of PADPRP increases the halflife of DNA strand breaks, so increasing the opportunity for SCEs. Recombinational repair of DNA double strand breaks would provide the mechanism for the PADPRP inhibitor dependent production of SCEs.

In conclusion, whilst the possibility that a small increase in DNA single strand break levels accounts for large increases in cytotoxicity, in cells co-treated with a DNA damaging agent and a PADPRP inhibitor cannot be excluded, it appears likely that there is no direct simple cause-effect relationship.

CHAPTER 6 : EFFECT OF LOW NMNAT ACTIVITY IN A TZ RESISTANT L1210 CELL LINE ON THE CELLULAR RESPONSE TO DNA DAMAGE

Due to the collaborative nature of the project, a number of results presented in this Chapter are shown with the kind permission of S. Jones, Cancer Research Unit, The University of Newcastle-upon-Tyne.

6.1 INTRODUCTION

2-β-D-ribofuranosylthiazole-4-carboxamide (Tiazofurin, TZ) is a C-nucleoside which exhibits significant oncolytic activity against a number of rodent and human tumour cell lines, e.g. murine leukaemias L1210 and P388, human leukaemia K562, Chinese hamster ovary CHO, and murine hepatoma 3924A. TZ was also found to be curative against the refractory Lewis lung carcinoma (Robins *et al*, 1982). Phase I and II clinical trials of TZ have shown a response rate of ~50% in the treatment of chronic myelogenous leukaemia (Tricot *et al*, 1989, Jayaram *et al*, 1992). However, a problem encountered with TZ treatment is the development of resistance.

Following treatment with TZ, the concentration of the guanine nucleotide pools, i.e. GMP, GTP, dGTP, were found to be decreased, and the activity of inosinate monophosphate dehydrogenase (IMPDH) was inhibited (Jayaram *et al*, 1982). IMPDH is the rate limiting enzyme in the biosynthesis of guanine, which converts IMP to xanthine monophosphate (XMP). The active metabolite of TZ was found to be an analogue of NAD^+, with the thiazole-4-carboxamide moiety of TZ replacing the nicotinamide group to form thiazole-4-carboxamide adenine dinucleotide (TAD) (Cooney *et al*, 1982,1983). The anabolism of TAD utilises an enzyme of the NAD^+ biosynthetic pathway. TZ is initially phosphorylated, by an as yet unidentified kinase to the 5'monophosphate, which is subsequently anabolised to the dinucleotide by nicotinamide

mononucleotide adenylyl transferase (NMNAT) which normally catalyses the reaction:- NMN + ATP → NAD^+ + PP_i. TAD inhibits IMPDH with a k_i of 1-2x10^{-7}M (Jayaram, 1985), which utilises NAD^+ as an obligatory co-factor. The IMPDH reaction procedes by a "bi-bi mechanism":- IMP binds to the enzyme followed by NAD^+, NADH is then released, followed by the product of the reaction, XMP (Yamada *et al*, 1988). TAD is considered to bind to the NADH site on IMPDH with the formation of an enzyme : XMP : TAD complex (Weber *et al*, 1985, Yamada *et al*, 1988).

The TZ related inhibition of IMPDH, and the subsequent depletion of the guanylate pools has been implicated as the cytotoxic mechanism of TZ. The administration of guanine prevents TZ induced cytotoxicity (Streeter & Robins, 1983). Both *de novo* synthesis and a salvage mechanism contribute to the guanylate pool. When either guanine and hypoxanthine are combined with the precursor PRPP, in the presence of hypoxanthine guanine phosphoribosyl transferase (HGPRT), guanylate and IMP are formed respectively. TZ does not directly affect this guanylate salvage pathway, but the increased concentrations of IMP due to inhibition of IMPDH are thought to result in a concentration dependent feedback inhibition of HGPRT (Weber *et al*, 1985). An added contribution to the high IMP concentrations could be due to activation of AMP deaminase following TZ treatment. High GTP concentrations usually repress this enzyme activity, which converts AMP to IMP (Weber *et al*, 1985).

Guanidine nucleotides are an important component of many metabolic reactions, e.g. RNA and DNA synthesis, the citric acid cycle, protein elongation, signal transduction (G proteins, cGMP). Therefore it is not surprising that guanylate depletion contributes to the cytotoxicity of TZ. Following TZ treatment, differentiation was observed subsequent to the down regulation of the proto-oncogenes, ras (Olah *et al*, 1988) and myc (Kharbanda *et al*, 1988). These encode for G proteins which couple membrane receptors to signal effector systems. GTPase activity leads to the production of the second messengers, i.e. cAMP and diacyl glycerol which subsequently activate regulatory enzymes, e.g. protein

kinase C, phospholipase C. When TZ was administered to HL60 cells with the prostoglandin PGE_2, a reduction in the concentration of cAMP was observed. However, when PGE_2 was substituted with forskolin, a compound that directly stimulates adenylate cyclase, the cAMP levels remained as control (Kharbanda *et al*, 1990). Also, mono-ADP-ribosylation by bacterial toxins, e.g. pertussis, cholera, was inhibited by TZ due to a depletion of the substrate, as the number of G protein sites occupied was reduced. However, the reduction in mono-ADPribosylation could also be explained by depletion of the NAD^+ concentration due to the presence of TAD (Kharbanda *et al*, 1990).

Prolonged TZ treatment can result in the development of resistance. Therefore, a number of cell lines resistant to TZ have been established, e.g. P388 (Jayaram *et al*, 1992), L1210 (Jayaram, 1985) and K562 (Jayaram *et al*, 1993) leukaemias, hepatoma 3924A (Jayaram *et al*, 1986), and CHO (Kuttan, 1989), using step-wise selection protocols, to examine possible resistance mechanisms. A major factor of a TZ resistant phenotype was found to be the impaired formation of the active metabolite, TAD, due to a reduction in the activity of NMNAT. Residual activities of <10% and <1% were found in K562 and L1210 resistant cells respectively. However, each cell line also presented individual resistance characteristics. The hepatoma 3924A TZ resistant cells showed a reduced uptake of TZ, together with an increase in the guanine salvage pathway (Jayaram *et al*, 1986), and the L1210 cells which also exhibited an increased salvage, and had an increased TADase activity, an enzyme function that was identified as responsible for the breakdown of TAD (Jayaram, 1985).

A wide range of naturally occurring sensitivities to TZ, both in murine and human cell lines (Ahluwalia *et al*, 1984, Jayaram, 1985), have been demonstrated. This appears to correlate with the TAD concentration which is regulated by the ratio of NMNAT activity (net formation of TAD) : TADase activity (net breakdown of TAD). In the more resistant cells the level of breakdown was increased and the level of formation decreased.

The P388 resistant cell line was found to exhibit collateral sensitivity to a variety of

cytocidal drugs, including a 5-10 fold increased sensitivity to 5FU as compared to the parental line. The reduced NAD^+ levels in DNA damaged, TZ resistant cells was proposed as a possible factor responsible for such sensitivities. Similar rates of repair of X ray induced DNA strand breaks were observed in the TZ sensitive and resistant cells, but in the presence of the PADPRP inhibitor 3AB, the resistant cells failed to repair the DNA strand breaks, suggestive of the involvement of PADPRP. The sensitive cells were still capable of strand break repair (Jayaram, 1985).

The NAD^+ biosynthetic enzyme NMNAT, has recently been purified to homogeneity from human placenta, utilising a variety of chromatographic procedures (e.g. absorption chromatography, FPLC) (Emanuelli *et al*, 1992). Previously, the yeast NMNAT enzyme had been the most characterised. The mammalian enzyme was characterised as a quaternary structure, possessing four apparently identical subunits of 33 KDa each. The mechanism of action was Bi-Bi, as for the yeast enzyme, with K_m values for NMN, ATP, NAD^+ and PP_i of 38μM, 23μM, 67μM, and 125μM respectively. ADP-ribose was found to inhibit the enzyme in a non-competitive manner, although the K_i was 950μM. Therefore, due to the high K_i, even under conditions of high ADP-ribose concentrations, e.g. damage to the DNA resulting from treatment with alkylating agents leading to the formation of ADP-ribose as a breakdown product of ADP-ribose polymer, it would be unlikely that ADP-ribose could significantly inhibit NMNAT activity.

6.2 AIMS

Characterisation of the TZ resistant phenotype has identified one of the main features as being the loss of NMNAT activity, the enzyme responsible for the formation of TAD. The intracellular role of NMNAT involves the final catalytic step in the formation of NAD^+, and therefore a reduction of NMNAT activity could lead to a depletion of the intracellular NAD^+ concentration. NAD^+ is important for the maintainance of the cellular redox potential, but ADP-ribosylation reactions, essentially poly(ADP-ribosyl)ation,

account for the majority of NAD^+ catabolism (see Section 1.1.1 and 1.1.2).

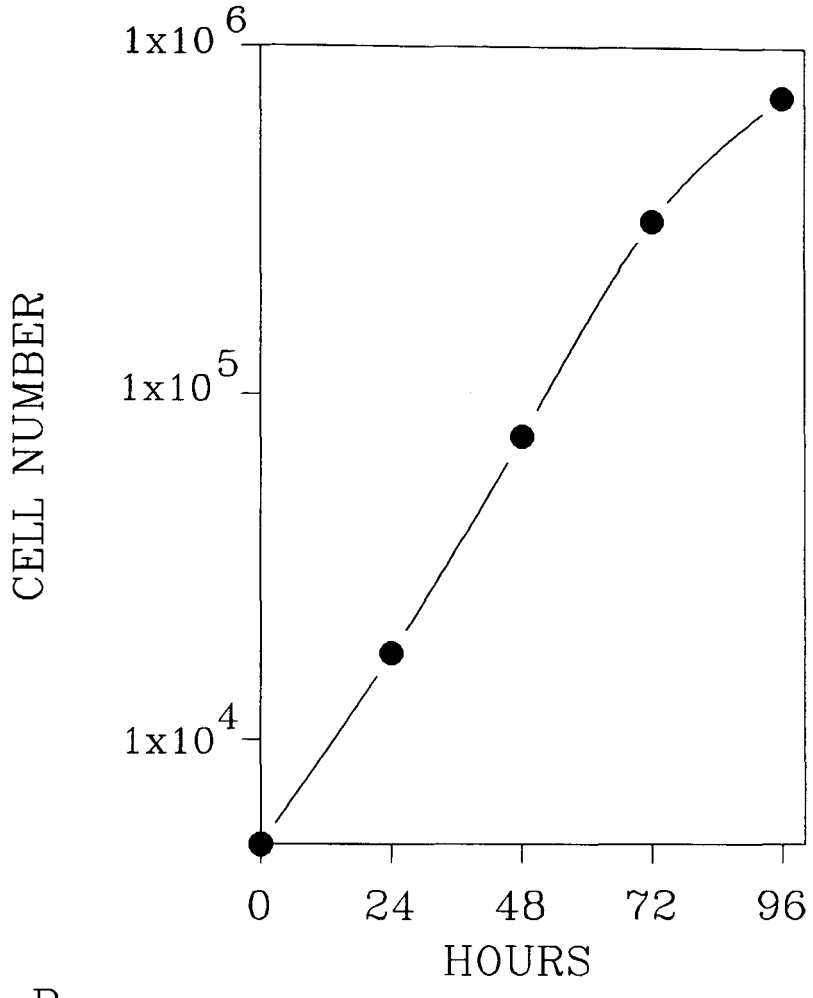
PADPRP is activated in response to breaks in the DNA strands, and has a well documented role in the DNA repair process (see Section 1.5). The use of PADPRP inhibitors in association with DNA damaging agents, e.g. alkylating agents (see Chapter 3, Section 3.6), ionising radiation (Lunec *et al*, 1984), has been observed to lead to a potentiation of cytotoxicity. The competitive inhibition by the PADPRP inhibitor was proposed to slow the release of PADPRP from the DNA strand break end. Subsequently, the inaccessibility of the break to DNA repair enzymes would be extended, resulting in an increased break level and cytotoxicity.

However, in some instances the presence of an inhibitor of PADPRP exhibits a protective effect, with an observed decrease in cytotoxicity (e.g. pancreatic islet cells treated with nitric oxide (NO) (Kallman *et al*, 1992, Heller *et al*, 1995), NO induced neurotoxicity (Zhang *et al*, 1994)). NO has been considered as a DNA damaging agent due to the formation of nitrite (NO_2^-) which can deaminate purines and pyrimidines. Zhang *et al* (1994) utilising rat brain extracts incubated with increasing concentrations of NO found PADPRP activity was increased upto 3 fold, as cell survival decreased. However, in the presence of the PADPRP inhibitors, BZ, 3AB, and 4AB, neurotoxicity was decreased in parallel with their potencies as PADPRP inhibitors (BZ, 3AB and 4AB increased cell survival by ~30%, ~20%, and ~2% respectively). The activation of PADPRP in response to DNA strand breaks results in a massive depletion of cellular NAD^+ (levels can be reduced to < 10% of control). The transfer of 1 ADP-ribose unit by PADPRP consumes 1 molecule of NAD^+, and 4 ATP molecules are subsequently required to regenerate the NAD^+ from nicotinamide. NAD^+ is required in numerous metabolic reactions, including the biosynthesis of ATP, the main energy form of the cell. In such circumstances, the "suicide" of the cell was predicted (Sims *et al*, 1982, 1983). A large reduction in NMNAT activity could also potentially result in the failure to resynthesise NAD^+ at the required rate, following NAD^+ depletion, and so result in a suicide response.

However, in the presence of a PADPRP inhibitor, the NAD^+ levels would be preserved, and cytotoxicity could be lowered.

The results presented in this Chapter utilised a TZ resistant L1210 cell line which we selected using a step wise selection protocol, and its parental L1210 counterpart (WT). The following investigations were then performed:- (1) The NMNAT activity and the intracellular NAD^+ concentration of the WT and TZR cell lines was measured to characterise the mechanism of resistance to TZ. (2) The survival of the TZR and WT L1210 cells treated with alkylating agents in the absence or presence of a PADPRP inhibitor was assessed to discover whether low NMNAT activity and consequently low NAD^+ levels would sensitise cells by reducing PADPRP function or exacerbate NAD^+ depletion. Two hypotheses could be predicted: (i) low NAD^+ levels would reduce PADPRP activity, and so sensitivity of the PADPRP inhibitors would increase. (ii) reduced NMNAT activity, and low NAD^+ levels in a situation of DNA damage could result in cell suicide, due to an inability to resynthesise NAD^+ at a sufficiently high rate. Therefore, PADPRP inhibitors would be protective. Both hypotheses could also predict that a reduced NMNAT activity would lead to increased sensitivity to the alkylating agent alone. (3) The effect of alkylation damage on the NAD^+ levels in the WT and TZR L1210 cells was considered, to determine if a reduced, starting concentration of NAD^+ and/or inability to resynthesise NAD^+ at high rates resulted in an altered pattern of NAD^+ depletion. (4) The NMNAT activity of WT L1210 cells exposed for increasing incubation periods to fixed concentrations of TM was assessed, as ADP-ribose was postulated as a possible, transient regulator of NMNAT activity (Emanuelli *et al*, 1992). A number of biological endpoints were utilised in the assessment including cytotoxicity studies, DNA strand break analysis, and characterisation of the NMNAT activities.

A.



B.

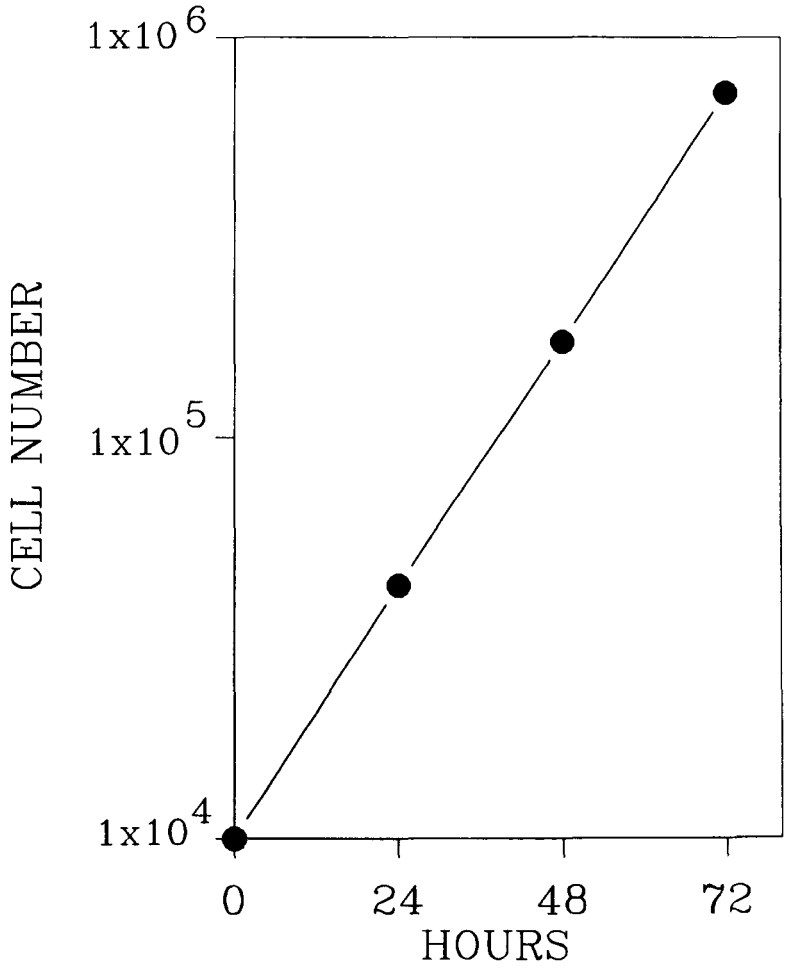


Figure 6.1: Growth curve analyses for A. L1210 cells B. TZR L1210 cells.. Each point is representative of triplicate samples.

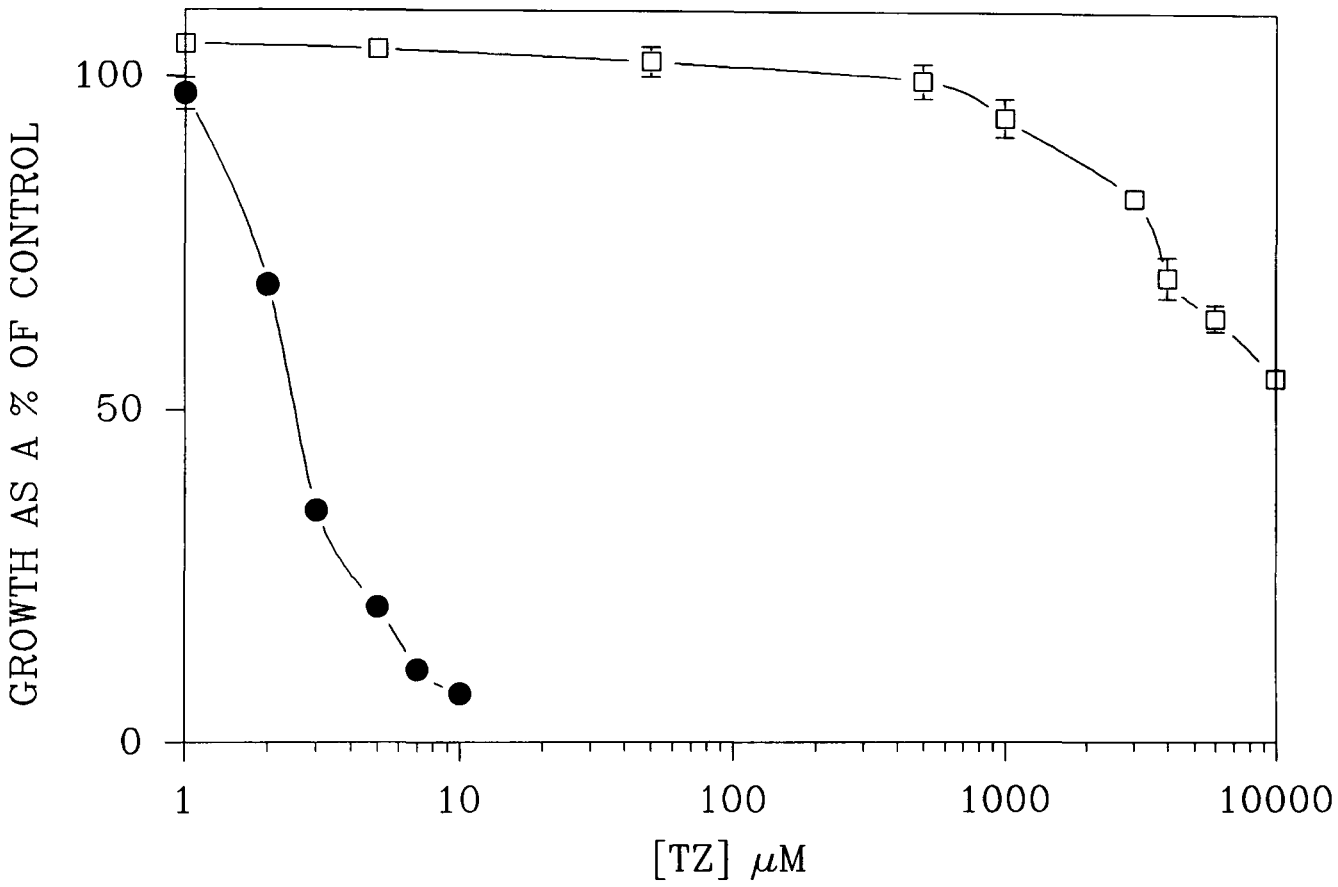


Figure 6.2: The growth inhibitory effect of a 48 hour continuous exposure to increasing concentrations of TZ on ● WT and □ TZR L1210 cells. The points are the average of three individually dosed samples from a single representative experiment.

6.3 ISOLATION AND CHARACTERISATION OF TZ RESISTANT L1210 CELLS.

6.3.1 Comparison of WT and TZR cell growth.

L1210 cells resistant to TZ were selected using a step-wise selection protocol as described in Chapter 2, Section 2.7. Resistance to TZ was achieved by subculturing the L1210 cells in RPMI-medium containing TZ. Initially the IC_{50} of 2.7μM TZ was used, but the TZ concentration was raised at intervals until the cells were capable of growth in 2mM TZ. This represents an ~750 fold increase in the concentration of TZ. An analysis of the growth rates for the two cell lines found a comparable level of cell growth, with both lines exhibiting a doubling rate of ~13 hours (see Figure 6.1, A & B).

Figure 6.2 (presented with permission from S. Jones) shows that a 48 hour incubation in the presence of increasing concentrations of TZ caused the growth of the sensitive L1210 cells, subsequently referred to as WT, and the TZ resistant L1210 cells, referred to as TZR, to decrease in a concentration dependent manner. However, the WT cell line had an IC_{50} to TZ of 2.7μM whereas the TZR cells exhibited an IC_{50} of >10000μM. This represented an ~4000 fold increase in resistance to TZ.

6.3.2 Measurement of NMNAT activity in WT and TZR cell lines.

The reduced activity of the biosynthetic enzyme, NMNAT was found to be one of the main characteristics of TZ resistance in the published TZ resistant cell lines (see Section 6.1). NMNAT catalyses the reaction: NMN + ATP → NAD^+ + PP_i. Therefore, the NMNAT activity of both the WT and the selected TZR cell lines was measured to determine whether our TZR L1210 line also exhibited a reduction in NMNAT activity. However, in the course of this work I discovered that the published assay for the measurement of NMNAT activity lacked sensitivity when transferred from tumour samples to tissue culture cells. Therefore, I have coupled together the preparative sections of the NMNAT assay with the NAD^+ colourimetric cycling assay which increased the level of sensitivity ~16 fold (see Chapter 2, Sections 2.14 and 2.15).

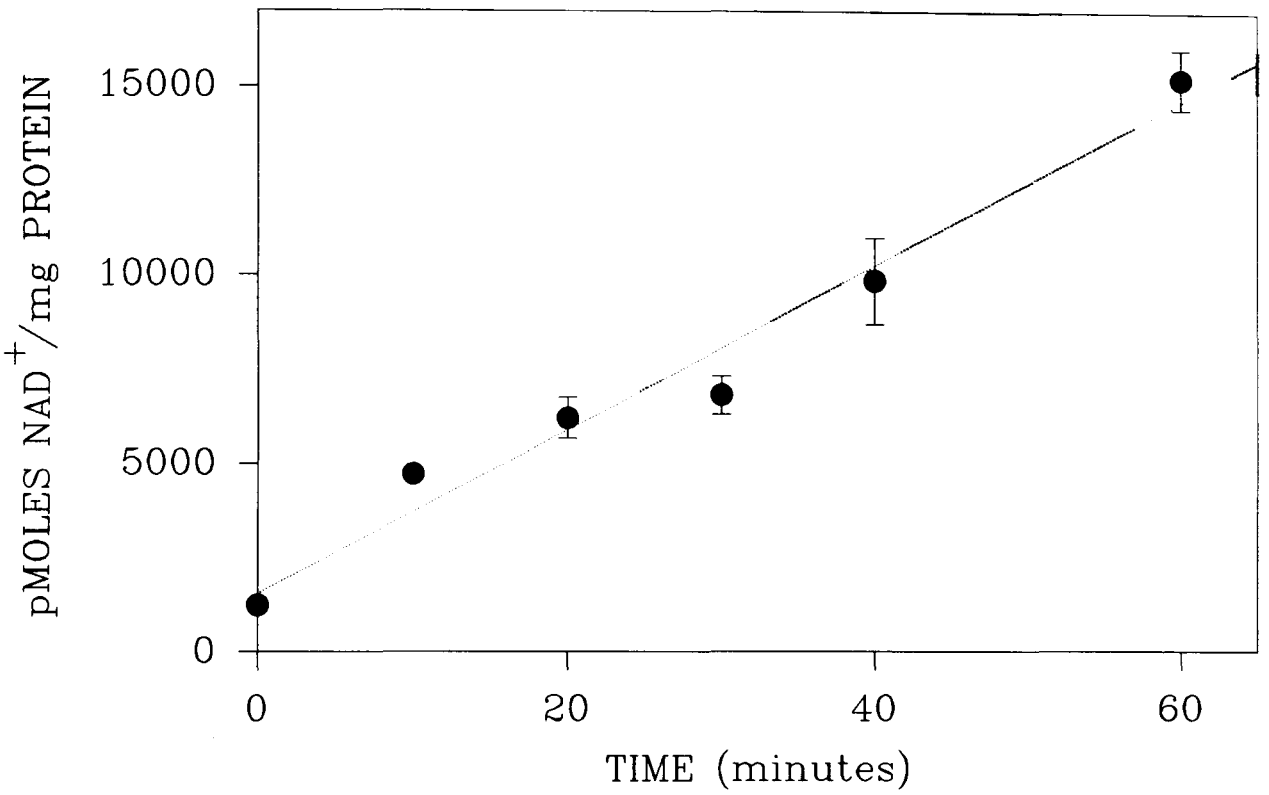


Figure 6.3: Linear regression analysis of the effect of time on the level of NAD^+ formed in the NMNAT assay. Each point represents the mean of triplicate samples.

Prior to the determination of NMNAT activity in the WT and TZR cell lines, several of the experimental variables were characterised. Initially, the length of the incubation period to determine the level of NMNAT activity was assessed. Figure 6.3 shows that pMoles NAD^+ formed/mg protein increased in a linear manner with time, up to 1 hour. An incubation period of 30 minutes was utilised for subsequent experiments. The effect NMN had on the NAD^+ cycling reaction was then assessed. ADH converts ethanol to the corresponding aldehyde, with the concomitant reduction of NAD^+ to NADH. The nicotinamide moiety of both NAD^+ and NADH forms several interactions with the dehydrogenase active site (Holbrook *et al*, 1975), therefore the nicotinamide moiety of NMN present in the reaction mix could also form possible interactions with the ADH, and so interfere in the reaction in the NAD^+ assay. However, NMN was found to have no effect on the rate of change of absorbance over the 5 minute reaction period, either in the absence (absorbance values were as control, i.e. -NAD^+, results not shown) or presence of the natural substrate NAD^+ (+NAD^+ = ΔAbs 0.15, +NAD^+ + NMN = ΔAbs 0.14).

Table 6.1 gives the NMNAT activities for the WT and TZR L1210 cells. In the WT cells, NMNAT activity was measured at ~400 pMoles NAD^+/mg protein/minute, whereas the activity of the NMNAT in the TZR cells was ~11 pMoles NAD^+/mg protein/minute. The activity of the NMNAT in the TZR cells was calculated as < 3% of that in the WT cells.

6.3.3 Measurement of intracellular NAD^+ levels in WT and TZR cell lines

As NMNAT catalyses the final reaction in the anabolism of NAD^+, it was postulated that the intracellular NAD^+ levels in the TZR cells could be reduced in comparison to the NAD^+ levels of the WT cells. Utilising the NAD^+ colourimetric assay (see Chapter 2, Sections 2.13 and 2.15) the intracellular NAD^+ content of the TZR cells was measured as 3768 pMoles NAD^+/mg protein, which represented an ~40% reduction of NAD^+ as

compared to the WT cells. The intracellular NAD^+ level for the WT cells was measured at 6070 pMoles NAD^+/mg protein (see Table 6.1) (shown with the kind permission of S. Jones).

TABLE 6.1

CELL LINE	NMNAT ACTIVITY pMoles NAD^+/mg protein/minute	CELLULAR NAD^+ LEVEL pMoles NAD^+/mg protein
WT	400.9 ± 27.0	6070 ± 195
TZR	10.7 ± 3.7	3768 ± 213

Table 6.1: The extractable NMNAT activity and the intracellular NAD^+ levels in WT and TZR L1210 cells was measured as described in Chapter 2, Sections 2.14 and 2.13, respectively. A sample of the cell extract was retained for determination of the protein concentrations (see Section 2.15, Chapter 2). The calculated values were averaged from three independent experiments ± SE.

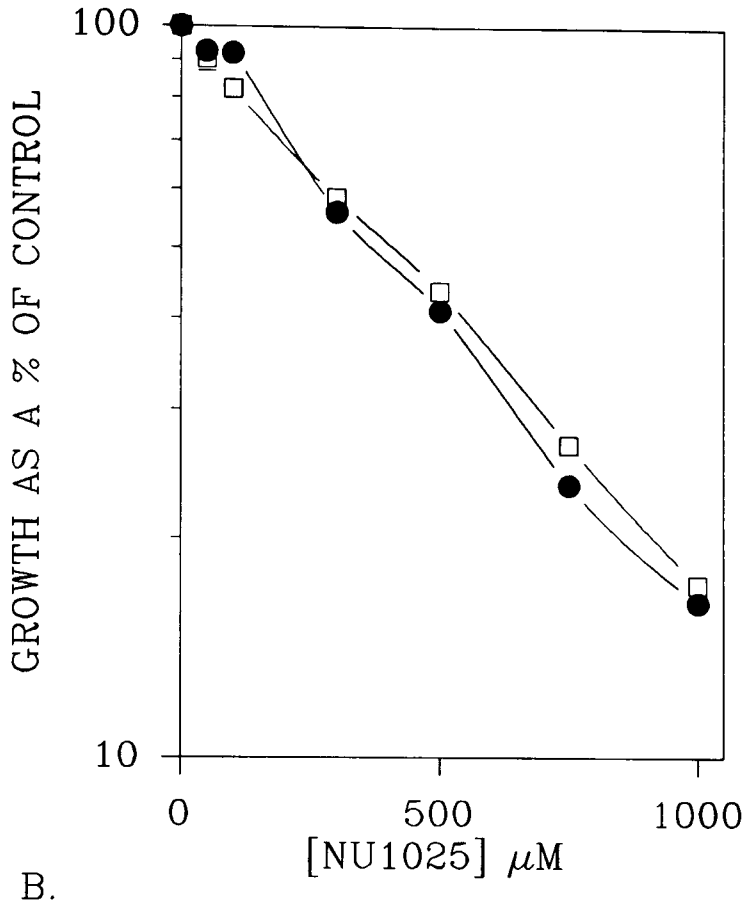
6.4 COMPARISON OF THE GROWTH INHIBITORY EFFECTS OF TM AND NU1025 ON WT AND TZR CELLS.

Data presented with the kind permission of S. Jones.

6.4.1 Effects of increasing NU1025 concentrations in the absence or presence of a fixed TM concentration.

The ability of the PADPRP inhibitor NU1025 to inhibit the growth of the WT and TZR L1210 cells was assessed. Previously, a 48 hour continuous exposure of L1210 cells to NU1025 was shown to reduce cell growth in a concentration dependent manner (see Chapter 3, Section 3.4). Figure 6.4A shows that the growth of the TZR cells following

A.



B.

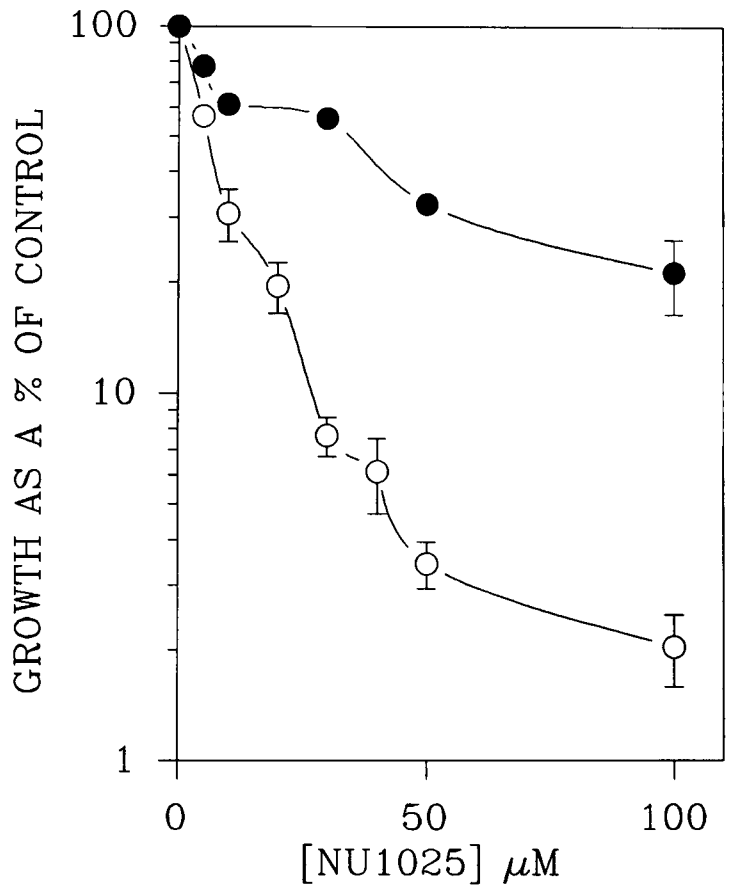
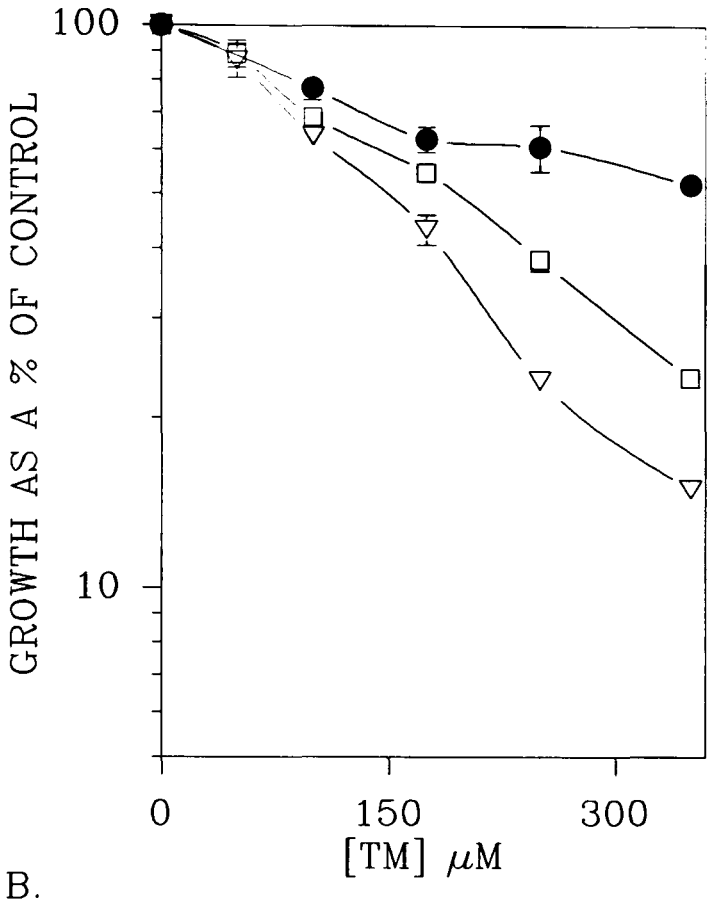


Figure 6.4: Growth inhibitory effects of a 48 hour continuous exposure of ● WT and □ TZR L1210 cells to A. increasing concentrations of NU1025 alone or B. in the presence of 100μM TM. Points were averaged from at least triplicate samples taken from two independent experiments.

A.



B.

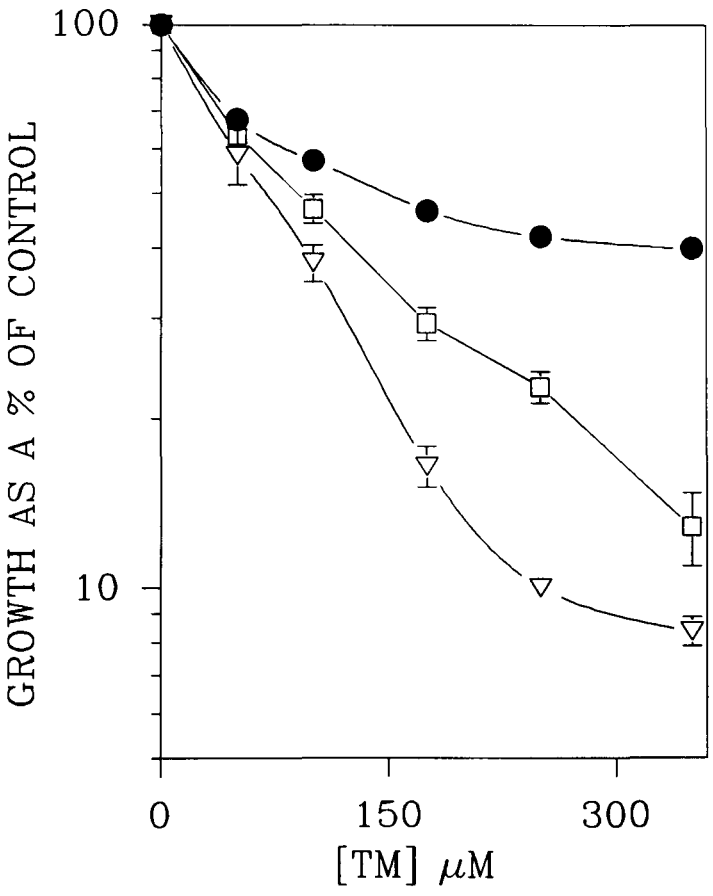


Figure 6.5: The growth inhibitory effect of a 48 hour continuous exposure of A. WT and B. TZR L1210 cells to increasing concentration of TM ● in the presence of fixed concentration of NU1025. A. □ 30μM NU1025, ▽ 50μM NU1025 B. □ 10μM. NU1025, ▽ 30μM NU1025. Points represent triplicate. samples from a single experiment.

a 48 hour continuous exposure, was also inhibited in a concentration dependent manner, with an similar IC_{50} value compared to the IC_{50} value for the WT cells (WT, ~370μM; TZR, ~368μM).

The subsequent analysis performed assessed the growth inhibitory effect of a 48 hour continuous coincubation with increasing concentrations of NU1025 in the presence of a fixed dose of TM on both the WT and TZR cells. 100μM TM was chosen as this allowed comparisons with the WT L1210 results already presented in Chapter 3, but note that this experiment was carried out in parallel for the purpose of a direct comparison. (In the subsequent Section 6.4.2 and Figure 6.5, A and B, a 48 hour incubation to TM alone resulted in a 3 fold greater inhibition of TZR cell growth as compared to the WT growth. However, for the experiments presented in Figure 6.4B the effect of 100μM TM alone on cell growth was normalised to 100%.) The growth of both the WT and TZR cell lines was inhibited in a concentration-dependent manner (see Figure 6.4B). However, unlike the analogous IC_{50} values for NU1025 alone, growth of the TZR cells was inhibited to a greater extent than the WT cells. In the presence of 100μM TM, growth of the WT cells was reduced ~10-fold from 370μM NU1025 to ~35μM, whereas the growth of the TZR cells was reduced ~70-fold, from ~368μM to ~6μM.

6.4.2 Effect of increasing TM concentrations in the absence or presence of NU1025.

The effect a 48 hour continuous exposure to TM alone had on the growth of WT and TZR L1210 cells was assessed. Figure 6.5A and B show that in both cell lines growth was inhibited in a concentration dependent manner. However the IC_{50} for the WT cells was ~385μM TM, whereas that for the TZR cells was ~156μM TM, representing an ~2.5 fold decrease in the TM concentration required to inhibit growth to a similar degree.

In Chapter 3, Section 3.6.2, it was demonstrated that growth inhibition of WT L1210 cells treated with increasing concentrations of TM in the presence of fixed concentrations of NU1025 was potentiated. This increase in the growth inhibition by NU1025 was

concentration dependent. Figure 6.5. A and B, compare the effect increasing concentrations of TM, in the presence of fixed concentrations of NU1025, had on the growth of WT and TZR L1210 cells. NU1025 potentiated the growth inhibitory effect of TM in both cell lines. A 3 fold lower concentration of NU1025 was required to reduce TZR cell growth to a similar level as the WT cells. Table 6.2 shows that 30μM NU1025 resulted in an ~2 fold enhancement of growth inhibition in WT cells whereas only 10μM NU1025 was required in the TZR cells. However, the difference observed between 50μM and 30μM NU1025 in the WT and TZR cells respectively was probably not significant. A statistical analysis of enhancement factors would be required, but this requires data taken from three independent experiments. The data presented in Table 6.2 was compiled from just a single experiment, albeit triplicate samples.

TABLE 6.2

CELL LINE	DOSING SCHEDULE	GROWTH INHIBITION IC_{50}	ENHANCEMENT FACTOR
WT	TM	385.4μM	
	TM + 30μM NU1025	181.9μM	~2.0
	TM + 50μM NU1025	139.6μM	~3.0
TZR	TM	156.6μM	
	TM + 10μM NU1025	84.1μM	~2.0
	TM + 30μM NU1025	65.0μM	~2.5

Table 6.2: The effect of a 48 hour continuous exposure to increasing concentrations of TM, in the absence or presence of fixed concentrations of NU1025, on the growth of WT and TZR L1210 cells. The data represents three independently dosed samples from a single experiment.

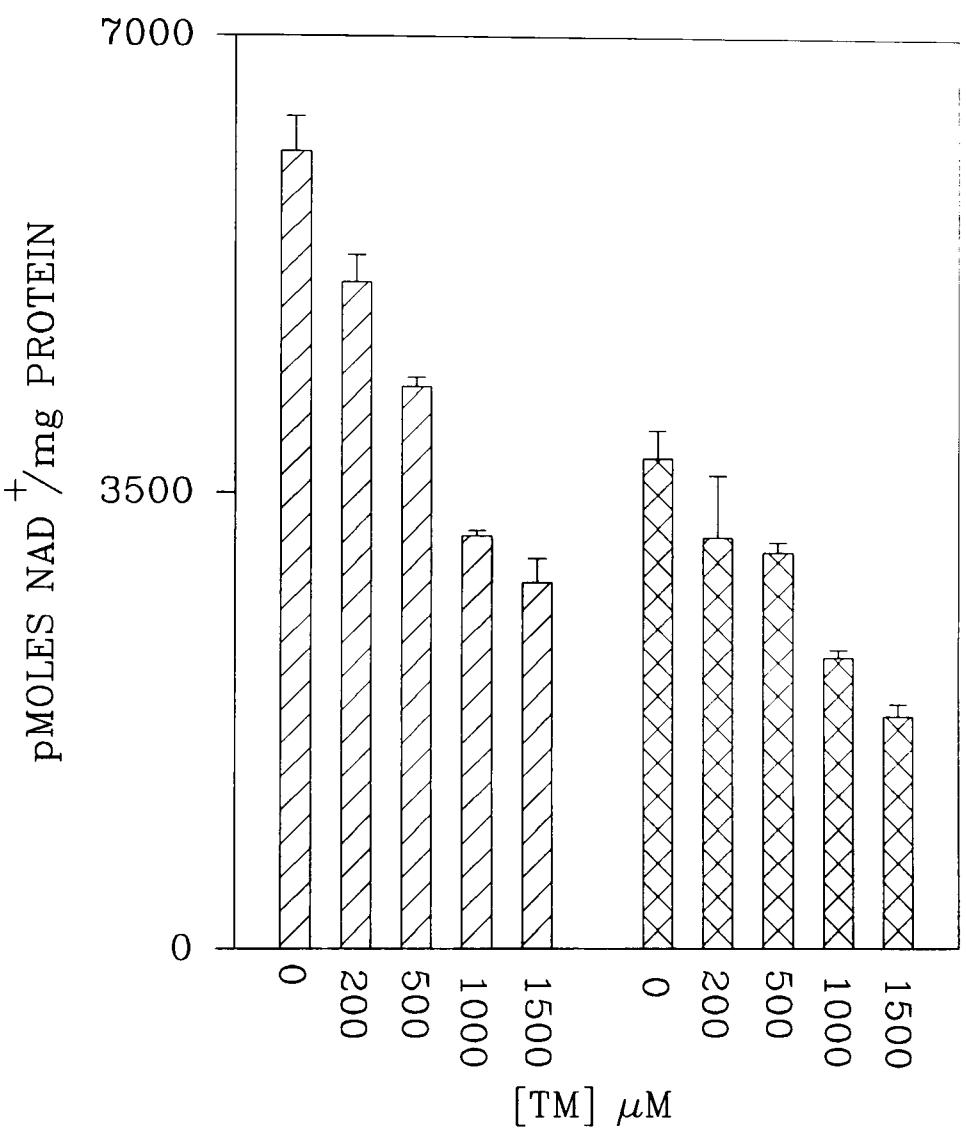


Figure 6.6: Effect of a 4 hour exposure to increasing concentrations of TM on intracellular NAD^+ concentrations of ▨ WT and ▩ TZR cell lines. Points were averaged from individually dosed, triplicate samples from a single experiment.

6.5 NAD^+ LEVELS OF TM TREATED WT AND TZR L1210 CELLS.

The intracellular NAD^+ levels of WT and TZR cells treated for a 4 hour continuous exposure to increasing concentrations of TM were determined. The NAD^+ levels in both cell lines was decreased in a concentration dependent manner (see Figure 6.6)(shown with permission from S. Jones). The initial NAD^+ level of the TZR cells was ~60% of that of the WT cells (see Table 6.1), but following TM treatment, proportionate NAD^+ depletion was observed for each TM concentration. For example a 50% NAD^+ depletion occured with 1mM TM in both the WT and the TZR cells (TZR = 2222pMoles NAD^+/mg protein as compared to control levels of 3778pMoles NAD^+/mg protein; WT = 3185pMoles NAD^+/mg protein as compared to control levels of 6111pMoles NAD^+/mg protein).

6.6 STRAND BREAK LEVELS IN DNA DAMAGED WT AND TZR L1210 CELLS.

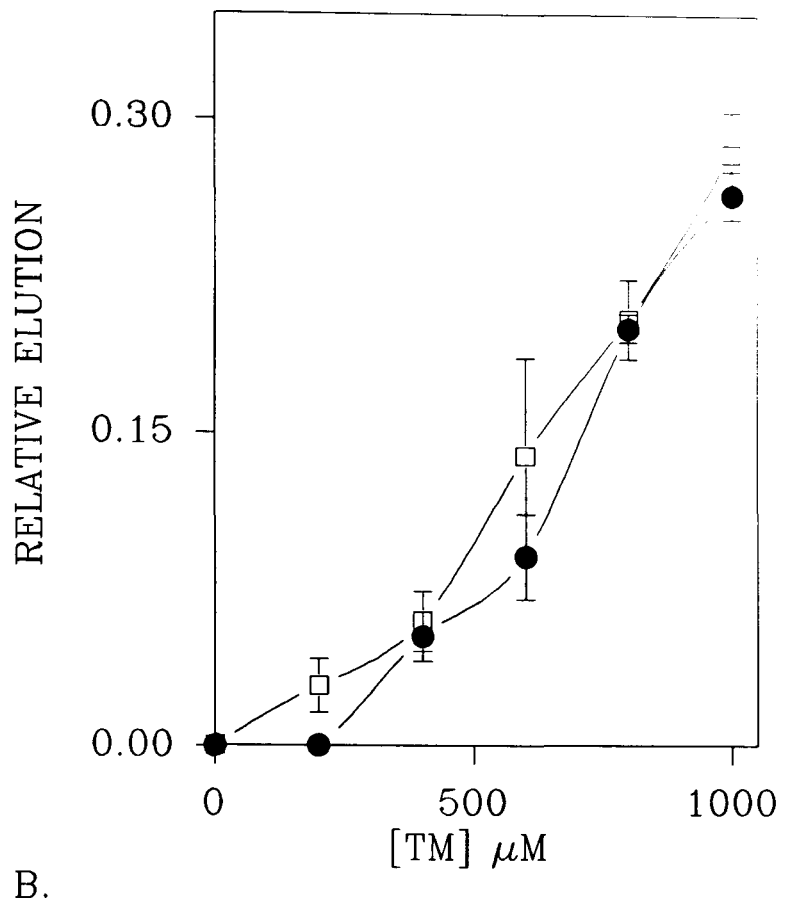
6.6.1 Effect of increasing concentrations of TM on the DNA strand break frequency in WT and TZR L1210 cells.

TM treatment results in DNA damage which is subsequently repaired by the BER process with AP endonuclease action leading to the formation of DNA single strand breaks. Chapter 4, Section 4.4 showed that R.E. values increased with increasing TM concentrations in treated WT cells. Figure 6.7A shows that the R.E. values for both the WT and TZR L1210 cells, in a head on comparison, treated with increasing concentrations of TM for an incubation of 1 hour, increased in a concentration dependent manner. There was a small, but not significant increase in the R.E. values of the TZR cell line compared to WT, indicating that TM treatment caused little or no additional increase in the DNA single strand break levels in the TZR cells as compared to the WT cell line.

6.6.2 The effect of NU1025 on a fixed level of TM induced damage in WT and TZR L1210 cells.

The R.E. profiles for both WT and TZR cells coincubated with 150μM TM in the

A.



B.

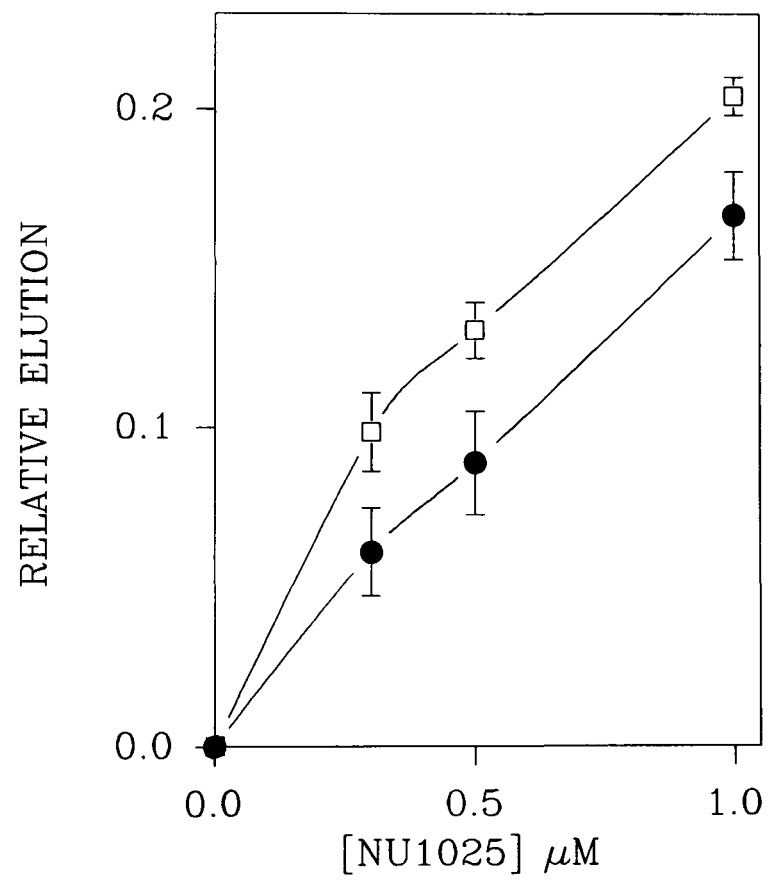


Figure 6.7: A. Relative elution values of the effect of increasing concentrations of TM on ● WT and □ TZR L1210 cells. B. Relative elution values of ● WT and □ TZR L1210 cells treated with 150μM TM in the presence of increasing concentrations of NU1025. The points represent the mean of three independent experiments.

presence of increasing concentrations of NU1025 are shown in Figure 6.7B. In Chapter 4, Section 4.6.1, when WT L1210 cells were coincubated with increasing NU1025 concentrations in the presence of 150μM TM, a concentration dependent increase in the R.E. values was observed. Figure 6.7B shows that the R.E. profile of TZR cells incubated with increasing concentrations of NU1025 and 150μM TM also increased in a concentration dependent manner. However, the rate of increase of DNA single strand break levels in the presence of the NU1025 dose was significantly increased at the lower concentrations of NU1025 in the TZR line as compared to the WT cells and this difference was maintained at the higher concentrations of NU1025. This is demonstrated by the concentrations of 0.74μM and 0.47μM NU1025 that were required by the WT and TZR L1210 cells respectively to give equivalent arbitrary R.E. values of 0.125. This represents an ~1.5-fold reduction in the concentration of NU1025 required to give the same level of DNA single strand breaks.

6.7 THE EFFECT OF TM TREATMENT ON NMNAT ACTIVITY.

TM treatment results in the formation of DNA single strand breaks due to BER of modified bases. The presence of the breaks in the DNA activates PADPRP which is the main consuming reaction of intracellular NAD^+. NMNAT, the enzyme responsible for the final reaction in the biosynthesis of NAD^+ is a chromatin bound enzyme with a frequency of 1 NMNAT enzyme/10-20 nucleosomes (Uhr & Smulson 1982). This is a similar frequency as observed for PADPRP. Therefore, because of its similar location to PADPRP, it was postulated that NMNAT activity may be up-regulated by some mechanism in response to PADPRP activation in order to increase the rate of NAD^+ biosynthesis, and so compensate for the NAD^+ catabolism during rapid (ADP-ribose) polymer formation in response to DNA damage.

Initially, the effect that a either 4 or 6 hour continuous exposure to increasing concentrations of TM had on NMNAT activity was determined (see Table 6.3). These

times were chosen as the peak level of single strand DNA breaks was observed at 4-6 hours, and >99% of the TM would have been degraded to MTIC at this time.

TABLE 6.3

[TM] μM	4 HOUR μMoles NAD^+/mg protein /min	% OF CONTROL	6 HOUR μMoles NAD^+/mg protein /min	% OF CONTROL
0	538.8 ± 10.7	100	692.9 ± 73.6	100
500	724.1 ± 19.7	134	966.6 ± 41.6	120
1000	624.7 ± 6.1	116	965.4 ± 23.4	140

Table 6.3: L1210 cells were treated with TM and the extractable NMNAT activity measured, following either a 4 or 6 hour incubation (see Section 2.14, Chapter 2). The values represent the average of three independently dosed samples in a single experiment ± SE.

At both 4 and 6 hours, at TM concentrations of 0.5 and 1mM there appeared to be an increase in the activity of NMNAT which was calculated as being statistically significant utilising a student's paired T test statistical analysis (p values < 0.05). However, the degradation period of TM (e.g. ~6 hours), and the low level of NAD^+ depletion, (e.g. a 50% NAD^+ depletion was observed only after 6 hours with 2mM TM, see Chapter 3, Section 3) suggested only low level activation of PADPRP. Therefore, the effect of MTIC, the more direct acting active metabolite of TM, was considered. MTIC has a half-life of ~4 minutes with an ~50% NAD^+ depletion observed after a 30 minute incubation with 1mM MTIC (see Chapter 3, Figure 3.9B).

A concentration of 1mM MTIC was chosen for the study, and the effect of time on NMNAT activity was determined. L1210 cells were incubated for increasing periods of

time, up to 24 hours, and the NMNAT activity was determined. A preliminary study indicated changes in NMNAT activity occurred at both 6 and 24 hours (control = 599 ± 40pMoles NAD^+/mg protein/minute, 6 hour = 481 ± 18pMoles NAD^+/mg protein/minute, 24 hour = 858 ± 104pMoles NAD^+/mg protein/minute), therefore further studies concentrated on these two time points. Table 6.4 shows that after a 6 hour incubation with 1mM MTIC there was no significant difference in NMNAT activity. However, after 24 hours in the presence of 1mM MTIC the activity of the NMNAT was increased to 134% of control. However, using a student's T test analysis this increase was not shown to be statistically significant (p value > 0.2).

TABLE 6.4

TIME (HOURS)	NMNAT ACTIVITY pMoles NAD^+/mg protein/minute	% OF CONTROL
0	502.9 ± 27.7	100
6	479.9 ± 13.7	95
24	673.6 ± 68.0	134

Table 6.4: The effect of either 6 or 24 hour continuous exposure to 1mM MTIC on the extractable NMNAT activity in WT L1210 cells. The values were averaged from three independent experiments ± SE.

6.8 DISCUSSION

PADPRP, when activated in response to breaks in the DNA, catalyses the formation of the polymer (ADP-ribose), which covalently modifies itself and other nuclear proteins, e.g. chromatin proteins, DNA repair enzymes (see Chapter 1, Section 1.2). The substrate for PADPRP is NAD^+, and (ADP-ribose) polymer formation accounts for the major

proportion of NAD^+ catabolism. >95% of the intracellular NAD^+ is confined to the nucleus (Rechsteiner *et al*, 1976).

The results presented in this Chapter have considered the effect of a reduced intracellular NAD^+ pool and biosynthetic capacity on the ability of PADPRP function in the response to DNA damage. Several reports have shown the intracellular NAD^+ levels of tumour cells to be reduced as compared to somatic cells (Lillian & Jedeikin, 1955; Branster & Morton, 1956; Glock & McLean, 1957). Also, solid tumours are known to contain a considerable proportion of hypoxic cells, which results in a large shift in the redox potential, with the NADH:NAD^+ ratios greatly increased (Kennedy *et al*, 1980). NADH is itself an inhibitor of PADPRP with a k_i of ~5μM (Ueda *et al*, 1982), as compared to the k_i for 3AB, of ~1.8μM (Purnell & Whish, 1980). Therefore, in the presence of lower NAD^+ levels PADPRP function would be impaired, resulting in an increased sensitisation to DNA damaging agents, which in the presence of a PADPRP inhibitor would be potentiated, due to competitive inhibition.

However, a second situation may well arise due to reduced NMNAT activity. PADPRP activation in DNA damaged cells would decrease already low NAD^+ levels, but the low NMNAT activity would be unable to resynthesise sufficient NAD^+, so cell suicide could result. A study of PADPRP function in reduced NAD^+ conditions could have significant biological implications, e.g. for cancer chemotherapeutic strategies.

The IC_{50} for TZ in L1210 cells, as determined by growth inhibition analysis was 2.7μM. Several reports in the literature have shown that resistance to TZ occurs due to the presence of lower TAD levels, resulting from a reduced NMNAT activity (Jayaram *et al*, 1992; Jayaram, 1985; Jayaram *et al*, 1993; Jayaram *et al*, 1986). This consequently reduces the intracellular NAD^+.

Using a step-wise selection protocol, we selected for TZ resistance in an L1210 cell line. By growth inhibition analysis, the TZR cell line exhibited a >4000-fold increased resistance to TZ as compared to the sensitive WT cells. A comparison of the NAD^+ levels

and the NMNAT activities in the WT and TZR cells showed that the NAD^+ level and NMNAT activity in the TZR cells was ~60% and <3% respectively of that of the WT. This reduced NMNAT activity compares well with the values published for other TZ resistant cell lines (Jayaram, 1985; Jayaram *et al*, 1993). The growth of the TZR cell line correlated extremely well with that of the WT cells, with both cell lines doubling approximately every 13 hours. This would suggest that the reduced NMNAT activity and intracellular NAD^+ pool sizes remained compatible with normal cell growth, and indicates that intracellular NAD^+ levels are in vast excess of requirements under normal growth conditions. Rechsteiner *et al* (1976) estimated that just 5% of the total NAD^+ pool was required for "unstressed" cell growth. The effect of "stress" in the form of damage to the DNA on the growth of TZR cells was then considered. TM results in base modifications which are repaired by both BER and mismatch repair processes. BER, due to AP endonuclease action results in the formation of DNA single strand breaks which activate PADPRP. TM treatment resulted in a concentration dependent inhibition of growth with both the WT and TZR cells. However, TZR cell growth was reduced to 50% of control utilising a 2.5-fold lower concentration of TM than required to reduce WT growth by 50% (IC_{50} ~156μM TM for TZR cells as compared to ~385μM in the WT cells). In the aims of this Chapter, two possible scenarios were postulated to explain the possible effects of low NMNAT activity on cell growth and survival. In the presence of a BER connected damaging agent, growth inhibition and cytotoxicity in the TZR cells was predicted to increase in both scenarios. This Chapter only considers the growth inhibitory effects. However, Chapter 3 demonstrated a close correlation between the growth inhibition and cytotoxicity studies resulting from TM treatment of L1210 cells. In the first scenario, it was suggested that lower levels of the substrate, NAD^+ (resulting from low NMNAT function), for PADPRP would impair the release of the enzyme from the DNA single strand break ends, as (ADP-ribose) polymer formation required for repulsion of PADPRP from the DNA would be slowed. Access of the repair enzymes to the damaged

site would be subsequently delayed. This would only be a viable theory if the NAD^+ levels fell to about the K_{mNAD+} of the enzyme (~50μM, Ueda *et al*, 1982), as until that point PADPRP would continue to function at a constant rate. However, below the K_m value the NAD^+ would become rate limiting, and only then would the activity of PADPRP be slowed. However, the second scenario suggested that if the NAD^+ biosynthetic capacity was reduced, then the activation of PADPRP in response to DNA damage would result in a more rapid "suicidal NAD^+ depletion" (Sims *et al*, 1982, 1983). The PADPRP inhibitor, NU1025 was used to identify the actual mechanism of action. If the first scenario was correct, then growth inhibition and cytotoxicity would be potentiated as PADPRP inhibition would slow further the release of the enzyme from the DNA strand break ends, but if the second scenario was true, then protection against growth inhibition and cytotoxicity could be afforded as PADPRP inhibition would preserve NAD^+ levels.

Initially the effect of NU1025 alone was considered. NU1025 decreased growth of both cell lines in a concentration dependent manner, with similar IC_{50} values of ~370μM determined. A possible explanation for the lack of difference between the WT and TZR cell lines could be that NU1025 treatment *per se* was not specifically targeting PADPRP, and that the growth inhibitory effects observed were due to effects on other metabolic reactions. The 3′substituted benzamides have been reported to affect purine metabolism (Milam & Cleaver, 1984; Milam *et al*, 1986) (see Chapter 1, Section 1.3.2, and Chapter 3, Section 3.8). A structural similarity between the benzamides and NU1025 is the presence of a carboxamide moiety, and all the compounds act in a competitive manner. Therefore, the possibility exists that NU1025 could exhibit similar non specific reactions. If NU1025 was directly affecting PADPRP, then in the TZR cell line, where NAD^+ concentrations are low, and the capacity for NAD^+ biosynthesis is reduced, NU1025, due to its competitive nature, could be expected to have greater inhibitory potential at lower concentrations. In fact, this was demonstrated in DNA damaged cells, where lower

concentrations of NU1025 were required in TZR cells compared to WT to obtain similar effects. The lack of such a differential when NU1025 growth inhibition *per se* is considered indicates that NU1025 is not targetting PADPRP at these high concentrations, but is exerting some other effect. The cotreatment of both WT and TZR cells with increasing concentrations of TM in the presence of fixed concentrations of NU1025 resulted in the potentiation of growth inhibition. However, a ~3 fold lower concentration of NU1025 was required by the TZR cell line to exhibit a similar potentiatory effect as observed in the WT cells (see Section 6.4.2 and Figure 6.5). These results would suggest the initial scenario to be correct. Due to the competitive nature of the PADPRP inhibitor (utilising a Lineweaver-Burke plot, NU1025 was shown to inhibit PADPRP in a competitive manner, J.K. Porteous, unpublished results, Cancer Research Unit, The University of Newcastle Upon Tyne) in reduced NAD^+ conditions the PADPRP inhibition would be more effective. Therefore, the repulsion of PADPRP from DNA single strand break ends would be slowed further as the inhibition of polymer formation would increase. This was further demonstrated when 100μM TM was incubated with increasing concentrations of NU1025. In the WT cells the IC_{50} for growth inhibition in the presence of TM was reduced ~10 fold as compared to NU1025 alone whereas in the TZR cells an ~70-fold reduction was observed (see Section 6.4.1).

Interestingly, the NAD^+ depletion in TZR cells treated for a 4 hour continuous exposure to increasing concentrations of TM was not relatively different to that of the NAD^+ depletion observed in TM treated WT cells. Although the initial NAD^+ level in the TZR cells was ~60% of that of WT, a proportionate depletion of NAD^+ was observed. However, when the absolute NAD^+ depletion of the two cell lines was compared a dissimilarity was found. At a TM concentration of 1000μM, the NAD^+ levels in the WT cells were reduced ~3000pMoles, whereas in the TZR cells an absolute reduction of ~1500pMoles was observed, representing a ~2.5 fold difference. This data would also indicate scenario (1) rather than scenario (2) to be more correct. In scenario (1), if the NAD^+ concentration fell below the K_{mNAD+} for PADPRP (~50μM, Ueda *et al*, 1982),

PADPRP function would be slowed, so reducing the level of NAD^- utilised, and consequently the level of NAD^+ depletion, as was observed.

As TM treatment results in the formation of DNA single strand breaks, the effect an increasing TM concentration had on DNA single strand break levels in the WT and TZR cells was determined. The potentiation of cytotoxicity of DNA damaging agents whose lesions were repairable via BER, by inhibitors of PADPRP was suggested to be due to an increased level of DNA single strand breaks. However, in Chapter 5 of this thesis, evidence for a possible dissociation between the concentration of NU1025 required to potentiate TM cytotoxicity and that required to increase the level of DNA single strand breaks was presented. The effect increasing concentrations of TM had on the level of DNA strand breaks in WT and TZR cells would also give support to the alternative theories described in the discussion of Chapter 5. The R.E. profiles for WT and TZR cell lines showed little or no difference, with DNA single strand breaks increasing in a concentration dependent manner. However, a 2.5 fold reduction in TM concentration gave the same growth inhibitory effect in TZR cells as for the WT cells. This was interpreted as due to a diminished PADPRP activity because the low NAD^+ levels were becoming rate limiting. Another reasoning (as previously discussed, see Section 5.6, Chapter 5) for this result would be that the cytotoxic event of TM treatment is not due solely to DNA single strand breaks. When the effect of increasing concentrations of NU1025 in the presence of 150μM TM was considered in both the WT and TZR cells, a concentration dependent increase in the R.E. values was observed, but in the TZR line it was enhanced ~2.5 fold. As NU1025 has been shown to be a competitive inhibitor of PADPRP, the decreased competition between the intracellular NAD^+ and the NU1025 for the active site of PADPRP, due to the depleted NAD^+ levels would result in a further delay of release of PADPRP from the break ends and hence increase the effect of NU1025 on strand break levels in TZR cells compared to WT.

The theory postulated in Chapter 5 was that the formation of long (ADP-ribose) polymers could act as a system to alert the cell to DNA damage, by targeting a cellular

component involved in cell cycle arrest, e.g. p53. The results presented above could also be interpreted in this light: In a situation of low intracellular NAD^+ leading to partial PADPRP inhibition the formation of long (ADP-ribose) polymers would be especially affected. However, the production of shorter polymer lengths, still capable of effecting repulsion of PADPRP from the DNA would result in the efficient repair of DNA single strand breaks. As described, both cell lines exhibited similar R.E. values to TM alone. In the presence of NU1025, the increased effectiveness of the inhibitor due to a lack of competition with intracellular NAD^+ would inhibit even the formation of the smaller polymer lengths, and so DNA single strand break repair would be slowed.

A functional relationship between the enzymes NMNAT and PADPRP was considered as NMNAT anabolises the NAD^+, which in the main is utilised by PADPRP in the formation of poly(ADP-ribose). Therefore, it was proposed that PADPRP could signal by some means to NMNAT, resulting in an increased level of activity, thereby replenishing the intracellular NAD^+ pool following DNA damage induced NAD^+ depletion. Possible mechanisms could include:- direct poly(ADP-ribosyl)ation of NMNAT, poly(ADP-ribose) modified chromatin could release the chromatin bound NMNAT, thereby activating it, NMNAT gene transcription could be induced by poly(ADP-ribosyl)ated chromatin. The NMNAT activity of WT L1210 cells was significantly increased when incubated for either 4 or 6 hours with 0.5-1mM TM. However, as a 50% NAD^+ depletion was only observed following a 6 hour incubation with 2mM TM, and the prolonged half-life of TM (~50 minutes in murine cells), the damaging agent was changed to MTIC. MTIC degrades with a half-life of ~4 minutes and a 30 minute incubation with 1mM MTIC depletes NAD^+ to 50% of control. Preliminary evidence suggested effects on NMNAT activity to occur at incubation periods of 6 and 24 hours. However, further investigation found an effect only at 24 hours, were NMNAT activity was increased to 134% of control (see Table 6.4). Improved experimental design could further the investigation into the possible association between NMNAT and

PADPRP activities. Following alkylation damage, NMNAT activity could be measured at the times when PADPRP activity was known to be at a high, or when the intracellular NAD^+ was depleted to a minimum. The effect of co-incubating the cells with an alkylating agent and a PADPRP inhibitor, on the activity of NMNAT would provide evidence as to whether alterations in NMNAT activity were dependent on PADPRP function.

Uhr & Smulson (1982) calculated that NMNAT enzyme molecules occured at a frequency of 1 enzyme/10-20 nucleosomes. This was an exceptionally large estimation, especially as the NAD^+ pool required for redox reactions represented just 5% of the total intracellular NAD^+ level. They speculated that not all the enzyme molecules were utilised at any one time, but in cases of an increased requirement, e.g. PADPRP activation, the reserve NMNAT capacity could be called upon. In the case of the TZR cell lines, with reduced capacity for NMNAT activity, the excess NMNAT present in the nucleus could well tolerate a loss of > 90% of the NMNAT with little observable effect on the biosynthetic capacity for NAD^+.

However, Ruggieri *et al* (1988) utilising yeast NMNAT suggested that PADPRP was capable of modifying the NMNAT so resulting in its inactivation. The identification of PADPRP activity in yeast has yet to be substantiated. This result is negated by the ability of cells to recover the NAD^+ pool. Figure 4.9B clearly demonstrates that after a 6 hour exposure to MTIC the NAD^+ levels are as for untreated controls whereas a 50% NAD^+ depletion was observed after just 30 minutes. The inhibition of NMNAT action by PADPRP modification would have negative implications for cell survival. If NAD^+ pools were not replenished, many essential metabolic processes involving redox reactions would be inhibited.

The data of Ruggieri *et al* (1988) could support an NAD^+ suicide hypothesis, as an inhibited NMNAT due to poly(ADP-ribosyl)ation would prevent restoration of the NAD^+ levels. However, the suicide hypothesis is not a valid explanation for the results presented

herein. The massive NMNAT loss could be interpreted as an analogous situation to the inhibition of NMNAT activity. However, Berger *et al* (1986) did not observe NAD^+ suicide in L1210 cells treated with MNNG, even when the intracellular NAD^+ levels fell to just 5% of control levels. Therefore, the hyperlethal doses of TM (e.g. 2mM TM) which were required to deplete the NAD^+ levels by only 50% of control would fail to deplete ATP levels, and so DNA, RNA and protein biosyntheses would continue, with the resulting survival of the cell.

Under normal growth conditions, the main route for the formation of intracellular NAD^+ is via nicotinamide. The reaction sequence is performed in two steps (see Chapter 1, Figure 1.1). The initial reaction catalysed by nicotinamide phosphoribosyl transferase is responsible for the formation of NMN from nicotinamide. The second reaction converts the NMN to the NAD^+. As previously stated this reaction is catalysed by NMNAT. The rate determining step of NAD^+ formation was found by Berger *et al* (1987) to be the initial reaction. The treatment of a purified T cell preparation with monoclonal antibodies to the T3 surface antigen which activates the T-cell receptor complex, and the phorbol ester TPA, induce T-cell mitogenesis with a concomitant increases in PADPRP activity and pyridine nucleotide metabolism. In TPA/antiT3 stimulated cells both nicotinamide phosphoribosyl transferase and NMNAT activities were increased and a comparison of the relative activities indicated the former to be the rate determining enzyme.

The role of NMNAT function in the cellular response to DNA damaging agents could vary between different cell lines, e.g. due to a dependency on the relative levels of NMNAT, nicotinamide phosphoribosyl transferase, PADPRP activity and the cellular NAD^+ levels. For example, in pancreatic islet cells treated with nitric oxide NAD^+ suicide effects predominate (Radons *et al*, 1994). A possible explanation for an increased probability that certain cell types enter on to a suicide pathway, could be that such cells exist in an arrested state (i.e. G_0 as opposed to cell cycling). G_0 arrested cells have been reported to contain lower NAD^+ levels than cycling cell populations (Sweigert *et al*,

1986). Therefore, these cells would have sufficient time to repair damage to the DNA, but consequential PADPRP activity could reduce NAD^+ levels below the point at which regeneration of the NAD^+ pool would be feasible, so leading to the suicide of the cell. Radons *et al* (1994) calculated 0.355pMoles $NAD^+/10^3$ pancreatic islet cells. In the presence of a NO donor, NAD^+ levels were reduced to ~30-40% of control and cells were observed to suicide. In an L1210 study, Berger *et al* (1986) found that a reduction of NAD^+ levels to just 5% of control did not induce a cell into suicide. However, L1210 cells contain ~3 fold greater NAD^+ concentration than the Islet cells (~1pMoles $NAD^+/10^3$ cells).

Overall this Chapter has indicated that cells with reduced NAD^+ levels show enhanced sensitivity to DNA damage resulting from TM treatment, and enhanced sensitivity to the PADPRP inhibitor, NU1025, in potentiating growth inhibition and DNA single strand break levels. Therefore, as tumour cells are reported to contain lower NAD^+ levels as compared to their somatic cell counterparts, and also higher NAD/NADH ratios, PADPRP inhibition would be a good candidate to target in chemotherapeutic regimes. In patients treated with TZ who develop a resistance to the drug, the use of TM and an inhibitor of PADPRP could subsequently be administered as the tumour cells would be predicted to be sensitised to this combination, based on the results presented in this Chapter.

CHAPTER 7 : SUMMARY AND FUTURE DIRECTIONS

7.1 SUMMARY

Utilising an *in vitro* PADPRP assay, the novel compounds NU1025 and PD 128763 were found to inhibit PADPRP, with an ~50 fold increase in potency as compared to the "classical" PADPRP inhibitors, 3AB and BZ. This increased inhibitory activity indicated a potential use of NU1025 and/or PD 128763 as resistance modifying agents in the treatment of cancer. However, prior to clinical trials, a comprehensive intact cell study was required. The data presented in Chapter 3 analysed the effect NU1025 and PD 128763 had on a number of biological endpoints known to be modulated by PADPRP function following DNA damage, e.g. growth inhibition, cytotoxicity studies, and intracellular NAD^+ levels, and compared them to the "classical" PADPRP inhibitors, 3AB and BZ. Overall, an excellent correlation of potency was observed between the *in vitro* and intact cell assays, with the relative inhibitor potencies (as compared to 3AB) for the intact cell studies, similar to those of the *in vitro* assay, i.e. 3AB:1, BZ:2, NU1025:43, and PD 128763:53. The concentrations of PADPRP inhibitor required to potentiate TM growth inhibition and cytotoxicity, e.g. 10-50μM NU1025, had little or no effect on the growth and cell survival *per se*. However, a similar concentration range of the PADPRP inhibitors abrogated the NAD^+ depletion resulting from TM treatment. Taken together, these results would suggest both NU1025 and PD 128763 exhibit excellent potential as resistance modifying agents and further *in vivo* studies prior to entrance onto clinical trials would be warranted.

The cytotoxicity of DNA damaging agents in the presence of PADPRP inhibitors was reportedly correlated with an increase in the DNA single strand break levels resulting from the repair of lesions in the DNA bases. Chapter 4 assessed the ability of increasing concentrations of the PADPRP inhibitors to increase the DNA single strand break levels resulting from TM treatment. To the best of my knowledge, this study represents the only

comprehensive and quantitative analysis of inhibitor concentration on the DNA strand break levels. All four inhibitors increased the DNA single strand breaks in a concentration dependent manner when in the presence of TM, with the relative potencies exhibiting an excellent correlation with the relative potencies determined for PADPRP inhibition in Chapter 3. However, when the concentrations of inhibitor required to enhance cytotoxicity were compared with those required to increase the DNA single strand break levels, a discrepency was observed. 3mM BZ and 50μM PD 128763 when in the presence of 150μM TM gave a >90% increase in cell kill, but there was little or no effect on DNA single strand break levels. The periods of incubation differed between the two biological endpoints, but preliminary data suggested increases in DNA strand breaks due to PADPRP inhibition were independent of time. A possible explanation discussed in Chapter 5, proposed that a subset of lesions were responsible for the increased cytotoxicity. However, other theories presented in Chapter 5 suggested that PADPRP could act as a cellular alarm system in situations of DNA damage, or that PADPRP acted upon two different cellular targets over different concentration ranges. In comparison, a good correlation between the concentrations of TM required to increase both cytotoxicity and DNA single strand break levels was observed, but due to subsets of lesions and differing exposure times, the increased cytotoxicity could not be unequivocally related to increases in DNA single strand break levels.

Chapter 6 determined the effect a lower intracellular NAD^+ concentrations and reduced NAD^+ biosynthetic capacity had on the activity of PADPRP in TM damaged cells in the absence or presence of the PADPRP inhibitor, NU1025. A L1210 cell line was made resistant to the IMPDH inhibitor, TZ using a step wise selection protocol. Characterisation of this TZR cell showed that NMNAT activity was <3% of the parental line, and that the NAD^+ levels were reduced by ~40%. The growth of TZR cells showed an ~3 fold increase in sensitivity to TM, and when co-incubated with NU1025, there was a dose dependent potentiation of the growth inhibition. However, an ~3 fold lower

concentration of NU1025 potentiated growth to a similar extent as in the WT cells. Both cell lines showed equal sensitivity to NU1025 alone. DNA single strand break levels resulting from TM treatment were equivalent in the WT and TZR cell lines, but a ~1.5 fold lower concentration of NU1025 potentiated the DNA single strand breaks in the TZR cells to a similar degree as the WT cells. Extrapolating from the data presented in Chapter 6, it is possible that a TM and PADPRP inhibitor regime in patients resistant to TZ would offer clinical benefit.

Finally, the effect of DNA damage on NMNAT activity was assessed. NMNAT is a chromatin bound enzyme, as is PADPRP, and is the final enzyme in the biosynthetic pathway of NAD^+. The possibility of PADPRP signalling the regulation of NMNAT activity was considered. In cells treated with MTIC for 24 hours, the activity of NMNAT was increased to 134% of control. This work is at a preliminary stage and will be discussed further in Section 7.2.

7.2 FUTURE DIRECTIONS

The most exciting and potentially useful data published in the literature recently was that PADPRP deficient mice are capable of normal development, and survive into adulthood (Wang *et al*, 1995). This provides an excellent system to identify the *in vivo* roles of PADPRP. However, an animal which is devoid of PADPRP is not analogous to one that has an inhibited or mutant enzyme, therefore the use of PADPRP inhibitors still retains a function in elucidating the cellular functions of PADPRP. This difference was initially shown utilising *in vitro* and intact cell systems. PADPRP is activated in response to DNA strand ends, to which it binds. Poly(ADP-ribose) polymer is the product of activation, and this in the main is used to auto/homo-modify PADPRP, which is subsequently repelled from the DNA. This mechanism is considered to result in the decondensation of the chromatin structure and facillitate access of repair enzymes to the damaged site. Utilising an *in vitro* cell free system, PADPRP was shown to be nonessential to the DNA repair

process. The existence of a PADPRP deficient mouse confirmed this observation. In the absence of PADPRP, the repair enzymes have constant access to the damaged sites. However, in the presence of PADPRP, an absolute requirement for NAD^+ was observed. When PADPRP is bound to a DNA break, until automodification of the enzyme occurs, repair is inhibited (Satoh & Lindahl, 1992; Satoh *et al*, 1993). When a recombinant DBD polypeptide was introduced into CV-1 cells, a trans-dominant inhibition of repair was also observed. This is an analogous system, in that the DBD polypeptide binds to the DNA strand break, but due to the lack of domains required for automodification, release of the DBD can not occur. Inhibitors of PADPRP are somewhat analogous in that they cause a transient delay of the repair process. Competition between the inhibitor and NAD^+, slows the automodification reaction and subsequent release of the enzyme.

PADPRP knockout mice could be an important tool that would benefit PADPRP research. A major problem associated with use of the PADPRP inhibitors is their lack of specificity. Several reports in the literature have demonstrated the involvement of the nicotinamide analogues, e.g. 3AB, BZ, in purine metabolism (see Section 1.3.2, Chapter 1), and this casts doubt on results obtained. The novel compounds, NU1025 and PD 128763, described in this thesis have yet to be fully characterised for inhibitory activity against mono ADP-ribosyl transferases and possible non-specific effects. The exposure of cells derived from a PADPRP deficient mouse to inhibitors of PADPRP in growth and/or cytotoxicity studies, would immediately answer the specificity argument. The targeting of reactions secondary to PADPRP would lead to continued growth inhibition and/or reduced survival, however, if the target was solely PADPRP, then no effects would be observed.

Chapter 5 reported a dissociation between the PADPRP inhibitor concentrations required to increase cytotoxicity and those required to increase the level of DNA strand breaks. Satoh *et al* (1994) proposed a dual function for ADP-ribose polymers: short polymers (<20 residues) were required for repair of damaged DNA, and long polymers were

required to to prevent homologous recombinational events. However, the data presented in this thesis, i.e. cytotoxicity increased at low inhibitor concentrations, whereas DNA repair was only inhibited at high concentrations, could suggest the long polymers were involved in alerting the cell to DNA damage, and the presence of short polymers was sufficient for repair. An elegant methodology for identifying polymer patterns utilises dihydroboronate affinity columns to isolate ADP-ribose polymers, which can be subsequently quantified by sequence gel analysis (Wielkens *et al*, 1981; Naegelli *et al*, 1989; Naegelli & Althaus, 1991, Malanga *et al*, 1995). A new methodology utilising a silver staining technique, which bypasses the need for radiolabelling the polymer has been developed (Malanga *et al*, 1995). An analysis of the effects of a range of inhibitor concentrations on the synthesis and size distributions of polymers in intact cells would be very imformative. For example, do low concentrations of inhibitor decrease the average length of the polymers, and how do the concentrations required correlate with the concentrations required to enhance cytotoxicity and inhibit DNA repair ?

At present, assessment of the role of PADPRP in DNA repair utilising the knockout$_{PADPRP}$ mice has been limited, with results from an indirect CAT plasmid assay and UDS assay only reported (see Section 1.5.7). Therefore, a direct comparison of homologous cell lines, one being proficient (+/+) and the other deficient (-/-) in PADPRP, in the presence and absence of PADPRP inhibitors, utilising a more quantitiative DNA repair assay, e.g. alkaline elution, could provide a greater understanding of PADPRP function.

In the discussion of Chapter 5, a possible target for the cellular signal was proposed. p53 is a trans-activating transcription factor which regulates several genes whose proteins are involved in the DNA damage induced G1 arrest. To investigate such a proposal, characterised cell lines for tolerance and p53 status would be required. The availability of an affinity column which would separate ADP-ribose polymers attached to their protein acceptors would be extremely advantageous (e.g. Adamietz & Rudolph, 1984).

Subsequent characterisation of the separated proteins by western blotting could identify p53 or other proteins as the signal target. Rather than covalent modification, noncovalent bonds between ADP-ribose polymer and p53 could be responsible for the DNA damage signal. Panzeter *et al* (1992) found that histone proteins interacted in a noncovalent manner with various sized linear and branched polymers. The domain of histone H1 responsible for this bonding was found to be that involved in the induction of higher chromatin structure.

As described in the discussion of Chapter 5, the cytotoxicity of DNA damaging agents could be due to single strand breaks formed during repair processes passing through the replication fork, so leading to the formation of double strand breaks (see Chapter 5, Section 5.6). Neutral elution measures such breaks, and could be utilised to assess the level of double strand breaks in both mature and nascent DNA following treatment with a DNA damaging agent in the presence and absence of PADPRP inhibitors. As double strand breaks represent a non-coded area of the DNA sequence, recombinational repair could be a possible mechanism by which cells could survive. Evaluation of double strand break levels over increasing periods of time could provide an indication of repair events.

The data from the intact cell study presented in this thesis would advocate the initiation of *in vivo* studies to assess the effects of the novel PADPRP inhibitors on tumour regression. The cell line used in this study, i.e. L1210 cells, would be a possible starting model, but further analyses would require the use of human tumour cells, e.g. the lung cancer cell line A549, to be representative of cancers found in the clinics. Nude mice would be implanted with the xenografts and co-treatments of TM and NU1025, or PD 128763 administered.

A reduction of intracellular NAD^+ concentration resulted in an increased sensitivity to the DNA damaging agent TM, when in the presence of the PADPRP inhibitor NU1025. However, the development of TZ resistance in a human cell line would provide a better model for preclinical studies. In cells with a lower starting NAD^+ level, a 50% reduction

in NAD^+ could lower the NAD^+ to or below the K_m concentration, and so PADPRP function would be impaired. Increased inhibition by PADPRP inhibitors would also result due to the decreased level of competition.

The possible interaction between PADPRP and NMNAT requires futher investigation as the results described in Chapter 6, Section 6.7 were at a preliminary stage. NMNAT activity was shown to be increased 24 hours after treatment with MTIC. However, an increase in PADPRP activity was observed to occur within a 30 minute incubation (as determined by NAD^+ depletion analysis, see Chapter 3, Section 3.7.1). Therefore, a possible interpretation could be that the upregulation of NMNAT activity was not attributable to PADPRP function. Coincubation with a PADPRP inhibitor in these experiments would provide an answer to this question.

REFERENCES

Adamietz P. & Rudolph A. (1984) ADP-ribosylation of Nuclear Proteins *in vivo*. **J.Biol.Chem.** 259:6841-6846.

Adolph K.W. (1987) ADPribosylation of Nuclear Proteins Labelled with [^{3}H]adenosine : Changes during the HeLa Cell Cycle. **Biochim.Biophys.Acta** 909:222-230.

Adolph K.W. & Song M.H. (1985a) Variations in ADP-ribosylation of Nuclear Scaffold Proteins During the HeLa Cell Cycle. **Biochem.Biophys.Res.Commun.** 126:840-847.

Adolph K.W. & Song M.H. (1985b) ADP-ribosylation of Metaphase and Interphase Nonhistones using [^{3}H]adenosine as a Radioactive Label. **Febbs Lett.** 182:158-162.

Agarwal S., Drysdale B., Shin H.S. (1988) Tumour Necrosis Factor-Mediated Cytotoxicity Involves ADP-Ribosylation. **J.Immunol.** 140:4187-4192.

Ahluwahlia G.S., Jayaram H.N., Plowman J.P., Cooney D.A., Johns D.G. (1984) Studies on the Mechanism of Action of 2-β-D-ribofuranosylthiazole-4-carboxamide-V. **Biochem.Pharmacol.** 33:1195-1203.

Alkhatib H.M., Chen D., Cherney B., Bhatia K., Notario V., Giri C., Stein G., Slattery E., Roeder R.G., Smulson M.E. (1987) Cloning and expression of cDNA for human poly(ADP-ribose)polymerase. **Proc.Natl.Acad.Sci.USA** 84:1224-1228.

Althaus F.R., Lawrence S.D., Sattler G.L., Pitot H.C. (1980) The Effect of Nicotinamide on Unsceduled DNA Synthesis in Cultured Hepatocytes. **Biochem.Biophys.Res.Commun.** 95:1063-1070.

Althaus F.R., Lawrence S.D., He Y., Sattler G.L., Tsukada Y., Pitot H.C. (1982) Effects of Altered [ADP-ribose]$_n$ Metabolism on Expression of Fetal Functions by Adult Hepatocytes. **Nature** 300:366-368.

Alvarez-Gonzalez R. & Althaus F.R. (1989) Poly(ADP-ribose) catabolism in mammalian cells exposed to DNA-damaging agents. **Mut.Res.** 218:67-74.

Alvarez-Gonzalez R. & Jacobson M.K. (1987) Characterisation of Polymers of Adenosine Diphosphate Ribose Generated in Vitro and in Vivo. **Biochemistry** 26:3218-3224.

Aoki K., Nishimura K., Abe H., Maruta H., Sakagami H., Hatano T., Okuda T., Yoshida T., Tsai Y., Uchiumi F., Tanuma S. (1993) Novel Inhibitors of Poly(ADP-ribose) Glycohydrolase. **Biochim.Biophys.Acta.** 1158:251-256.

Arundel-Suto C.M., Scavone S.V., Turner W.R., Suto M.J., Sebolt-Leopold J.S. (1991) Effects of PD 128763, a New Potent Inhibitor of Poly(ADP-Ribose) Polymerase, on X-ray-Induced Cellular Recovery Processes in Chinese Hamster V79 Cells. **Rad.Res.** 126:367-371.

Aubin R.J., Frechette A., de Murcia G., Mandel P., Grondin A.L.G., Poirier G.G. (1983) Correlation between Endogenous Nucleosomal Hyper(ADP-ribosyl)ation of Histone H1 and the Induction of Chromatin Relaxation. **The EMBO J.** 2:1685-1693.

Baer J.C., Freeman A.A., Newlands E.S., Watson A.J., Rafferty J.A., Margison G.P. (1993) Depletion of O^6-alkylguanine-DNA alkyltransferase Correlates with Potentiation of Temozolomide and CCNU toxicity in Human Tumour Cells. **Br.J.Cancer** 67: 1299-1302.

Baksi K., Alkhatib H., Smulson M.E. (1987) *In vivo* Characterisation of the Poly(ADP-ribosylation) of SV40 Chromatin and Large T Antigen by Immunofractionation. **Exp.Cell Res.** 172:110-123.

Banasik M., Komura H., Shimoyama M., Ueda K. (1992) Specific Inhibitors of Poly(ADP-Ribose) Synthetase and Mono(ADP-Ribosyl) Transferase. **J.Biol.Chem.** 267: 1569-1575.

Barak Y., Juven T., Haffner R., Oren M. (1993) *mdm2* Expression is Induced by Wild Type p53 Activity. **The EMBO J.** 12:461-468.

Bardwell J., Bardwell L., Tomkinson A.E., Freidberg E.C. (1994) Specific Cleavage of Model Recombination and Repair Intermediates by the Yeast Rad1-Rad10 DNA Endonuclease. **Science** 265: 2082-2085.

Barrows L.R. & Magee P.N. (1982) Nonenzymic Methylation of DNA by S-adenosylmethionine *in vitro*. **Carcinogenesis** 3:349-351.

Baumgartner M., Schneider R., Auer B., Herzog H., Schweiger M., Hirsch-Kauffmann M. (1992) Fluoresence in situ mapping of the human nuclear NAD^+ ADP-ribosyltransferase gene (ADPRT) and two secondary sites to human chromosomal bands 1q42, 13q34 and 14q24. **Cytogenet.Cell Genet.** 61:172-174.

Ben-Hur E., Lindquist K., Elkind M.M. (1985) Poly(ADP-ribose) and the Recovery From Damage in

Chinese Hamster Cells due to 5-Bromodeoxyuridine Photolysis. **Int.J.Radiat.Biol.** 48:33-43.

Benjamin R.C. & Gill D.M. (1980) Poly(ADP-ribose) Synthesis *in vitro* Programmed by Damaged DNA. **J.Biol.Chem.** 255: 10502-10508.

Berger N.A., Weber G., Kaichi A.S. (1978) Characterisation and Comparison of Poly(Adenosine Diphosphoribose) Synthetase and DNA Synthesis in Nucleotide-Permeable Cells. **Biochim.Biophys.Acta** 519:87-104.

Berger N.A., Sikorski G.W., Petzold S.J., Kurohara K.K. (1979) Association of Poly(Adenosine Diphosphoribose) Synthesis with DNA Damage and Repair in Normal Human Lymphocytes. **J.Clin.Invest.** 63:1164-1171.

Berger N.A. & Sikorski G.W. (1980) Nicotinamide Stimulates Repair of Damage in Human Lymphocytes. **Biochem.Biophys.Res.Commun.** 95:67-72.

Berger N.A., Catino D.M., Vietti T.J. (1982) Synergistic Antileukemic Effect of 6-Aminonicotinamide and 1,3-Bis(2-chloroethyl)-1-nitrosourea on L1210 Cells *in vitro*. **Cancer Res.** 42:4382-4386.

Berger N.A., Berger S.J., Catino D.M., Petzold S.J., Robins R.K. (1985) Modulation of Nicotinamide Adenine Dinucleotide and Poly(Adenosine Diphosphoribose) Metabolism by the Synthetic "C" Nucleoside Analogs, Tiazofurin and Selenazofurin. **J.Clin.Invest.** 75:702-709.

Berger S.J., Sudar D.C., Berger N.A. (1986) Metabolic Consequences of DNA Damage: DNA Damage Induces Alterations in Glucose Metabolism by Activation of Poly(ADP-ribose)Polymerase. **Biochem.Biophys.Res.Commun.** 134:227-232.

Berger S.J., Manory I., Sudah D.C., Berger N.A. (1987) Induction of the Pyridine Nucleotide Synthesis Pathway in Mitogen-Stimulated Human T-Lymphocytes. **Exp.Cell Res.** 169:149-157.

Berridge M.J. & Irvine R.F. (1989) Inositol Phosphatases and Cell Signalling. **Nature** 341:197-205.

Birnboim H.C. & Jevcak J.J. (1981) Fluorometric Method for Rapid Detection of DNA Strand Breaks in Human White Blood Cells Produced by Low Doses of Radiation. **Cancer Res.** 41:1889-1892.

Blaho J.A., Michael N., Kang V., Aboul-Ela N., Smulson M.E., Jacobson M.K., Roizman B. (1992) Differences in the Poly(ADP-Ribosyl)ation Patterns of ICP4, the Herpes Simplex Virus Major Regulatory

Protein, in Infected Cells and in Isolated Nuclei. **J.Virol.** 66:6398-6407.

Bohr V.A., Smith C.A., Okumoto D.S., Hanawalt P.C. (1985) DNA Repair in an Active Gene: Removal of Pyrimidine Dimers from the DHFR Gene of CHO Cells Is Much More Efficient than in the Genome Overall. **Cell** 40: 359-369.

Boiteux S., O'Connor T.R., Laval J. (1987) Formamidopyrimidine-DNA Glycosylase of *Escherichia coli*: Cloning and Sequencing of the *fpg* Structural Gene and Overproduction of the protein. **EMBO J.** 6:3177-3183.

Boorstein R.J. & Pardee A.B. (1984) 3-Aminobenzamide Is Lethal to MMS-Damaged Human Fibroblasts Primarily During S Phase. **J.Cell.Physiol.** 120: 345-353.

Boorstein R.J., Levy D.D., Teebor G.W. (1987) Toxicity of 3-Aminobenzamide to Chinese Hamster Cells Containing 5-Hydroxymethyluracil in Their DNA. **Cancer Res.** 47:4372-4377.

Boorstein R.J., Chiu L., Teebor G.W. (1992) A Mammalian Cell Line Deficient in Activity of the DNA Repair Enzyme 5-Hydroxymethyluracil-DNA Glycosylase Is Resistant to the Toxic Effects of the Thymidine Analog 5-Hydroxymethyl-2'-Deoxyuridine. **Mol.Cell Biol.** 12:5536-5540.

Boulikas T. (1993) Poly(ADP-ribosyl)ation, DNA Strand Breaks, Chromatin and Cancer. **Tox.Lett.** 67:129-150.

Boyce R.P. & Howard-Flanders P. (1964) Release of Ultraviolet Light-Induced Thymine Dimers From DNA in *E.coli K-12*. **Biochemistry** 51:293-300.

Bradford M.M. (1976) A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. **Ann.Biochem.** 72:248-254

Branster M.V. & Morton R.K. (1956) Comparative Rates of Synthesis of Diphosphopyridine Nucleotide by Normal and Tumour Tissue from Mouse Mammary Gland: Studies with Isolated Nuclei. **Biochem.J.** 63:640-646.

Braun S.A., Panzeter P.L., Collinge M.A., Althaus F.R. (1994) Endoglycosidic Cleavage of Branched Polymers by Poly(ADP-ribose) Glycohydrolase. **Eur.J.Biochem.** 220:369-375.

Brochu G., Duchaine C., Thibeault L., Lagueux J., Shah G.M., Poirier G.G. (1994) Mode of Action of

Poly(ADP-ribose) Glycohydrolase. **Biochim.Biophys.Acta.** 1219:342-350.

Brown D.M., Horsman M.R., Hirst D.G., Brown J.M. (1984) Enhancement of Melphalan Cytotoxicity *in vivo* and *in vitro* by Inhibitors of Poly(ADP-ribose) Polymerase. **Int.J.Rad.Oncol.Biol.Phys.** 10:1665-1668.

Buch L., Streeter D., Halpern R.M., Simon L.N., Stout M.G., Smith R.A. (1972) Inhibiton of Transfer Ribonucleic Acid Methylase Activity from Several Human Tumours by Nicotinamide and Nicotinamide Analogs. **Biochemistry** 11:393-397.

Burzio L. & Koide S.S. (1972) *In Vitro* Effects of NAD on DNA Synthesis in Isolated Nuclei from Regenerating Rat Liver and Novikoff Hepatoma. **FEBS Lett.** 20:29-32.

Burzio L. & Koide S.S. (1973) Activation of the Template Activity of Isolated Rat Liver Nuclei for DNA Synthesis and Its Inhibition by NAD. **Biochem.Biophys.Res.Commun.** 53:572-579.

Burzio L., Reich L., Koide S.S. (1975) Poly(Adenosine Diphosphoribose) Synthetase Activity of Isolated Nuclei of Normal and Leukaemic Leucocytes (38930). **Proc.Natl.Acad.Sci.USA.** 149:933-938.

Butt T.R., Brothers J.F., Giri C.P., Smulson M.E. (1978) A nuclear protein-modifying enzyme is responsive to ordered chromatin structure. **Nuc.Acid Res.** 5:2775-2788.

Byrnes J.J., Downey K.M., Black V.L., So A.G. (1976) A New Mammalian DNA Polymerase with 3′ to 5′ Exonuclease Activity: DNA Polymerase δ. **Biochemistry** 15:2817-2823.

Cantoni O., Murray D., Meyn R.E. (1986) Effect of 3-aminobenzamide on DNA strand-break Rejoining and Cytotoxicity in CHO Cells Treated with Hydrogen Peroxide. **Biochim.Biophys.Acta.** 867:135-143.

Cardenas-Corona M.E., Jacobson E,L., Jacobson M.K. (1987) Endogenous Polymers of ADP-Ribose Are Associated with the Nuclear Matrix. **J.Biol.Chem.** 262:14863-14866.

Cassel D. & Pfeuffer T. (1976) Mechanism of Cholera Toxin Action: Covalent Modification of the Guanyl Nucleotide-binding Protein of the Adenylate Cyclase System. **Proc.Natl.Acad.Sci.USA** 75:2669-2673.

Chambon P., Weill J.D., Mandel P. (1963) Nicotinamide Mononucleotide Activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. **Biochem.Biophys.Res.Commun.** 11:39-43.

Chambon P., Weill J.D., Doly J., Strosser M.T., Mandel P. (1966) On the formation of a novel adenylic

compound by enzymatic extracts of liver nuclei. **Biochem.Biophys.Res.Commun.** 25:638-643.

Chase J.W. & Richardson C.C. (1974) Exonuclease VII of *Escherichia coli*. **J.Biol.Chem.** 249:4553-4561.

Chen T.R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. **Exp.Cell.Res.** 104:255-262.

Cherney B.W., McBride O.W., Chen D., Alkhatib H., Bhatia K., Hensley P., Smulson M.E. (1987) cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase. **Proc.Natl.Acad.Sci.USA** 84:8370-8374.

Chung H. W., Phillips J.W., Winegar R.A., Preston R.J., Morgan W.F. (1991) Modulation of Restriction Enzyme-Induced Damage by Chemicals That Interfere with Cellular Responses to DNA Damage: A Cytogenetic and Pulsed-Field Gel Analysis. **Radiat.Res.** 125:107-113.

Clapper D.L., Walseth T.F., Dargie P.J., Lee H.C. (1987) Pyridine Nucleotide Metabolites Stimulate Calcium Release from Sea Urchin Egg Microsomes Desensitised to Inositol Triphosphate. **J.Biol.Chem.** 262:9561-9568.

Clark J.B., Ferris G.M., Pinder S. (1971) Inhibiton of Nuclear NAD Nucleosidase and Poly ADP-Ribose Polymerase Activity from Rat Liver by Nicotinamide and 5'-Methyl nicotinamide. **Biochim.Biophys.Acta** 238:82-85.

Clayson D.B. (1989) Can a Mechanistic Rationale be Provided for Non-genotoxic Carcinogens Identified in Rodent Bioassays? **Mut.Res.** 221:53-67.

Cleaver J.E. & Kraemer K.H. (1984) Xeroderma Pigmentosum. In: Metabolic Basis of Inheritted disease, 6th Edition. Eds. Stanbury J.B., Wyngaarden J.B., Frederickson D.S., Goldstein J.L., Brown M.S. New York: McGraw Hill. pp2949-2971.

Cleaver J.E., Bodell W.J., Morgan W.F., Zelle B. (1983) Differences in the Regulation by Poly(ADP-ribose) of Repair of DNA Damage from Alkylating Agents and Ultraviolet Light According to Cell Type. **J.Biol.Chem.** 258:9059-9068.

Cleaver J.E. (1985) Increased Repair Replication in Human Lymphoid Cells by Inhibition of Polyadenosine Diphosphoribose Synthesis with No Increase in Patch Sizes. **Cancer Res.** 45:1163-1169.

Cleaver J.E. & Morgan W.F. (1985) Poly(ADP-ribose) Synthesis is Involved in the Toxic Effects of Alkylating Agents But Does Not Regulate DNA Repair. **Mut.Res.** 150:69-76.

Cleaver J.E, Milam K.M, Morgan W.F. (1985) Do Inhibitor Studies Demonstrate a Role for Poly(ADP-ribose) in DNA Repair? **Radiation Res.** 101:16-28.

Cook P.R. & Brazell I.A. (1973) Supercoils in Human DNA. **J.Cell Sci.** 19:261-279.

Cooney D.A., Jayaram H.N., Gebeyehu G., Betts C.R., Kelley J.A., Marquez V.E., Johns D.G. (1982) The Conversion of 2-β-D-ribofuranosylthiazole-4-carboxamide to an Analogue of NAD with Potent IMP Dehydrogenase-inhibitory Properties. **Biochem.Pharmacol.** 31:2133-2136.

Cooney D.A., Jayaram H.N., Glazer R.L., Kelley J.A., Marquez V.E., Gebeyehu L.A., Van Cott A.C., Zwelling L.A., Johns D.G. (1983) Studies on the Mechanism of Action of Tiazofurin Metabolism to an Analog of NAD with Potent IMP Dehydrogenase-Inhibitory Activity. **Adv.Enzyme Reg.** 21:271-303.

Coquerelle T., Dosch J., Kaina B. (1995) Overexpression of *N*-methylpurine-DNA Glycosylase in Chinese Hamster Ovary Cells Renders them More Sensitive to the Production of Chromosomal Aberrations by Methylating Agents - a Case of Imbalanced DNA Repair. **Mut.Res.** 336:9-17.

Coulondre C. & Miller J.H. (1977) Genetic Studies if the lac Repressor III. Aditional Correlation of Mutational Sites with Specific Amino Acid Residues. **J.Mol.Biol.** 117:525-567.

Creissen D and Shall S. (1982) Regulation of DNA ligase activity by poly(ADP-ribose). **Nature** 296:271-272.

Darby M.K., Schmitt B., Jongstra-Bilen J., Vosberg H.P. (1985) Inhibiton of calf thymus type II DNA topoisomerase by poly(ADP-ribosylation) **The EMBO J.** 4:2129-2134.

Das S.K., Lau C.C., Pardee A.B. (1984) Comparative Analysis of Caffeine and 3-aminobenzamide as DNA Repair Inhibitors in Syrian Baby Hamster Kidney Cells. **Mut.Res.** 131: 71-79.

Demple B. & Linn S. (1980) DNA *N*-Glycosylases and UV Repair. **Nature** 287:203-208.

Demple B. & Karran P. (1983) Death of an Enzyme: Suicide Repair of DNA. **TIBS** 8:137-139.

de Murcia G., Jongstra-Bilen J., Ittel M., Mandel P., Delain E. (1983) Poly(ADP-ribose) Polymerase Auto-modification and Interaction with DNA: Electron Microscopic Visualisation. **The EMBO J.** 2:543-548.

de Murcia G., Huletsky A., Lamarre D., Gaudreau A., Pouyet J., Daune M., Poirier G.G. (1986) Modulation of Chromatin Superstructure Induced by Poly(ADP-ribose) Synthesis and Degradation. **J.Biol.Chem.** 261:7011-7017.

de Murcia G., Menissier de Murcia J., Schreiber V. (1991) Poly(ADP-ribose) Polymerase: Molecular Biological Aspects. **Bioessays** 13:455-462.

de Murcia G. & Menissier de Mucia J. (1994) Poly(ADP-ribose) Polymerase: A Molecular Nick-Sensor. **TIBS** 19:172-176.

Deng W.P. & Nickoloff J.A. (1994) Mismatch Repair of Heteroduplex DNA Intermediates of Extrachromosomal Recombination in Mammalian Cells. **Mol.Cell Biol.** 14: 400-406.

Desmarais Y., Menard L., Lagueux J., Poirier G.G. (1991) Enzymological properties of poly(ADP-ribose) polymerase: characterisation of automodification sites and NADase activity. **Biochem.Biophys.Acta.** 1078:179-186.

Dianov G., Price A., Lindahl T. (1992) Generation of Single-Nucleotide Repair Patches Following Excision of Uracil Residues from DNA. **Mol.Cell.Biol.** 12:1605-1612.

Dianov G., Sedgwick B., Daly G., Olsson M., Lovett S., Lindahl T. (1994) Release of 5'-terminal Deoxyribose-phosphate Residues from Incised Abasic Sites in DNA by the *Escherichia coli* RecJ Protein. **Nuc.Acid Res.** 22:993-998.

Ding R., Pommier Y., Kang V.H., Smulson M. (1992) Depletion of Poly(ADP-ribose) Polymerase by Antisense RNA Expression Results in a Delay in DNA Strand Break Rejoining. **J.Biol.Chem.** 267:12804-12812.

Ding R. & Smulson M. (1994) Depletion of Nuclear Poly(ADP-Ribose) Polymerase by Antisense RNA Expression: Influences on Genomic Stability, Chromatin Organisation, and Carcinogenic Cytotoxicity. **Cancer Res.** 54:4627-4634.

Dive C. & Hickman J.A. (1991) Drug-Targeted Interactions: Only the First step in the Commitment to a Programmed Cell Death? **Br.J.Cancer** 64:192-196.

Donehower L.A., Harvey M., Slagle B.L., McArther M.J., Montgomery C.A., Butel J.S, Bradley A. (1992) Mice Deficient for p53 are Developmentally Normal but Susceptible to Spontaneous Tumours. **Nature**

356:215-221.

Dowd D.R. & Lloyd R.S. (1990) Biological Significance of Facillitated Diffusion in Protein-DNA Interactions. **J.Biol.Chem.** 265: 3424-3431.

Duncan B.K. (1985) Isolation of Insertion, Deletion, and Nonsense Mutations of the Uracil-DNA Glycosylase (*ung*) Gene of *Escherichia coli* K-12. **J.Bacteriol.** 164:689-695.

Durkacz B.W., Omidiji O., Gray D.A., Shall S. (1980) $(ADP\text{-}ribose)_n$ Participates in Excision Repair. **Nature** 283:593-596.

Durkacz B.W., Irwin J., Shall S. (1981a) Inhibiton of $(ADP\text{-}ribose)_n$ Biosynthesis Retards DNA Repair But Does Not Inhibit DNA Repair Synthesis. **Biochem.Biophys.Res.Commun.** 101:1433-1441.

Durkacz B.W., Irwin J., Shall S. (1981b) The Effect of Inhibition of $(ADP\text{-}ribose)_n$ Biosynthesis on DNA Repair Assayed by the Nucleoid Technique. **Eur.J.Biochem.** 121:65-69.

Durkacz B.W., Lunec J., Grindley H., Griffin S., Horner O., Simm A. (1992) Murine Melanoma Cell Differentiation and Melanogenesis Induced by Poly(ADP-ribose) Polymerase Inhibitors. **Exp.Cell Res.** 202:287-291.

Durrant L.G., Margison G.P., Boyle J.M. (1981) Effects of 5-methylnicotinamide on Mouse L1210 Cells Exposed to N-methyl-N-nitrosourea: Mutation Induction, Formation and Removal of Methylation Products in DNA, and Unscheduled DNA Synthesis. **Carcinogenesis** 2:1013-1017.

Einck L. & Bustin M. (1985) The Intracellular Distribution and Function of the High Mobility Group Chromosomal Proteins. **Exp.Cell Res.** 156:295-310.

El-Deiry W., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer E., Kinzler K.W., Vogelstein B. *WAF1*, a Potential Mediator of p53 Tumour Supression. **Cell** 75:817-825.

Emanuelli M., Natalini P., Raffaelli N., Ruggieri S., Vita A., Magni G. (1992) NAD Biosynthesis in Human Placenta: Purification and Characterisation of Homogeneous NMN Adenyltransferase. **Arch.Biochem.Biophys.** 298:29-34.

Farzaneh F., Zalin R., Brill D., Shall S. (1982) DNA Strand Breaks and ADP-ribosyl Transferase Activation During Cell Differentiation. **Nature** 300:362-366.

Farzaneh F., Meldrum R., Shall S. (1987a) Transient Formation of DNA Strand Breaks During the Induced Differentiation of a Human Promyelocytic Leukaemic Cell Line, HL-60. **Nuc.Acid Res.** 15:3493-3502.

Farzaneh F., Feon S., Lebby R.A., Brill D., David J., Shall S. (1987b) DNA Repair in Human Promyelocytic Cell Line, HL-60. **Nuc.Acid Res.** 15:3503-3513.

Farzaneh F., Panayotou G.N., Bowler L.D., Hardas B.D., Broom T., Walther C., Shall S. (1988) ADP-ribosylation is Involved in the Integration of Foreign DNA into the Mammalian Genome. **Nuc.Acid Res.** 16:11319-11326.

Ferro A.M. & Olivera B.M. (1982) Poly(ADP-ribosylation) *in vitro* -reaction parameters and enzyme mechanism. **J.Biol.Chem.** 257:7808-7813.

Ferro A.M., Higgins N.P., Olivera B.M. (1983) Poly(ADP-ribosylation) of a DNA Topoisomerase. **J.Biol.Chem.** 258:6000-6003.

Ferro A.M. & Olivera B.M. (1984) Poly(ADP-ribosylation) of DNA Topoisomerase I from Calf Thymus. **J.Biol.Chem.** 259:547-554.

Fornace A.J. & Little J.B. DNA Crosslinking Induced by X-rays and Chemical Agents. **Biochim.Biophys.Acta** 477:343-355.

Franklin W.A. & Lindahl T. (1988) DNA Deoxyribophosphodiesterase. **EMBO J.** 7:3617-3622.

Friedberg E.C. (1985) DNA repair. Publishers: W.H. Freeman & Co.

Friedberg E.C., Bardwell A.J., Bardwell L., Wang Z., Dianov G. (1994) Transcription and Nucleotide Excision Repair - Reflection, Considerations and Recent Biochemical Insights. **Mut.Res.** 307:5-14.

Fujimura S., Hasegawa S., Shimizu Y., Sugimura T. (1967) Polymerisation of the Adenosine 5'-Diphosphate-Ribose Moiety of Nicotinamide-Adenine Dinucleotide by Nuclear Enzyme. I. Enzymic Reactions. **Biochim.Biophys.Acta** 145:247-259.

Gill D.M., Caplan R.A., Bredlau C.E., Meren R. (1974) The Structure and Function of Poly(ADP-ribose). **Fogarty Int.Centre Proc.** 26:85-99.

Gill D.M. & Meren R. (1978) ADP-Ribosylation of Membrane Proteins Catalysed by Cholera Toxin: Basis

of the Activation of Adenylate Cyclase. **Proc.Natl.Acad.Sci.USA** 75:3050-3054.

Giner H., Simonin S., de Murcia G., Menissier-de-Murcia J. (1992) Overproduction and Large-Scale Purification of the Human Poly(ADP-ribose) Polymerase Using a Baculovirus Expression System. **Gene** 114:279-283.

Giri C.P., West M.H.P., Ramierez M.L., Smulson M. (1978) Nuclear Protein Modification and Cromatin Substructure. 2. Internucleosomal Localisation of Poly(ADP-ribose) Polymerase. **Biochemistry** 17:3501-3504.

Glock G.E. & McLean P. (1957) Levels of Oxidised and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Tumours. **Biochem.J.** 65:413-416.

Goldman N., Brown M., Khoury G. (1981) Modification of SV40 T Antigen by Poly ADP-Ribosylation. **Cell** 24:567-572.

Gossard F. & Verly W.G. (1978) Properties of the Main Endonuclease Specific for Apurinic Sites of *Escherichia coli* (Endonuclease VI). 82:321-332.

Gradwohl G., Mazen A., de Murcia G. (1987) Poly(ADP-ribose) Polymerase Forms Loops with DNA. **Biochem.Biophys.Res.Commun.** 148:913-919.

Gradwohl G., Menissier-de Murcia J., Molinete M., Simonin F., Koken M., Hoeijmakers J.H.J., de Murcia G. (1990) The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA. **Proc.Natl.Acad.Sci.USA** 87:2990-2994.

Grasso P., Sharratt M., Cohen A.J. (1991) Role of Persistent Non-geneotoxic Tissue Damage in Rodent Cancer and Relevance to Humans. **Annu.Rev.Pharmacol.Toxicol.** 31:253-287.

Griffin R.J., Pemberton L.C., Rhodes D., Bleasdale C., Bowman K., Calvert H., Curtin N.J., Durkacz B.W., Newell D.R., Porteous J.K., Golding B.T. (1995) Novel Potent Inhibitors of the DNA Repair Enzyme Poly(ADP-ribose) Polymerase (PARP). **Anticancer Drug Design** "in press".

Grosso L.E. & Pitot H.C. (1984) Modulation of C-myc Expression in the HL-60 Cell Line. **Biochem.Biophys.Res.Commun.** 119:473-480.

Gruskin E.A. & Lloyd R.S. (1988) Molecular Analysis of Plasmid DNA Repair within Ultraviolet-irradiated

Escherichia coli. **J.Biol.Chem.** 263: 12738-12743.

Guzder S.N., Sung P., Bailly V., Prakash L., Prakash S. (1994) RAD25 is a DNA Helicase Required for DNA Repair and RNA Polymerase II Transcription. **Nature** 369: 578-581.

Habraken Y., Sung P., Prakash L., Prakash S. (1993) Yeast Excision Repair Gene *RAD2* Encode a Single-stranded DNA endonuclease. **Nature** 366: 365-368.

Haines M.E., Johnston I.R., Mathias A.P., Ridge D. (1969) The Synthesis of Nicotinamide-Adenine Dinucleotide and Poly(Adenosine Diphosphate Ribose) in Various Classes of Rat Liver Nuclei. **Biochem.J.** 115:881-887.

Hall P.A., McKee P.H., Menage H., Dover R., Lane D.P. (1993) High Levels of p53 Protein in UV-Irradiated Normal Human Skin. **Oncogene** 8:203-207.

Halldorsson H., Gray D.A., Shall S. (1978) Poly(ADP-ribose) Polymerase Activity in Nucleotide Permeable Cells. **FEBS lett.** 85:349-352.

Harper J.W., Adami G.R., Wei N., Keyomarsi K., Elledge S.J. (1993) The p21 Cdk-Interacting Protein Cip1 is a Potent Inhibitor of G1 Cyclin-Dependent Kinases. **Cell** 75:805-816.

Harrap K.R. & Furness M.E. (1973) The Cytotoxicity of Chlorambucil and its Associated Effects on NAD Metabolism. **Eur.J.Cancer** 9:343-351.

Harris C.C. (1991) Chemicla and Physical Carcinogenesis: Advances and Perspectives for the 1990's. **Cancer Res.(Suppl.)** 51:5023s-5044s.

Hartwell L.H. & Kastan M.B. (1994) Cell Cycle Control and Cancer. **Science** 266:1821-1828.

Hatakeyama K., Nemoto Y., Ueda K., Hayaishi O. (1986) Purification and Characterisation of Poly(ADP-ribose) Glycohydrolase - different modes of action on large and small poly(ADP-ribose). **J.Biol.Chem.** 261:14902-14911.

Hayakawa H., Koike G., Sekiguchi M. (1990) Expression and Cloning of Complementary DNA for a Human Enzyme that Repairs O^6-Methylguanine in DNA. **J.Mol.Biol.** 213: 739-747.

Heller B., Wang Z., Wagner E.F., Radons J., Burkle A., Fehsel K., Burkart V., Kolb H. (1995) Inactivation

of the Poly(ADP-ribose) Polymerase Gene Affects Oxygen Radical and Nitric Oxide Toxicity in Islet Cells. **J.Biol.Chem.** 270:11176-11180.

Hendricks W.M. (1991) Pellagra and Pellagralike Dermatoses: Etiology, Differential Diagnosis, Dermatopathology, and Treatment. **Semim.Dermatol.** 10:282-292.

Hepburn P.A. & Tisdale M.J. (1991) Antitumour Imidazotetrazines-XXIV. Growth Suppression by DNA from Cells Treated with Imidazotetrazinones. **Biochem.Pharmacol.** 41:339-343.

Hermeking H. & Eick D. (1994) Mediation of c-Myc-Induced Apoptosis by p53. **Science** 265:2091-2093.

Hickman J.A., Beere H.M., Wood A.C., Waters C.A., Parmar R. (1993) Mechanisms of Cytotoxicity Caused by Antitumour Drugs. **Toxicol.Lett.** 65:553-561.

Higashitani A., Tabata S., Endo H., Hotta Y. (1990) Purification of DNA Ligases from Mouse Testis and their Behaviour During Meiosis. **Cell Struct.Funct.** 15:67-72.

Hilz H. & Kittler M. (1971) Lack of Correlation Between Poly ADP-Ribose Formation and DNA Synthesis. **Hoppe-Seyler's Z.Physiol.Chem.** 352:1693-1704.

Hilz H., Bredehorst R., Nolde S., Kittler M. (1972) On the Biological Significance of Poly(ADP-ribose). **Hoppe Seyler's Z.Physiol.Chem.** 353:848-849.

Hilz H., Wielkens K., Bredehorst R. (1982) Quantitation of Mono(ADP-ribosyl) and Poly(ADP-ribosyl) Proteins. In:ADP-ribosyation Reactions. pp 305-321. Eds. Hayaishi O. & Ueda K. Academic Press Inc.

Hogeboom G.H. & Schneider W.C. (1952) VI. The Synthesis of Diphosphopyridine Nucleotide by Liver Cell Nuclei. **Cytochemical Studies** 6:611-620.

Holbrook J.J., Liljas A., Steindel S.J., Rossman M.G. (1975) Lactate Dehydrogenase. **Enzymes (3rd edition)** 11:191.

Holliday R. & Grigg G.W. (1993) DNA Methylation and Mutation. **Mut.Res.** 285: 61-67.

Hollstein M.C., Brooks P., Linn S., Ames B.N. (1984) Hydroxymethyluracil DNA glycosylase in Mammalian Cells. **Proc.Natl.Acad.Sci.** 81:4003-4007.

Honjo T., Nishizuka Y., Hayaishi O. (1968) Diptheria Toxin-Dependent Adenosine Phosphate Ribosylation of Aminoacyl Transferase II and Inhibition of Protein Synthesis. **J.Biol.Chem.** 243:3553-3555.

Hori T. (1981) High Incidence of Sister Chromatid Exchanges and Chromatid Interchanges in the Conditions of Lowered Activity of Poly(ADP-ribose) Polymerase. **Biochem.Biophys.Res.Commun.** 102:38-45.

Horsman M.R. & Chaplin D.J. (1994) Enhancement of Cyclophosphamide Cytotoxicity *in vitro* by the Benzamide analogue Pyrazinamide. **Br.J.Cancer** 69:648-654.

Hough C.J. & Smulson M.E. (1984) Association of Poly(adenosine diphosphate ribosylated) Nucleosomes with Transcriptionally Active and Inactive Regions of Chromatin. **Biochemistry** 23:5016-5023.

Huang J., Hsu D.S., Kazantsev A., Sancar A. (1994) Substrate Spectrum of Human Excinuclease: Repair of Abasic Sites, Methylated Bases, Mismatches, and Bulky Adducts. **Proc.Natl.Acad.Sci.USA** 91:12213-12217.

Huet J. & Laval F. (1985) Potentiation of Cell Killing by Inhibitors of Poly(Adenosine Diphosphate-Ribose) Synthesis in Bleomycin-treated Chinese Hamster Ovary Cells. **Cancer Res.** 45:987-991.

Huletsky A., de Murcia G., Muller S., Hengartner M., Menard L., Lamarre D., Poirier G.G. (1989) The Effect of Poly(ADP-ribosyl)ation on Native and H1-depleted Chromatin. **J.Biol.Chem.** 264:8878-8886.

Hunter T. (1993) Braking the Cycle. **Cell** 75:839-841.

Hunting D.J., Gowans B.J., Henderson J.F. (1985) Specificity of Inhibitors of Poly(ADP-ribose) Synthesis - Effects on Nucleotide Metabolism in Cultured Cells. **Mol.Pharmacol.** 28:200-206.

Hunting D.J. & Gowans B.J. (1988) Inhibition of Repair Patch Ligation by an Inhibitor of Poly(ADP-ribose) Synthesis in Normal Human Fibroblasts Damaged with Ultraviolet Radiation. **Mol.Pharmacol.** 33:358-362.

Huppi K., Bhatia K., Siwarski D., Klinman D., Cherney B., Smulson M. (1989) Sequence and organisation of the mouse poly(ADP-ribose) polymerase gene. **Nucl.Acid Res.** 17:3387-3401.

Ikai K., Ueda K., Fukushima M., Nakamura T., Hayaishi O. (1980a) Poly(ADP-ribose) Synthesis, a Marker of Granulocyte Differentiation. **Proc.Natl.Acad.Sci.USA** 77:3682-3685.

Ikai K., Ueda K., Hayaishi O. (1980b) Immunohistochemical Demonstration of Poly(Adenosine Diphosphate-Ribose) in Nuclei of Various Rat Tissues. **J.Histochem.Cytochem.** 28:670-676.

Ikeda M., Tsuji H., Nakamura S., Ichiyama A., Nishizuka O. (1965) Studies on the Biosynthesis of Nicotinamide Adenine Dinucleotide. **J.Biol.Chem.** 240:1395-1401.

Ikejima M., Marsischky G., Gill D.M. (1987) Direction of Elongation of Poly(ADP-ribose) Chains - addition of residues at the polymerase-proximal terminus. **J.Biol.Chem.** 262:17641-17650.

Ikejima M., Noguchi S., Yamashita R., Ogura T., Sugimura T., Gill D.M., Miwa M. (1990) The Zinc fingers of Human Poly(ADP-ribose) Polymerase Are Differentially Required fot the Recognition of DNA Breaks and Nicks and the Consequent Enzyme Activation. **J.Biol.Chem.** 265:21907-21913.

Institoris E., Fox B.W., Palyi I. (1992) Benzamide Potentiation of the Cytotoxicity of Bifunctional Galacticol in Resistant P388 Leukemia Correlates with Inhibition of DNA Ligase II. **Cancer Chemother.Pharmacol.** 30:325-329.

Ionov Y., Peinado M.A., Malkhosyan S., Shibata D., Perucho M. (1993) Ubiquitous Somatic Mutations in Simple Repeated Sequences Reveal a New Mechanism for Colonic Carcinogenesis. **Nature** 363:558-561.

Ittel M.E., Jongstra-Bilen J., Rochette-Egly C., Mandel P. (1983) Involvement of PolyADP-ribose Polymerase in the Initiation of Phytohemagglutinin Induced Human Lymphocyte Proliferation. **Biochem.Biophys.Res.Commun.** 116:428-434.

Jacobson E.L., Antol K.M., Juarez-Salinas H., Jacobson M.K. (1983) Poly(ADP-ribose) Metabolism in Ultraviolet Irradiated Human Fibroblasts. **J.Biol.Chem.** 258:103-107.

Jacobson E.L., Smith J.Y., Mingmuang M., Meadows R., Sims J.L. (1984) Effect of Nicotinamide Analogues on Recovery from DNA Damage in C3H10T1/2 Cells. **Cancer Res.** 44: 2485-2492.

Jacobson E.L., Meadows R., Measel J. (1985a) Cell Cycle Perturbations Following DNA Damage in the Presence of ADP-ribosylation Inhibitors. **Carcinogenesis** 6: 711-714.

Jacobson E.L., Smith Y., Wielckens K., Hilz H., Jacobson M.K. (1985b) Cellular Recovery of Dividing and confluent C3H10T1/2 Cells from N-methyl-N'-nitro-N-nitrosoguanidine in the presence of ADP-ribosylation inhibitors. **Carcinogenesis** 6:715-718.

James M.R. & Lehmann A.R. (1982) Role of Poly(adenosine diphosphate ribose) in Deoxyribonucleic Acid Repair in Human Fibroblasts. **Biochemistry** 21:4007-4013.

Janakidevi K. & Koh C. (1974) Synthesis of Polyadenosine Diphosphate Ribose by Isolated Nuclei of Swine Aortic Tissue. **Biochemistry** 13:1327-1330.

Jaxel C., Capranico G., Kerrigan D., Kohn K.W., Pommier Y. (1991) Effect of Local DNA Sequence on Topoisomerase I Cleavage in the Presence or Absence of Camptothecin. **J.Biol.Chem.** 266:20148-20423.

Jayaram H.N., Cooney D.A., Glazer R.I., Dion R.L., Johns D.G. (1982) Mechanism of Resistance to the OncolyticC-Nucleoside2-β-D-Ribofuranosylthiazole-4-Carboxamide(NSC-286193).**Biochem.Pharmacol.** 31:2557-2560.

Jayaram H.N. (1985) Biochemical Mechanisms of Resistance to Tiazofurin. **Adv.Enzyme Reg.** 24:67-89.

Jayaram H.N., Pillwein K., Lui M.S., Faderan M.A., Weber G. (1986) Mechanism of Resistance to Tiazofurin in Hepatoma 3924A. **Biochem.Pharmacol.** 35:587-593.

Jayaram H.N., Lapis E., Tricot G., Kneebone P., Paulik E., Zhen W., Engeler G.P., Hoffman R., Weber G. (1992) Clinical Pharmacokinetic Study of Tiazofurin Administered as a 1-Hour Infusion. **Int.J.Cancer** 51:182-188.

Jayaram H.N., Zhen W., Gharebaghi K. (1993) Biochemical Consequences of Resistance to Tiazofurin in Human Myelogenous Leukemic K562 Cells. **Cancer Res.** 53:2344-2348.

Jedeikin L.A. & Weinhouse S. (1955) Metabolism of Neoplastic Tissue VI. Assay of Oxidised and Reduced Diphosphopyridine Nucleotide in Normal and Neoplastic Tissues. **J.Biol.Chem.** 213:271-280.

Jessberger R. & Berg P. (1991) Repair of Deletions and Double-strand Gaps by Homologous Recombination in a Mammalian in vitro System. **Mol.Cell Biol.** 11:445-457.

Jessberger R., Produst V., Hubscher U., Berg P. (1993) A Mammalian Protein Complex that Repairs Double-strand Breaks and Deletions by Recombination. **J.Biol.Chem.** 286:15070-15079.

Johnson G.S. (1981) Benzamide and Its Derivatives Inhibit Nicotinamide Methylation as Well as ADP-ribosylation. **Biochem.Int.** 2:611-617.

Johnson A.W. & Demple B. (1988) Yeast DNA 3'-Repair Diesterase Is the Major Cellular Apurinic/Apyrimidinic Endonuclease: Substrate Specificity and Kinetics. **J.Biol.Chem.** 263:18017-18022.

Johnstone A.P. & Williams G.T. (1982) Role of DNA Breaks and ADP-ribosyl Transferase Activity in Eukaryotic Differentiation Demonstrated in Human Lymphocytes. **Nature** 300:368-370.

Jongstra-Bilen J., Ittel M., Neidergang C., Vosberg H., Mandel P. (1983) DNA Topoisomerase I from Calf Thymus is Inhibited *in vitro* by Poly(ADP-ribosylation). **Eur.J.Biochem.** 136:391-396.

Juarez-Salinas H., Sims J.L., Jacobson M.K. (1979) Poly(ADP-ribose) Levels in Carcinogen-treated Cells. **Nature** 282:740-741.

Jung S., Miranda E.A., Menissier de Murcia J., Niedergang C., Delarue M., Schulz G.E., de Murcia G.M. (1994) Crystallisation and X-ray Crystallographic Analysis of Recombinant Chicken Poly(ADP-ribose) Polymerase Catalytic Domain Produced in Sf9 Insect Cells. **J.Mol.Biol.** 244:114-116.

Kallman B., Burkart V., Kroncke K.D., Kolb-Bachofen V., Kolb H. (1992) Toxicity of Chemically Generated Nitric Oxide Towards Pancreatic Islet Cells Can Be Prevented by Nicotinamide. **Life Sciences** 51:671-678.

Kameshita I., Matsuda Z., Taniguchi T., Shizuta Y. (1984) Poly(ADP-ribose) Synthetase. Separation and Identification of Three Proteolytic Fragments as the Substrate Binding Domain, the DNA-Binding Domain, and the Automodification Domain. **J.Biol.Chem.** 259:4770-4776.

Kane C.M. & Linn S. (1981) Purification and Characterisation of an Apurinic/Apyrimidinic Endonuclease from HeLa Cells. **J.Biol.Chem.** 256:3405-3414.

Karran P., Lindahl T., Ofsteng I., Evensen G.B., Seeberg E. (1980) *Escherichia coli* Mutants Deficient in 3-Methyladenine-DNA Glycosylase. **J.Mol.Biol.** 140:101-127.

Karran P. & Bignami M. (1994) DNA Damage Tolerance, Mismatch Repair and Genome Instability. **Bioessays** 16:833-839.

Kasid U.N., Halligan B., Liu L.F., Dritschilo A., Smulson M. (1989) Poly(ADP-ribose)-mediated Post-translational Modification of Chromatin-associated Human Topoisomerase I. **J.Biol.Chem.** 264:18687-18692.

Kastan M.B., Onyekwere O., Sidransky D., Vogelstein B., Craig R.W. (1991) Participation of p53 Protein in the Cellular Response to DNA Damage. **Cancer Res.** 51:6304-6311.

Kastan M.B., Zhan Q., El-Diery W., Carrier F., Jacks T., Walsh W.V., Plunkett B.S., Vogelstein B, Fornace A.J. (1992) A Mammalian Cell Cycle Checkpoint Pathway Utilising p53 and GADD45 is Defective in Ataxia-Telangiectasis. **Cell** 71:587-597.

Kaufmann S.H., Desnoyers S., Ottaviano Y., Davidson N.E., Poirier G.G. (1993) Specific Proteolytic Cleavage of Poly(ADP-ribose) Polymerase: An Early Marker of Chemotherapy-Induced Apoptosis. **Cancer Res.** 53:3976-3985.

Kawaichi M., Ueda K., Hayaishi O. (1981) Multiple Autopoly(ADP-ribosyl)ation of Rat Liver Poly(ADP-ribose) Synthetase - Mode of modification and properties of automodified synthetase. **J.Biol.Chem.** 256:9483-9489.

Keith G., Desgres J., de Murcia G. (1990) Use of Two-Dimensional Thin-Layer Chromatography for the Components Study of Poly(adenosine diphosphate ribose). **Anal.Biochem.** 191:309-313.

Kelley M.R., Venugopal S., Harless J., Deutsch W.A. (1989) Antibody to a Human DNA Repair Protein Allows for Cloning of a *Drosophila* cDNA That Encodes an Apurinic Endonuclease. **Mol.Cell.Biol.** 9:965-973.

Kenedy K.A., Teicher B.A., Rockwell S., Sartorelli A.C. (1980) The Hypoxic Cell: A Target for Selective Cancer Chemotherapy. **Biochem.Pharmacol.** 29:1-8.

Kern S.E., Kinzler K.W., Bruskin A., Jarosz D., Friedman P., Prives C., Vogelstein B. (1991) Identification of p53 as a Sequence-Specific DNA-Binding Protein. **Science** 252:1708-1711.

Kern S.E., Pietenpol J.A., Thiagalingam S., Seymour A., Kinzler K.W., Vogelstein B. (1992) Oncogenic Forms of p53 Inhibit p53-Regulated Gene Expression. **Science** 256:827-830.

Kharbanda S.M., Sherman M.L., Spriggs D.R., Kufe D.W. (1988) Effects of Tiazofurin on Protooncogene Expression during HL-60 Cell Differentiation. **Cancer Res.** 48:5965-5968.

Kharbanda S.M., Sherman M.L., Kufe D.W. (1990) Effects of Tiazofurin on Guanine Nucleotide Binding Regulatory Proteins in HL-60 Cells. **Blood** 75:583-588.

Kidwell W.R. & Mage M.G. (1976) Changes in Poly(adenosine diphosphate-ribose) Polymerase in Synchronous HeLa Cells. **Biochemistry** 15:1213-1217.

Kidwell W.R., Nolam N., Stone P.R. (1982) Variation in Poly(ADP-ribose) and Poly(ADP-ribose) Synthetase in Synchronously Dividing Cells. In:ADP-ribosylation Reactions. Eds: Hayaishi O. & Ueda K. (Academic Press, New York).

Kiehlbauch C.C., Aboul-Ela N., Jacobson E.L., Ringer D.P., Jacobson M.K. (1993) High Resolution Fractionation and Characterisation of ADP-Ribose Polymers. **Anal.Biochem.** 208:26-34.

Kim H., Jacobson E.L., Jacobson M.K. (1993) Position of Cyclisation in Cyclic ADP-Ribose. **Biochem.Biophys. Res.Commun.** 194:1143-1147.

Kohn.K.W., Ewig R.A.G., Erickson L.C., Zwelling L.A. (1981) Measurement of Strand Breaks and Crosslinks by Alkaline Elution. In Freidburg,E.C & Hanawalt.P.C (eds.) DNA Repair: A Laboratory Manuel of Research Procedures, Vol.1 part B, pp379-401. Marcel Dekker, New York & Basel.

Kornberg A. (1950) Reversible Enzymatic Synthesis of Diphosphopyridine Nucleotide and Inorganic Pyrophosphate. **J.Biol.Chem.** 182:779-793.

Koshiyama H., Lee H.C., Tashjian A.H. (1991) Novel Mechanism of Intracellular Calcium Release in Pituitary Cells. **J.Biol.Chem.** 266:16985-16988.

Kriek E. (1972) Persistant Binding of a New Reaction Product of the Carcinogen *N*-Hydroxy-*N*-2-Acetylaminofluorene with Guanine in Rat Liver DNA *in vivo*. **Cancer Res.** 32:2042-2048.

Kuerbitz S.J., Plunkett B.S., Walsh W.V., Kastan M.B. (1992) Wild-Type p53 is a Cell Cycle Checkpoint Determinant Following Irradiation. **Proc.Natl.Acad.Sci.USA** 89:7491-7495.

Kuttan R. (1989) Unique Bioactivation of Tiazofurin - Studies with Resistant Cells. **Indian J.Biochem.Biophys.** 26:160-165.

Lahue R.S., Au K.G., Modrich P. (1989) DNA Mismatch Correction in a Defined System. **Science** 245:160-164.

Lautier D., Hoflack J., Kirkland J.B., Poirier D., Poirier G.G. (1994) The Role of Poly(ADP-ribose) Metabolism in Response to Active Oxygen Cytotoxicity. **Biochim.Biophys.Acta** 1221:215-220.

Lazebnik Y.A., Kaufmann S.H., Desnoyers S., Poirier G.G., Earnshaw W.C. (1994) Cleavage of Poly(ADP-ribose) Polymerase by a Proteinase with Properties like ICE. **Nature** 371:346-347.

Leach F.S., Nicolaides N.C., Papadopoulos N., Liu B., Jen J., Parsons R., Peltyomaki P., Sistonen P., Aaltonen L.A., Nystrom-Lahti M., Guan X.Y., Zhang J., Meltzer P.S., Yu J., Kao F., Chen D.J., Cerosaletti K.M., Fournier R.E.K., Todd S., Lewis T., Leach R.J., Naylor S.L., Weissenbach J., Mecklin J., Jarvinen H., Petersen G.M., Hamilton S.R., Green J., Jass J., Watsonm P., Lynch H.T., Trent J.M., Chapelle A., Kinzler K.W., Vogelstein B. (1993) Mutations of a *mutS* Homolog in Hereditary Nonpolyposis Colorectal Cancer. **Cell** 75:1215-1225.

Le Cam E., Fack F., Menissier- de Murcia J., Cogmet JJ.A.H., Barbin A., Sarantoglou V., Revet B., Delain E., de Murcia G. (1994) Conformational Analysis of a 139 Base-pair DNA Fragment Containing a Single-stranded Break and Its Interaction with Human Poly(ADP-ribose) Polymerase. **J.Biol.Chem.** 235:1062-1071.

Leduc Y., Lawrence J., de Murcia G., Poirier G.G. (1988) Cell Cycle Regulation of Poly(ADP-ribose) Synthetase in FR3T3 Cells. **Biochim.Biophys.Acta** 968:275-282.

Lee H.C., Walseth T.F., Bratt G.T., Hayes R.N., Clapper D.L. (1989) Structural Determination of a Cyclic Metabolite of NAD^+ with Intracellular Ca^{2+} -Mobilising Activity. **J.Biol.Chem.** 264:1608-1615.

Legerski R. & Peterson C.(1992) Expression Cloning of a Human DNA Repair Gene Involved in Xeroderma Pigmentosum Group C. **Nature** 359:70-73.

Lehmann A.R. & Karren P. (1981) DNA Repair. **Exp.Rev.Cyto.** 72:101-146.

Lehmann A.R. & Broughton B.C. (1984) Poly(ADP-ribosylation) Reduces the Steady-state Level of Breaks in DNA Following Treatment of Human Cells with Alkylating Agents. **Carcinogenesis** 5:117-119.

Leiber U., Kittler M., Hilz H. (1973) Enzymes of Poly(ADPR) Metabolism in Proliferating and Nonproliferating Liver Tissues. **Hoppe-Seyer's Z Physiol.Chem.** 354:1347-1350.

Li L., Elledge S.J., Peterson C.A., Bales E.S., Legerski R.J. (1994) Specific Association Between the Human DNA Repair Proteins XPA and ERCC1. **Proc.Natl. Acad.Sci.USA** 91: 5012-5016.

Lin J. & Sancar A. (1992) Active Site of (A)BC Excinuclease. **J.Biol.Chem.** 267:17688-17692.

Lindahl T. & Andersson A. (1972) Rate of Chain Breakage at Apurinic Sites in Double-Stranded Deoxyribonucleic Acid. **Biochemistry** 11:3618-3623.

Lindahl T. & Nyberg B. (1972) Rate of Depurination of Native Deoxyribonucleic Acid. **Biochemistry** 11:3610-3618.

Lindahl T. & Nyberg B. (1974) Heat-Induced Deamination of Cytosine Residues in Deoxyribonucleic Acid. **Biochemistry** 13:3405-3410.

Lindahl T., Sedgwick B., Sekiguchi M., Nakabeppu Y. (1988) Regulation and Expression of the Adaptive Response to Alkylating Agents. **Ann.Rev.Biochem.** 57:133-157.

Lindahl T. (1990) Repair of Intrinsic DNA Lesions. **Mut.Res.** 238:305-311.

Lindahl T. (1993) Instability and Decay of the Primary Structure of DNA. **Nature** 362:709-715.

Linn S. (1982) Nucleases Involved in DNA Repair. In: Nucleases, 3rd Edition. Editors: Linn S.M. & Roberts R.J. Cold Spring Harbour Laboratory, New York. pp 59-83.

Lippke J.A., Gordon L.K., Brash D.E., Haseltine W.A. (1981) Distribution of UV Light-Induced Damage in a Defined Sequence of Human DNA: Detection of Alkaline-Sensitive Lesions at Pyrimidine Nucleoside-Cytidine Sequences. **Proc.Natl.Acad.Sci.USA** 78:3388-3392.

Little J.B. (1968) Delayed Initiation of DNA Synthesis in Irradiated Human Diploid Cells. **Nature** 218:1064-1065.

Loeb L.A. (1989) Endogenous Carcinogenesis: Molecular Oncology into the Twenty-first Century-Presidential Address. **Cancer Res.** 49:5489-5496.

Loeb L.A. (1994) Microsatellite Instability: Marker of a Mutator Phenotype in Cancer. **Cancer Res.** 54:5059-5063.

Lonn U. & Lonn S. (1985) Accumulation of 10-kilobase DNA Replication Intermediates in Cells Treated with 3-aminobenzamide. **Proc.Natl.Acad.Sci.USA** 82: 104-108.

Lowe S.W., Ruley H.E., Jacks T., Housman D.E. (1993) p53-Dependent Apoptosis Modulates the Cytotoxicity of Anticancer Agents. **Cell** 74:957-967.

Lunec J., George A.M., Hedges M., Cramp W.A., Whish W.J.D., Hunt B. (1984) Post-irradiation Sensitisation with the ADP-ribosyltransferase Inhibitor 3-acetamidobenzamide. **Br.J.Cancer** 49:19-25.

Lunn J.M. & Harris A.L. (1988) Cytotoxicity of 5-(3-methyl-1-triazeno)imidazole-4-carboxamide(MTIC) on Mer^+, Mer^-Rem^- and Mer^- cell lines: Differential Potentiation by 3-acetamidobenzamide. **Br.J.Cancer** 57:54-58.

MacInnes M.A., Dickson J.A., Hernandez R.R., Learmonth D., Lin G.Y., Mudgett J.S., Park M.S., Schauer S., Reynolds R.J., Strniste G.F., Yu J.Y. (1993) Human *ERCC5* cDNA-Cosmid Complementation for Excision Repair and Bipartite Amino Acid Domains Conserved with RAD Proteins of *Saccaromyces cerevisiae* and *Schizosaccaromyces pombe*. **Mol.Cell Biol.** 13:6393-6402.

Madhani H.D., Bohr V. A., Hanawalt P.C. (1986) Differential DNA Repair in Trascriptionally Active and Inactive Proto-oncogenes: c-*abl* and c-*mos*. **Cell** 45: 417-423.

Malanga M. & Althaus F.R. (1994) Poly(ADP-ribose) Molecules Formed During DNA Repair *in vivo*. **J.Biol.Chem.** 269:17691-17696.

Malanga M., Bachmann S., Panzeter P.L., Zweifel B., Althaus F.R. (1995) Poly(ADP-ribose) Quantification at the Femtomole Level in Mammalian Cells. **Anal.Biochem.** "in press".

Masuda H., Ozols R.F., Lai G., Fojo A., Rothenberg M., Hamilton T.C. (1988) Increased DNA Repair as a Mechanism of Acquired Resistance to *cis*-Diamminedichloroplatinum(II) in Human Ovarian Cancer Cell Lines. **Cancer Res.** 48:5713-5716.

Masuda H., Tanaka t., Matsuda H., Kusaba I. (1990) Increased Removal of DNA-Bound Platinum in a Human Ovarian Cancer Cell Line Resistant to *cis*-Diamminedichloroplatinum(II). **Cancer Res.** 50:1863-1866.

Mathis G. & Althaus F.R. (1987) Release of Core DNA from Nucleosomal Core Particles Following $(ADP\text{-}ribose)_n$-modification *in vitro*. **Biochem.Biophys.Res.Commun.** 143:1049-1054.

Mathis G. & Althaus F.R. (1990) Uncoupling of DNA Excision Repair and Nucleosomal Unfolding in Poly(ADP-ribose)-Depleted Mammalian Cells. **Carcinogenesis** 11:1237-1239.

Mattern M.R., Mong S., Bartus H.F., Mirabelli C.K., Crooke S.T., Johnson R.K. (1987) Relationship Between the Intracellular Effects of Camptothecin and the Inhibition of DNA Topoisomerase I in Cultured

L1210 Cells. **Cancer Res.** 47:1793-1798.

Mattern M.R., Hofmann G.A., McCabe F.L., Johnson R.K. (1991) Synergistic Cell Killing by Ionising Radiation and Topoisomerase I Inhibitor Topotecan (SK&F 104864). **Cancer Res.** 51:5813-5816.

Mazen A., Menissier-de Murcia J., Molinete M., Simonin F., Gradwohl G., Poirier G.G, de Murcia G. (1989) Poly(ADP-ribose) polymerase: a novel finger protein. **Nuc.Acid Res.** 17:4689-4698.

McConkey D.J., Hartzell P., Nicotera P., Orrenius S. (1989) Calcium-activated DNA Fragmentation Kills Immature Thymocytes. **FASEB J.** 3:1843-1849.

McCurry L.S. & Jacobson M.K. (1981) Poly(ADP-ribose) Synthesis Following DNA Damage in Cells Heterozygous of Homozygous for the Xeroderma Pigmentosum Genotype. **J.Biol.Chem.** 256:551-553.

McGrathR.A. & Williams R.W. (1966) Reconstruction *in vivo* of Irradiated *Escherichia coli* Deoxyribonucleic acid; the Rejoining of Broken Pieces. **Nature** 212:534-535.

Medcalf A.S.C. & Lawley P.D. (1981) Time Course of O^6-methylguanine Removal from DNA of *N*-methyl-*N*-nitrosourea-treated Human Fibroblasts. **Nature** 289:796-798.

Mellon I., Bohr V.A., Smith C.A., Hanawalt P.C. (1986) Preferential DNA Repair of an Active Gene in Human Cells. **Proc.Natl.Acad.Sci.USA** 83: 8878-8882.

Mellon I. & Hanawalt P.C. (1989) Induction of the *Escherichia coli* Lactose Operon Selectively Increases Repair of Its Transcribed DNA Strand. **Nature** 342: 95-98.

Menissier-de Murcia J., Molinete M., Gradwohl G., Simonin F., de Murcia G. (1989) Zinc-binding Domain of Poly(ADP-ribose) polymerase Participates in the Recognition of Single Strand Breaks on DNA. **J.Mol.Biol.** 210:229-233.

Meuth M., Aufreitter E., Reichard P. (1976) Deoxyribonucleotide Pools in Mouse-Fibroblast Cell Lines with Altered Ribonucleotide Reductase. **Eur.J.Biochem.** 71:39-43.

Milam K.M. & Cleaver J.E. (1984) Inhibitors of Poly(Adenosine Diphosphate-Ribose) Synthesis: Effect on Other Metabolic Processes. **Science** 223:589-591.

Milam K.M., Thomas G.H., Cleaver J.E. (1986) Disturbances in DNA Precursor Metabolism Associated

with Exposure to an Inhibitor of Poly(ADP-ribose) Synthetase. **Exp.Cell Res.** 165:260-268.

Miller E.G. (1975) Effect of Deoxyribonuclease I on the Number and Length of Chains of Poly(ADP-ribose) Synthesised, *in vitro*. **Biochem.Biophys.Res.Commun.** 66:280-286.

Minaga T., Romashcin A.D., Kirsten E., Kun E. (1979) The *in Vivo* Distribution of Immunoreactive Larger Than Tetrameric Polyadenosine Diphosphoribose in Histone and Non-Histone Protein Fractions of Rat Liver. **J.Biol.Chem.** 254:9663-9668.

Minaga T. and Kun E. (1983) Probable Helical Conformation of Poly(ADP-ribose) - the effect of cations on spectral properties. **J.Biol.Chem.** 258:5726-5730.

Mitchell R.B. & Dolan M.E. (1993) Effect of Temozolomide and Dacarbazine on O^6-alkylguanine-DNA alkyltransferase Activity and sensitivity of Human Tumour Cells and Xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea. **Cancer Chemother.Pharmacol.** 32:59-63.

Miwa M., Tanaka M., Matsushima T., Sugimura T. (1974) Purification and Properties of a Glycohydrolase from Calf Thymus Splitting Ribose-Ribose Linkages of Poly(Adenosine Diphosphate Ribose). **J.Biol.Chem.** 249:3475-3482.

Miwa M., Saikawa N., Yamaizumi Z., Nishimura S., Sugimura T. (1979) Structure of Poly(adenosine diphosphate ribose): Identification of 2′-[1″-ribosyl-2″-(or3″-)(1‴-ribosyl)]adenosine - 5′, 5″, 5‴-tris(phosphate) as a Branch Linkage. **Proc.Natl.Acad.Sci.USA** 76:595-599.

Miwa M., Kanai M., Kondo T., Hoshino H., Ishihara K., Sugimura T. (1981) Inhibitors of Poly(ADP-ribose) Polymerase Enhance Unsceduled DNA Synthesis in Human Peripheral Lymphocytes. **Biochem.Biophys.Res.Commun.** 100:464-470.

Modrich P. (1991) Mechanisms and Biological Effects of Mismatch Repair. **Annu.Rev.Genet.** 25:229-253.

Molinete M., Vermeulen W., Burkle A., Menissier-de Murcia J., Kupper J.H., Hoeijmakers J.H.J., de Murcia G. (1993) Overproduction of the Poly(ADP-ribose) Polymerase DNA-Binding Domain Blocks Alkylation-Induced DNA Repair Synthesis in Mammalian Cells. **EMBO J.** 12:2109-2117.

Morgan W.F. & Cleaver J.E. (1982) 3-Aminobenzamide Synergistically Increases Sister-chromatid Exchanges in Cells Exposed to methyl methanesulfonate but not to Ultraviolet Light. **Mut.Res.** 104:361-366.

Morgan W.F. & Cleaver J.E. (1983) Effect of 3-Aminobenzamide on the Rate of Ligation During Repair of Alkylated DNA in Human Fibroblasts. **Cancer Res.** 43:3104-3107.

Morton R.K. (1958) Enzymic Synthesis of Coenzyme I In Relation to Chemical Control of Cell Growth. **Nature** 181:540-542.

Mosbaugh D.W. & Linn S. (1982) Characterisation of the Action of *Escherichia coli* DNA Polymerase I at Incisions Produced by Repair Endodeoxyribonucleases. **J.Biol.Chem.** 257:575-583.

Moses K., Harris A.L., Durkacz B.W. (1988) Synergistic Enhancement of 6-Thioguanine Cytotoxicity by ADP-Ribosyltransferase Inhibitors. **Cancer Res.** 48:5650-5654.

Moses K., Willmore E., Harris A.L., Durkacz B.W. (1990) Correlation of Enhanced 6-Mercaptopurione Cytotoxicity with Increased Phosphoribosylpyrophosphate Levels in Chinese Hamster Ovary Cells Treated with 3-Aminobenzamide. **Cancer Res.** 50:1992-1996.

Moss J. & Vaughan M. (1979) Activation of Adenylate Cyclase by Choleragen. **Ann.Rev.Biochem.** 48:581-600.

Mullins D.W., Giri C.P., Smulson M. (1977) Poly(adenosine diphosphate-ribose) Polymerase: The Distribution of a Chromosome-Associated Enzyme within the Chromatin Substructure. **Biochemistry** 16:506-513.

Naegelli H., Loetscher P., Althaus F.R. (1989) Poly ADP-ribosylation of Proteins - processivity of a post-translational modification. **J.Biol.Chem.** 264: 14382-14385.

Naegelli H. and Althaus F.R. (1991) Regulation of Poly(ADP-ribose) Polymerase - histone specific adaptations of reaction products. **J.Biol.Chem.** 266:10596-10601.

Nasmyth K. & Hunt T. (1993) Dams and Sluices. **Nature** 366:634-635.

Nduka N., Skidmore C.J., Shall S. (1980) The Enhancement of Cytotoxicity of *N*-Methyl-*N*-nitrosourea and of γ-Radiation by Inhibitors of Poly(ADP-ribose) Polymerase. **Eur.J.Biochem.** 105:525-530.

Neidergang C.P., de Murcia G., Ittel M., Pouyet J., Mandel P. (1985) Time Course of Polynucleosome Relaxation and ADP-ribosylation. **Eur.J.Biochem.** 146:185-191.

Nelson W.G. & Kastan M.B. (1994) DNA Strand Breaks: the DNA Template Alterations That Trigger p53-Dependent DNA Damage Response Pathways. **Mol.Cell Biol.** 14:18125-1823.

Newbold R.F., Warren W., Medcalf A.S.C., Amos J. (1980) Mutagenicity of Carcinogenic Methylating Agents is Associated with a Specific DNA Modification. **Nature** 283:596-599.

Newlands E.S., Blackledge G., Slack J.A., Goddard C., Brindley C.J., Holden L., Stevens M.F. (1985) Phase I Clinical Trial of Mitozolomide. **Cancer Treat.Rep.** 69:801-805.

Newlands E.S., Blackledge G.R.P., Slack J.A., Rustin G.J.S., Smith D.B., Stuart N.S.A., Quarterman C.P., Hoffman R., Stevens M.F.G., Brampton M.H., Gibson A.C. (1992) Phase I Trial of Temozolomide (CCRG 81045: M&B 39831: NSC 362856). **Br.J.Cancer** 65:287-291.

Nishizuka Y., Ueda K., Nakazawa K., Hayaishi O. (1967) Studies on the Polymer of Adenosine Diphosphate Ribose. **J.Biol.Chem.** 242:3164-3171.

Nossieri C., Coppola S., Ghibelli L. (1994) Possible Involvement of Poly(ADP-Ribosy) Polymerase in Triggering Stress-Induced Apoptosis. **Exp.Cell Res.** 212:367-373.

O'Donovan A., Davies A.A., Moggs J.G., West S.C., Wood R.D. (1994) XPG Endonuclease makes the 3' Incision in Human DNA Nucleotide Excision Repair. **Nature** 371: 432-435.

Oei S.L., Herzog H., Hirsch-Kauffman M., Schneider R., Auer B., Schweiger M. (1994) Transcriptional regulation and autoregulation of the human gene for ADP-ribosyltransferase. **Mol.Cell Biochem** 138:99-104.

Ogura T., Nyunoya H., Takaahashi-MasutaniM., Miwa M., Sugimura T., Esumi H. (1990). Characterisation of a putative promotor region of the human poly(ADP-ribose) polymerase gene: structural similarity to that of the DNA ploymerase β gene. **Biochem.Biophys.Res.Commun.** 167:701-710.

Ohashi Y., Ueda K., Kawaichi M., Hayaishi O. (1983) Activation of DNA Ligase by Poly(ADP-ribose) in Chromatin. **Proc.Natl.Acad.Sci.USA** 80:3604.

Oikawa A., Tohda H., Kanai M., Miwa M., Sugimura T. (1980) Inhibitors of Poly(Adenosine diphoshate ribose) Polymerase Induce Sister Chromatid Exchanges. **Biochem.Biophys.Res.Commun.** 97:1311-1316.

Olah E., Natsumeda Y., Ikegami T., Kote Z., Horanyi M., Szelenyi J., Paulik E., Kremmer T., Hollan S.R.,

Sugar J., Weber G. (1988) Inductionof Erythroid Differentiation and Modulation of Gene Expression by Tiazofurin in K-562 Leukemia Cells. **Proc.Natl.Acad.Sci.USA** 85:6533-6537.

Oliner J.D., Pietenpol J.A., Thiagalingam S., Gyuris J., Kinzler K.W., Vogelstein B. (1993) Oncoprotein MDM2 Conceals the Activation Domain of Tumour Suppressor p53. **Nature** 362:857-860.

Olson D.C., Marechal V., Momand J., Chen J., Romocki C., Levine A.J. (1993) Identification and Characterisationof Multiple mdm-2 Proteins and mdm-2-p53 Protein Complexes. **Oncogene** 8:2353-2360.

Olsson A., Olofsson T., Pero R.W. (1993) Specific Binding and Uptake of Extracellular Nicotinamide in Human Leukemic K-562 Cells. **Biochem. Pharmacol.** 45:1191-1200.

Ostling O. & Johanson K.J. (1984) Microelectrophoretic Study of Radiation-Induced DNA Damages in Individual Mammalian Cells. **Biochem.Biophys.Res.Commun.** 123:291-298.

Panzeter P.L. & Althaus F.R. (1990) High Resolution Size Analysis of ADP-ribose Polymers Using Modified DNA Sequencing Gels. **Nuc.Acids Res.** 18:2194

Panzeter P.L., Realini C.A., Althaus F.R. (1992) Noncovalent Interactions of Poly(adenosine diphosphate ribose) with Histones. **Biochemistry** 31:1379-1385.

Panzeter P.L., Zweifel B., Malanga M., Waser S.H., Richard M., Althaus F.R. (1993) Targeting of Histone Tails by Poly(ADP-ribose). **J.Biol.Chem.** 268:17662-17664.

Panzeter P.L. & Althaus F.R. (1994) DNA Strand Break-Mediated Partioning of Poly(ADP-ribose) Polymerase Function. **Biochemistry** 33:9600-9605.

Pardoll D.M., Vogelstein B., Coffrey D.S. (1980) A Fixed Site of DNA Replication in Eukaryotic Cells. **Cell** 19:527-536.

Park C. & Sancar A. (1994) Formation of a Ternary Complex by Human XPA, ERCC1, and ERCC4(XPF) Excision Repair Proteins. **Proc.Natl.Acad.Sci.USA** 91: 5017-5021.

Parsons R., Li G., Longley M.J., Fang W., Papadopoulos N., Jen J., Chapelle A., Kinzler K.W., Vogelstein B., Modrich P. (1993) Hypermutability and Mismatch Repair Deficiency in RER$^+$ Tumour Cells. **Cell** 75:1227-1236.

Pathak M.A., Kramer D.M., Fitzpatrick T.B. (1974) Photobiology and Photochemistry of Foucoumarins (Psoralems). In: Sunlight and Man. Editor: Fitzpatrick T.B. pp 335-368.

Pedraza-Reyes M. & Alvarez-Gonzalez R. (1990) Oligo(3'-deoxyADP-ribosyl)ation of the Nuclear Matrix Lamins from Rat Liver Utilising 3'-deoxyNAD as a Substrate. **Febbs Lett.** 277:88-92.

Pegg A.E., Scicchitano D., Dolan M.E. (1984) Comparison of the Rates of Repair of O^6-Alkylguanines in DNA by Rat Liver and Bacterial O^6-Alkylguanine-DNA Alkyltransferase. **Cancer Res.** 44:3806-3811.

Pegg A.E. (1990) Mammalian *O^6*-Alkylguanine-DNA Alkyltransferase: Regulation and Importance in Response to Alkylating Carcinogenic and Therapeutic Agents. **Cancer Res.** 50:6119-6129.

Picksley S.M. & Lane D.P. (1993) The p53-mdm2 Autoregulatory Feedback Loop: A Paradigm for the Regulation by p53? **Bioessays** 15:689-690.

Pine J. (1994) Arresting Developments in Cell-Cycle Control. **Tibs** 19:143-145.

Poirier G.G., Neidergang C., Champagne M., Mazen A., Mandel P. (1982a) Adenosine Diphosphate Ribosylation of Chicken-Erythrocyte Histones H1, H5 and High-Mobility-Group Proteins by Purified Calf-Thymus Poly(adenosine diphosphate-ribose) Polymerase. **Eur.J.Biochem.** 127:437-442.

Poirier G.G., de Murcia G., Jongstra-Bilen J., Neidergang C., Mandel P. (1982b) Poly(ADP-ribosyl)ation of Polynucleosomes causes Relaxation of Chromatin Structure. **Proc.Natl.Acad.Sci.USA** 79:3423-3427.

Porteous J.W. & Pearson C.K. (1982) Poly(ADP-ribose) and Differentiation of Mammalian Intestinal Epithelium. In:ADP-ribosylation Reactions. pp 423-437. Eds. Hayaishi O. & Ueda K. Academic Press Inc.

Potvin F., Roy R.J., Poirier G.G., Guerin S.L. (1993). The US-1 element from the gene encoding rat poly(ADP-ribose) polymerase binds the transcription factor SP1. **Eur.J.Biochem.** 215:73-80.

Prakash S., Sung P., Prakash L. (1993) DNA Repair Genes and Proteins of *Saccharomyces cerevisiae*. **Ann.Rev.Genet.** 27:33-70.

Preiss J., Schlaeger R., Hilz H. (1971) Specific Inhibitors of Poly ADPribose Polymerase by Thymidine and Nicotinamide in HeLa Cells. **Febbs Lett.** 19:244-246.

Purnell M.R. & Whish W.J.D. (1980) Novel Inhibitors of Poly(ADP-Ribose) Synthetase. **Biochem.J.**

185:775-777.

Purnell M.R., Kidwell W.R., Minshall L., Whish W.J.D. (1985) In: Althaus F.R. Hilz H. Shall S.(eds) ADP-Ribosylation of Proteins pp98-105. Springer-Verlag, Berlin, Heidelberg.

Radman M. & Wagner R. (1993) Mismatch recognition in Chromosomal Interactions and Speciation. **Chromosoma** 102:369-373.

Rankin P.W., Jacobson E.L., Benjamin R.C., Moss J., Jacobson M.K. (1989) Quantitative Studies of Inhibitors of ADP-Ribosylation *in vitro* and *in vivo*. **J.Biol.Chem.** 264:4312-4317.

Rayssiguier C., Thaler D.S., Radman M. (1989) The Barrier to Recombination Between *Escherichi coli* and *Salmonella typhimurium* is Disrupted in Mismatch-repair Mutants. **Nature** 342:396-401.

Realini C.A. & Althaus F.R. (1992) Histone Shuttling by Poly(ADP-ribosylation). **J.Biol.Chem.** 267:18858-18865.

Rechsteiner M., Hillyard D., Olivera B.M. (1975) Turnover of Nicotinamide Adenine Dinucleotide in Cultures of Human Cells. **J.Cell Physiol.** 88:207-215.

Rechsteiner M., Hillyard D., Olivera B.M. (1976) Magnitude and Significance of NAD Turnover in Human Cell Line D98/AH2. **Nature** 259:695-696.

Reed J.C. (1994) Bcl-2 and the Regulation of Programmed Cell Death. **J.Cell Biol.** 124:1-6.

Riazuddin S. & Lindahl T. (1978) Properties of 3-methyladenine-DNA Glycosylase from *Escherichia coli*. **Biochemistry** 17:2110-2118.

Rice W.G., Hillyer C.D., Harten B., Schaeffer C.A., Dorminy M., Lackey D.A., Kirsten E., Mendeleyev J., Buki K.G., Hakam A., Kun E. (1992) Induction of Endonuclease-mediated Apoptosis in Tumour Cells by C-Nitroso-Substituted Ligands of Poly(ADP-ribose) Polymerase. **Proc.Natl.Acad.Sci.USA** 89:7703-7707.

Robins R.K., Srivastava P.C., Narayanan V.L., Plowman J., Paull K.D. (1982) 2-β-Ribofuranosylthiazole-4-carboxamide, a Novel Potential Antitumour Agent for Lung Tumours and Metastases. **J.Med.Chem.** 25:107-108.

Robins P., Jones C.J., Biggerstaff M., Lindahl T., Wood R.D. (1991) Complementation of DNA Repair in Xeroderma Pigmentosum Group A Cell Extracts by a Protein with Affinity for Damaged DNA. **The EMBO J.** 10: 3913-3921.

Rochette-Egly C., Ittel M.E., Bilen J., Mandel P. (1980) Effect of Nicotinamide on RNA and DNA Synthesis and on Poly(ADP-ribose) Polymerase Activity in Normal and Phytohemagglutinin Stimulated Human Lymphocytes. **FEBS.Lett.** 120:7-11.

Roitt I.M. (1956) The Inhibition of Carbohydrate Metabolism in Ascites-Tumour Cells by Ethyleneimines. **Biochemistry** 63:300-307.

Ruffer J.T. & Morgan W.F. (1992) Potentiation of DNA Damage by Inhibition of Poly(ADP-ribosyl)ation: A Test of the Hypothesis for Random Nuclease Action. **Exp.Cell Res.** 200:506-512.

Ruggieri S., Gregori L., Natalini P., Vita A., Magni G. (1988) Recent Observations on the Structure and Properties of the Yeast NMN Adenyltransferase. **Experientia** 44:27-29.

Rydberg B. & Lindahl T. (1982) Nonenzymic Methylation of DNA by the Intracellular Methyl Group Donor S-Adenosyl-L-Methionine is a Potentially Mutagenic Reaction. **EMBO J.** 1:211-216.

Rydberg B., Hall J., Karran P. (1990) Active Site Amino Acid Sequence of the Bovine O^6-Methylguanine-DNA Methyltransferase. **Nuc.Acid Res.** 18:17-21.

Sakumi K., Nakabeppu Y., Yamamoto Y., Kawabata S., Iwanaga S., Sekiguchi M. (1986) Purification and Structure of a 3-Methyladenine-DNA Glycosylase I of *Escherichia coli*. **J.Biol.Chem.** 261:15761-15766.

Sancar A. & Sancar G.B. (1988) DNA Repair Enzymes. **Ann.Rev.Biochem.** 57:29-67.

Sancar A. (1994) Mechanisms of DNA Excision Repair. **Science** 266:1954-1956.

Sandell L.L. & Zakian V.A. (1993) Loss of a Yeast Telomere: Arrest, Recovery, and Chromosome Loss. **Cell** 75:729-739.

Satoh M.S. & Lindahl T. (1992) Role of Poly(ADP-ribose) Formation in DNA Repair. **Nature** 356:356-358.

Satoh M.S., Poirier G.G., Lindahl T. (1993) NAD^+-dependent Repair of Damaged DNA by Human Cell

Extracts. **J.Biol.Chem.**
268:5480-5487.

Satoh M.S., Poirier G.G., Lindahl T. (1994) Dual Function for Poly(ADP-ribose) Synthesis in Response to DNA Strand Breakage. **Biochemistry** 33:7099-7106.

Savard P., Poirier G.G., Sheinin R. (1981) Poly(ADP-Ribose) Polymerase Activity in Mouse Cells which Exhibit Temperature-Sensitive DNA Synthesis. **Biochim.Biophys.Acta** 653:271-275.

Schein P.S., Cooney D.A., McMenamin M.G., Anderson T. (1973) Streptozotocin Diabetes - Further Studies on the Mechanism of Depression of Nicotinamide Adenine Dinucleotide Concentrations in Mouse Pancreatic Islets and Liver. **Biochem.Pharmacol.** 22:2625-2631.

Scherly D., Nouspikel T., Corlet J., Ulca C., Bairoch A., Clarckson S.G. (1993) Complementation of the DNA Repair Defect in Xeroderma Pigmentosum Group G Cells by a Human cDNA Related to Yeast *RAD2*. Nature **363**:182-185.

Schlotterer C. & Tautz D. (1992) Slippage Synthesis of Simple Sequence DNA. **Nucl.Acid Res.** 20:211-215.

Schreiber V., Molinete M., Boeuf H., de Murcia G., Menissier-de Murcia J. (1992) The human poly(ADP-ribose) polymerase nuclear localisation signal is a bipartite element functionally separate from DNA binding and catalytic activity. **The EMBO J.** 11:3263-3269.

Schwartz J.L., Morgan W.F., Kapp L.N., Wolff S. (1983) Effects of 3-Aminobenzamide on DNA Synthesis and Cell Cycle Progression in Chinese Hamster Ovary Cells. **Exp.Cell.Res.** 143:377-382.

Sebolt-Leopold J.S. & Scavone S.V. (1992) Enhancement of Alkylating Agent Activity *In Vitro* by PD128763, a Potent Poly(ADP-Ribose) Synthetase Inhibitor. **Int.J.Radiation.Oncology Biol.Phys.** 22:619-621.

Selby C.P. & Sancar A. (1993) Molecular Mechanism of Transcription-Repair Coupling. **Science** 260:53-57.

Selby C.P., Witkin E.M., Sancar A. (1991) *Escherichia coli mfd* Mutant Deficient in "Mutation Frequency Decline" Lacks Strand-specific Repair: *In vitro* Complementation with Purified Coupling Factor. **Proc.Natl.Acad.Sci.USA** 88: 11574-11578.

Sestili P., Spadoni G., Balsamini C., Scovassi I., Cattabeni F., Duranti E., Cantoni O., Higgins D., Thomson C. (1990) Structural Requirements for Inhibitors of Poly(ADP-ribose) Polymerase. **J.Cancer Res.Clin.Oncol.** 116:615-622.

Setlow R.B. & Carrier W.L. (1964) The Disappearance of Thymine Dimers from DNA: An Error-Correcting Mechanism. **Biochemistry** 51:226-231.

Shall S. (1975) Experimental Manipulation of the Specific Activity of Poly(ADP-Ribose) Polymerase. **J.Biochem.** 77 2p.

Shall S. (1984) ADP-Ribose in DNA Repair: A New Component of DNA Excision Repair. **Adv.Rad.Biol.** 11:1-69.

Shaw I.C. & Jones H.B. (1994) Mechanisms of Non-genotoxic Carcinogenesis. **TIPS** 15:89-93.

Shibutani S., Takeshita M., Grollman A.P. (1991) Insertion of Specific Bases During DNA Synthesis Past the Oxidation-Damaged Base 8-oxodG. **Nature** 349:431-434.

Shima H., Nakaysau M., Aonuma S., Sugimura T., Nagao M. (1989) Loss of the *MYC* Gene Amplified in Human HL-60 Cells After Treatment with Inhibitors of Poly(ADP-ribose) Polymerase or with Dimethyl Sulphoxide. **Proc.Natl.Acad.Sci.USA** 86:7442-7445.

Shivji M.K.K., Kenny M.K., Wood R.D. (1992) Proliferating Cell Nuclear Antigen is Required for DNA Excision Repair. **Cell** 69: 367-374.

Simonin F., Menissier-de Murcia J., Poch O., Muller S., Gradwohl G., Molinete M., Penning C., Keith G., de Murcia G. (1990) Expression and Site-directed Mutagenesis of the Catalytic Domain of Human Poly(ADP-ribose) polymerase in *Escherichia coli.* Lysine 893 is critical for activity. **J.Biol.Chem.** 265:19249-19256.

Simonin F., Poch O., Delarue M., de Murcia G. (1993a) Identification of Potential Active-site Residues in the Human Poly(ADP-ribose) Polymerase. **J.Biol.Chem.** 268:8529-8535.

Simonin F., Hofferer L., Panzeter P.L., Muller S., de Murcia G., Althaus F.R. (1993b) The Carboxyl-terminal Domain of Human Poly(ADP-ribose) Polymerase - overproduction in *Escherichia coli*, large scale purification and characterisation. **J.Biol.Chem.** 268:13454-13461.

Sims J.L., Sikorski G.W., Catino D.M., Berger S.J., Berger N.A. (1982) Poly(adenosinediphosphoribose) Polymerase Inhibitors Stimulate Unsceduled Deoxyribonucleic Acid Synthesis in Normal Human Lymphocytes. **Biochemistry** 21:1813-1821.

Sims J.L., Berger S.J., Berger N.A. (1983) Poly(ADP-ribose) Polymerase Inhibitors Preserve Nicotinamide Adenine Dinucleotide and Adenosine 5′-Triphosphate Pools in DNA-Damaged Cells: Mechanism of Stimulation of Unsceduled DNA Synthesis. **Biochemistry** 22:5188-5194.

Singh N., Leduc Y., Poirier G., Cerutti P. (1985) Non-histone Chromosomal Protein Acceptors for Poly(ADP)-ribose in phorbol-12-myristate-13-acetate Treated Mouse Embryo Fibroblasts (C3H10T1/2). **Carcinogenesis** 6:1489-1494.

Skidmore C.J., Davies M.I., Goodwin P.M., Halldorsson H., Lewis P.J., Shall S., Zia'ee A. (1979) The Involvement of Poly(ADP-ribose) Polymerase in the Degradation of NAD Caused by γ-Radiation and *N*-Methyl-*N*-Nitrosourea. **Eur.J.Biochem.** 101:135-142.

Slattery E., Dignam J.D., Matsui T., Roeder R.G. (1983) Purification and Analysis of a Factor Which Supresses Nick-induced Transcription by RNA Polymerase II and Its Identity with Poly(ADP-ribose) Polymerase. **J.Biol.Chem.** 258:5955-5959.

Smith M.L., Chen I.T., Zhan Q., Bae I., Chen C.Y., Gilmer T. M., Kastan M.B., O'Connor P.M., Fornace A.J. (1994) Interaction of the p53-Regulated Protein Gadd45 with Proliferating Cell Nuclear Antigen. **Science** 266:1376-1379.

Smulson M., Stark P., Gazzoli M., Roberts J. (1975) Release of Template Restriction for DNA Synthesis by Poly (ADP-ribose) Polymerase During the HeLa Cell Cycle. **Exp.Cell.Res.** 90:175-182.

Snyder R.D. (1984) 3-Aminobenzamide does not Alter Repair in Human Fibroblasts Through Modulation of Deoxynucleoside Triphosphate Pools. (1984) **Biochem.Biophys.Res.Commun.** 124:457-461.

Stevens M.F.G., Hickman J.A., Langdon S.P., Chubb D., Vickers L., Stone R., Baig G., Goddard C., Gibson N.W., Slack J.A., Newton C., Lunt E., Fizames C., Lavelle F. (1987) Antitumour Activity and Pharmokinetics in Mice of 8-Carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (CCRG 81045; M & B 39831), a Novel Drug with Potential as an Alternative to Dacarbazine. **Cancer Res.** 47:5846-5852.

Stevnsner T., Ding R., Smulson M., Bohr V.A. (1994) Inhibition of Gene-Specific Repair of Alkylation Depleted of Poly(ADP-ribose) Polymerase. **Nuc.Acid Res.** 22:4620-4624.

Stratling W.H., Muller U., Zentgraf H. (1978) The Higher Order Repeat Structure of Chromatin is Built Up of Globular Particles Containing Eight Nucleosomes. **Exp.Cell Res.** 117:301-311.

Streeter D.G. & Robins R.K. (1983) Comparative *in vitro* Studies of Tiazofurin and a Selenazole Analog. **Biochem.Biophys.Res.Commun.** 115:544-550.

Sung P., Reynolds P., Prakash L., Prakash S. (1993) Purification and Characterisation of the *Saccharomyces cerevisiae* RAD1/RAD10 Endonuclease. **J.Biol.Chem.** 268: 26391-26399.

Suto M.J., Turner W.R., Arudel-Suto C.M., Werbel L.M., Sebolt-Leopold J.S. (1991) Dihydroisoquinolinones: the Design and Synthesis of a New Series of Potent Inhibitors of Poly(ADP-ribose) Polymerase. **Anti-Cancer Drug Design** 7:107-117.

Sweigert S.E., Petzold S.J., Surowy C.S., Berger S.J., Dethlefsen L.A., Berger N.A. (1986) Poly(ADP-ribose) Polymerase Activity in Proliferating and Quiescent Murine Mammary Carcinoma Cells. **Radiat.Res.** 105:219-226.

Takada T., Okazaki I.J., Moss J. (1994) ADP-ribosylarginine Hydrolases. **Mol.Cell.Biochem.** 138:119-122.

Takano S., Nakabeppu Y., Sekiguchi M. (1988) Functional Sites of the Ada Regulatory Protein of *Escherichia coli*. **J.Mol.Biol.** 201:261-271.

Takasawa S., Nata K., Yonekura H., Okamoto H. (1993a) Cyclic ADP-ribose in Insulin Secretion from Pancreatic β Cells. **Science** 259:370-373.

Takasawa S., Tohgo A., Noguchi N., Koguma T., Nata K., Sugimoto T., Yonekura H., Okamoto H. (1993b) Synthesis and Hydrolysis of Cyclic ADP-Ribose by Human Leukocyte Antigen CD38 and Inhibition of the Hydrolysis by ATP. **J.Biol.Chem.** 268:26052-26054.

Tanaka Y., Yoshihara K., Itaya A., Kamiya T., Koide S.S (1984) Mechanism of the Inhibition of Ca^{2+}/Mg^{2+}-dependent Endonuclease of Bull Seminal Plasma Induced by ADP-ribosylation. **J.Biol.Chem.** 259:6579-6585.

Tanaka K, Miura N, Satokata I, Miyamoto I, Yoshida M.C, Satoh Y, Kondo S, Yasui A, Okayama H, Okada Y. (1990) Analysis of a Human DNA Excision Repair Gene Involved in Group A Xeroderma Pigmentosum and Containing a Zinc-Finger Domain. **Nature** 348:73-76.

Taniguchi T, Suzuki S, Shizuta Y. (1985) Poly(ADP-ribose) polymerase of RNA Polymerase II from Wheat Germ. **Biochem.Biophys.Res.Commun.** 127:526-532.

Tanizawa A, Kubota M, Hashimoto H, Shimizu T. Takimoto T, Kitoh T, Akiyama Y, Mikawa H. (1989) VP-16-Induced Nucleotide Pool Changes and Poly(ADP-ribose) Synthesis: The Role of VP-16 in Interphase Death. **Exp.Cell Res.** 185:237-246.

Tanuma S, Enomoto T, Yamada M. (1979) Effect of Thymidine on Poly(ADP-ribosyl)ation *in vivo*. **J.Biol.Chem.** 254:4960-4962.

Tanuma S & Johnson G.S. (1983a) ADP-ribosylation of Nonhistone High Mobility Group Proteins in Intact Cells. **J.Biol.Chem.** 258:4067-4070.

Tanuma S, Johnson L.D, Johnson G.S. (1983b) ADP-ribosylation of Chromosomal Proteins and Mouse Mammary Tumour Virus Gene Expression. **J.Biol.Chem.** 258:15371-15375.

Tanuma S., Kawashima K., Endo H. (1987) Evidence of a Regulatory Role of the Level of Poly(ADP-ribose) in Chromosomal Proteins in Metallothionein Gene Expression by Glucocorticoids But Not by Heavy Metals. **Bichim.Biophys.Acta.** 910:197-201.

Tavassoli M., Tavassoli M.H., Shall S. (1983) Isolation and purification of poly(ADP-ribose) glycohydrolase from pig thymus. **Eur.J.Biochem.** 135:449-455.

Tenniswood M.P., Guenette R.S., Kakins J., Mooibroek M., Wong P., Welsh J. (1992) Active Cell Death in Hormone-Dependent Tissues. **Cancer Metastasis Rev.** 11:197-220.

Thibeault L., Hengartner M., Lagueux J., Poirier G.G., Muller S. (1992) Rearrangement of the Nucleosomal Structure in Chromatin by Poly(ADP-ribose). **Biochim.Biophys.Acta** 1121:317-324.

Thibodeau J., Gradwohl G., Dumas C., Clairoux-Moreau S., Brunet G., Penning C., Poirier G.G., Moreau P. (1989) Cloning of rodent cDNA encoding the poly(ADP-ribose) polymerase catalytic domain ans analysis of mRNA levels during the cell cycle. **Biochem.Cell.Biol.** 67:653-660.

Thi Man N. & Shall S. (1982) The Alkylating Agent, Dimethyl Sulphate, Stimulates ADP-Ribosylation of Histone H1 and Other Proteins in Permeabilised Mouse Lymphoma (L1210) Cells. **Eur.J.Biochem.** 126:83-88.

Thomas K.R., Folger K.R., Capecchi M.R. (1986) High Frequency Targeting of Genes to Specific Sites in the Mammalian Genome. **Cell** 44:419-428.

Thomassin H., Menard L., Hengartner C., Kirkland J.B., Poirier G.G. (1992) Poly(ADP-ribosyl)ation of Chromatin in an *in-vitro* Poly(ADP-ribose)-Turnover System. **Biochim.Biophys.Acta** 1137:171-181.

Thompson L.H., Brookman K.W., Weber C.A., Salazar E.P., Reardon J.T., Sancar A., Deng Z., Siciliano M.J. (1994) Molecular Cloning of the Human Nucleotide-Excision-Repair Gene *ERCC4*. **Proc.Natl.Acad.Sci.USA** 91:6855-6859.

Thraves P.J., Kasid U., Smulson M.E. (1985) Selective Isolation of Domains of Chromatin Proximal to Both Carcinogen-Induced DNA Damage and Poly-Adenosine Diphosphate-Ribosylation. **Cancer Res.** 45:386-391.

Tisdale M.J. (1988) Antitumour Imidazotetrazines and Gene Expression. **Acta Oncol.** 27:511-516.

Tomkinson A.E., Roberts E., Daly G., Totty N.F., Lindahl T. (1991) Three Distinct DNA Ligases in Mammaliam Cells. **J.Biol.Chem.** 266:21728-21735.

Tomkinson A.E., Bardwell A.J., Bardwell L., Tappe N.J., Freidberg E.C. (1993) Yeast DNA Repair and Recombination Proteins Rad1 and Rad10 Constitute a Single-stranded-DNA Endonuclease. **Nature** 362: 860-862.

Tricot G.J., Jayaram H.N., Lapis E., Natsumeda Y., Nichols C.R., Kneebone P., Heerema N., Weber G., Hoffman R. (1989) Biochemically Directed Therapy of Leukemia with Tiazofurin, a Selective Blocker of Inosine 5'-phosphate dehydrogenase activity. **Cancer Res.** 49:3696-3701.

Tsang L.L.H., Quarterman C.P., Gescher A., Slack J.A. (1991) Comparison of the Cytotoxicity in vitro of Temozolomide and Dacarbazine, Prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. **Cancer Chemother.Pharmacol.** 27:342-346.

Tsai Y., Aoki T., Maruta H., Abe H., Sakagami H., Hatano T., Okuda T., Tanuma S. (1992) Mouse Mammary Tumour Virus Gene Expression is Suppressed by Oligomeric Ellagitannins, Novel Inhibitors of Poly(ADP-ribose) Glycohydrolase. **J.Biol.Chem.** 267:14436-14441.

Tsuchiya M. & Shimoyama M. (1994) Target Protein for Eukaryotic Arginie-specific ADP-ribosyltransferase. **Mol.Cell.Biochem.** 138:113-118.

Uchida K., Morita T., Sato T., Ogura T., Yamashita R., Noguchi S., Suzuki H., Nyunoya H., Miwa M., Sugimura T. (1987) Nucleotide Sequence of a Full-Length cDNA for Human Fibroblast Poly(ADP-ribose) Polymerase. **Biochem.Biophys.Res.Commun.** 148:617-622.

Uchida K., Suzuki H., Maruta H., Abe H., Aoki K., Miwa M., Tanuma S. (1993a) Preferential Degradation of Protein-bound $(ADP\text{-}ribose)_n$ by Nuclear Poly(ADP-ribose) Glycohydrolase from Human Placenta. **J.Biol.Chem.** 265:3194-3200.

Uchida K., Hanai S., Ishikawa K., Ozawa Y., Uchida M., Sugimura T., Miwa M. (1993b) Cloning of cDNA encoding Drosophila poly(ADP-ribose) polymerase: Leucine zipper in the auto-modification domain. **Proc.Natl.Acad.Sci.USA** 90:3481-3485.

Ueda K., Kawaichi M., Hayaishi O. (1982) Poly(ADP-ribose) Synthetase. In: ADP-ribosylation Reactions. Editors: Hayaishi O. & Ueda K. Academic Press, New York. pp 117-155.

Ueda K. & Hayaishi O. (1985) ADP-ribosylation. **Ann.Rev.Biochem.** 54:73-100.

Ueda K., Hayaishi O., Oka J., Komura H., Nakanishi K. (1985) 5'-ADP-3''-Deoxypentos-2''-ulose. A Novel Product of ADP-Ribosyl Protein Lyase. ADP-Ribosylation of Proteins (ed. by F.R Althaus, H.Hilz and S.Shall) publishers Spinger-Verlag Berlin Heidelberg 1985.

Uhr M.L. & Smulson M. (1982) NMN Adenyltransferase: Its Association with Chromatin and with Poly(ADP-ribose) Polymerase. **Eur.J.Biochem.** 128:435-443.

Utakoji T., Hosoda K., Umezawa K., Sawamura M., Matsushima T., Miwa M., Sugimura T. (1979) Induction of Sister Chromatid Exchanges by Nicotinamide in Chinese Hamster Lung Fibroblasts and Human Lymphoblastoid Cells. **Biochem.Biophys.Res.Commun.** 90:1147-1152.

Van Duain M., de Wit J., Odijk H., Westerveld A., Yasui A., Koken M.H.M., Hoeijmakers J.H.J., Bootsma D. (1986) Molecular Characterisation of the Human Excision Repair Gene *ERCC-1*: cDNA Cloning and Amino Acid Homology with the Yeast DNA Repair Gene *RAD10*. **Cell** 44:913-923.

Varsney U., Hutcheon T., vab de Sande J.H. (1988) Sequence Analysis, Expression, and Conservation of *Escherichia coli* Uracil DNA Glycosylase and Its Gene (ung). **J.Biol.Chem.** 265:7776-7784.

Waga S., Hannon G.J., Beach D., Stillman B. (1994) The p21 Inhibitor of Cyclin-dependent Kinases Controls DNA Replication by Interaction with PCNA. **Nature** 369:574-578.

Waldman B.C. & Waldman A.S. (1990) Illegitimate and Homologous Recombination in Mammalian Cells: Differential Sensitivity to an Inhibitor of Poly(ADP-ribosylation). **Nuc.Acid.Res.** 18:5981-5988.

Wang R.Y.H., Kuo K.C., Gehrke C.W., Huang L., Ehrlich M. (1982) Heat- and Alkali-Induced Deamination of 5-Methylcytosine and Cytosine Residues in DNA. **Biochim.Biophys.Acta** 697:371-377.

Wang J.C. (1987) Recent Studies of DNA Topoisomerases. **Biochim.Biophys.Acta** 909:1-9.

Wang Z., Auer B., Stingl L., Berghammer H., Haidacher D., Schweiger M., Wagner E.F. (1995) Mice Lacking ADPRT and Poly(ADP-ribosyl)ation Develop Normally But are Susceptible to Skin Disease. **Genes & Develop.** 9:509-520.

Ward J.F. (1975) Molecular Mechanisms of Radiation-Induced Damage to Nucleic Acids. **Adv.Rad.Biol.** 5:181-239.

Weber C.A., Salazar E.P., Stewart S.A., Thompson L.H. (1990) *ERCC2*: cDNA Cloning and Molecular Characterisation of a Human Nucleotide Excision Repair Gene with High Homology to Yeast *Rad3*. **The EMBO J.** 9:1437-1447.

Weber G., Natsumeda Y., Pillwein K. (1985) Targets and Markers of Selective Action of Tiazofurin. **Adv.Enzyme Reg.** 24:45-65.

Weeda G., Van Ham R.C.A., Vermeulen W., Bootsma D., Van der Eb A.J., Hoeijamakers J.H.J. (1990) A Presumed DNA Helicase Encoded by *ERCC-3* is Involved in the Human Repair Disorders Xeroderma Pigmentosum and Cockayne's Syndrome. **Cell** 62:777-791.

Weinert T.A. & Hartwell L.H. (1988) The RAD9 Gene Controls the Cell Cycle Response to DNA Damage in *Saccharomyces cerevisiae*. **Science** 241:317-322.

Weinstein I.B., Jeffrey A.M., Jennette K.W., Blobstein S.H., Harvey R.G., Harris C., Autrup H., Kasai H., Nakanishi K. (1976) Benzo(a)pyrene Diol Epoxides as Intermediates in Nucleic Acid Binding in vitro and in vivo. **Science** 193:592-595.

Weisinger G. & Sachs L. (1983) DNA-binding Protein that Induces Cell Differentiation. **The EMBO J.** 2:2103-2107.

Weisinger G., Korn A.P., Sachs L. (1986) Protein that Induces Cell Differentiation Causes Nicks in Double-stranded DNA. **Febs Lett.** 200:107-110.

Weiss B. & Grossman L. (1987) Phosphodiesterases Involved in DNA Repair. **Adv. Enzymol.** 60:1-34.

Welsh K.M., Lu A., Clark S., Modrich P. (1987) Isolation and Characterisation of the *Escherichia coli mutH* Gene Product. **J.Biol.Chem.** 262:15624-15629.

Wesierska-Gadek J. & Sauermann G. (1985) Modification of Nuclear Matrix Proteins by ADP-ribosylation, Association of Nuclear ADP-ribosyltransferase with the Nuclear Matrix. **Eur.J.Biochem.** 153:421-428.

Wielkens K., Bredehorst R., Adamietz P., Hilz H. (1981) Protein-Bound Polymeric and Monomeric ADP-Ribose Residues in Hepatic Tissues. **Eur.J.Biochem.** 117:69-74.

Wielckens K., Schmidt A., George E., Bredehorst R., Hilz H. (1982) DNA Fragmentation and NAD depletion - their relation to the turnover of endogenous mono(ADP-ribosyl) and poly(ADP-ribosyl) proteins. **J.Biol.Chem.** 257:12872-12877.

Willmore E. & Durkacz B.W. (1993) Cytotoxic Mechanisms of 5-fluropyrimidines. **Biochem.Pharmacol.** 46: 205-211.

Witmer M.V., Aboul-Ela N., Jacobson M.J., Stamato T.D. (1994) Increased Sensitivity to DNA-Alkylating Agents in CHO Mutants with Decreases Poly(ADP-ribose) Polymerase Activity. **Mut.Res.** 314:249-260.

Woll P.J., Crowther D., Johnson P.W.M., Soukop M., Harper P.G., Harris M., Brampton M.H. (1995) Phase II Trial of Temozolomide in Low-grade non-Hodgkin's Lymphoma. **Br.J.Cancer** 72:183-184.

Worcel A. & Benyajati C. (1977) Higher Order Coiling of DNA in Chromatin. Cell 12:83-100.

Wyllie A.H., Beattie G.J., Hargreaves A.D. (1981a) Chromatin Changes in Apoptosis. **Histochem.J.** 13:681-692.

Wyllie A.H., Kerr J.F.R., Currie A.R. (1981b) Cell Death: The Significance of Apoptosis. **Int.Rev.Cytol.** 68:251-306.

Xiong Y., Zhang H., Beach D. (1993) Subunit Rearrangement of the Cyclin-Dependent Kinases is Associated with Cellular Transformation. **Genes Dev.** 7:1572-1583.

Yamada Y., Natsumeda Y., Weber G. (1988) Action of the Active Metabolites of Tiazofurin and Ribavirin on Purified IMP Dehydrogenase. **Biochemistry** 27:2193-2196.

Yingnian Y., Ming F., Yifan D., Xingruo C. (1989) On The Relationship Between Adenosine Diphosphoribosyl Transferase and S Phase DNA Synthesis in Cultured Mammalian Cells. **Proc.CAMS.PUMC** 4:157-161.

Yoshihara K., Tanigawa Y., Burzio L., Koide S.S. (1975) Evidence for Adenosine Diphosphate Ribosylation of Ca^{2+}/Mg^{2+}-dependent Endonuclease. **Proc.Natl.Acad.Sci.USA** 72:289-293.

Yoshihara K., Hashida T., Yoshihara H., Tanaka Y., Ohgushi H. (1977) Enzyme-bound Early Product of Purified Poly(ADP-ribose) Polymerase. **Biochem.Biophys.Res.Commun.** 78:1281-1288.

Yoshihara K., Itaya A., Tanaka Y., Ohashi Y., Ito K., Teroka H., Tsukada K., Matsukage A., Kamiya T. (1985) Inhibition of DNA Polymerase α, DNA Polymerase β, Terminal Deoxynucleotidyl Transferase, and DNA Ligase II By Poly(ADP-ribosyl)ation reaction In Vitro. Biochem. **Biophys.Res.Commun.** 128:61-67.

Zahradka P. & Ebisuzaki K. (1982) A Shuttle Mechanism for DNA-Protein Interactions. The Regulation of Poly(ADP-ribose) Polymerase. **Eur.J.Biochem.** 127: 579-585.

Zauberman A., Barek Y., Ragimov N., Levy N., Oren M. (1993) Sequence-Specific DNA Binding by p53: Identification of Target Sites and Lack of Binding to p53-MDM2 Complexes. **The EMBO J.** 12:2799-2808.

Zhang J., Dawson V.L., Dawson T.M., Snyder S.H. (1994) Nitric Oxide Activation of Poly(ADP-Ribose) Synthetase in Neurotoxicity. **Science** 263:687-689.

Zimmermann S.B. & Levin C.J. (1975) DNA Ligase Activity on Chromatin and Its Analogs Rejoining of DNA Strands in Polylysine-DNA Complexes and in Reconstituted Chromatins. **Biochemistry** 14:1671-1677.

Zucchetti M., Catapano C.V., Filippeschi S., Erba E., D'Incalci M. (1989) Temozolomide Induced Differentiation of K562 Leukemia Cells is Not Mediated by Gene Hypomethylation. **Biochem.Pharmacol.** 38:2069-2075.

British Journal of Cancer (1995) 72, 849-856

Potentiation of temozolomide-induced cytotoxicity: a comparative study of the biological effects of poly(ADP-ribose) polymerase inhibitors

S Boulton[1], LC Pemberton[2], JK Porteous*, NJ Curtin[1], RJ Griffin[1,2], BT Golding[2] and BW Durkacz[1]

[1]*Cancer Research Unit and* [2]*Department of Chemistry, The University, Newcastle upon Tyne NE2 4HH, UK.*

Summary Four poly(ADP-ribose) polymerase (PADPRP) inhibitors [3-aminobenzamide, benzamide, 3,4-dihydro-5-methoxyisoquinolin-1(2*H*)-one (PD 128763) and 8-hydroxy-2-methylquinazolin-4(3*H*)-one (NU1025)] were compared with respect to their effects on a number of biological end points. The following parameters were assessed: their ability to inhibit the enzyme in permeabilised L1210 cells; their ability to potentiate the cytotoxicity of temozolomide (including the cytotoxicity of the compounds *per se*); their ability to increase net levels of temozolomide-induced DNA strand breaks and inhibit temozolomide-induced NAD depletion. PD 128763 and NU1025 were equipotent as PADPRP inhibitors, and 40- and 50-fold more potent than benzamide and 3-aminobenzamide respectively. All the compounds acted in a concentration-dependent manner to potentiate the cytotoxicity and increase DNA strand break levels in cells treated with temozolomide. There was an excellent correlation between the potency of the compounds as PADPRP inhibitors and their effects on cell survival and DNA repair. Temozolomide treatment caused a decrease in cellular NAD levels, and this was abolished by the PADPRP inhibitors. In conclusion, the new generation of PADPRP inhibitors are at least 50-fold more effective than 3-aminobenzamide as chemopotentiators, and can be used at micromolar rather than millimolar concentrations in intact cells.

Keywords: poly(ADP-ribose) polymerase; temozolomide; DNA repair; NAD; 3-aminobenzamide

Poly(ADP-ribose) polymerase (PADPRP, EC 2.4.2.30) is an abundant chromatin-bound enzyme which is activated by DNA strand breaks. PADPRP function has been implicated in a variety of biological processes, including DNA repair and cellular survival following DNA damage, recombination and regulation of gene expression and development (for reviews see Boulikas, 1991; de Murcia and Ménissier-de Murcia, 1994). Although the role of PADPRP is best understood in DNA repair, this is still an area of contention regarding the precise molecular mechanisms involved. Two general mechanisms have been proposed, based on accumulated evidence. Poly(ADP-ribose) synthesis leads to a dramatic structural modification of chromatin, resulting from the electrostatic repulsion of the negatively charged covalently modified proteins (mainly PADPRP itself and histones) from the DNA. This may allow access of repair enzymes to damaged sites on DNA (Althaus *et al.*, 1993). Alternatively, unmodified PADPRP binds tightly to free DNA ends, thus preventing further processing; upon automodification, PADPRP is released from DNA, allowing gap filling and ligation to occur (Satoh and Lindahl, 1992). An additional function of poly(ADP-ribose) synthesis may be to modulate directly the activity or function of covalently modified acceptor proteins (enzymes, transcription factors). For example, poly (ADP-ribosylation) of DNA polymerases, ligase and topoisomerase I causes inhibition of these enzymes *in vitro* (Ferro *et al.*, 1983; Yoshihara *et al.*, 1985). However, little work has been carried out to establish whether these proteins can act as acceptors in intact cells.

A range of PADPRP inhibitors, of which 3-aminobenzamide (3AB) has been the most widely used, was first developed by Purnell and Whish (1980). The demonstration that they could modulate cellular DNA repair and survival responses (Durkacz *et al.*, 1980) has led to a search for more potent inhibitors for use in cancer chemotherapy (Suto *et al.*, 1991; Banasik *et al.*, 1992). There has been a long-standing dispute concerning the specificity of benzamide and its derivatives as PADPRP inhibitors (particularly regarding their effects on *de novo* purine synthesis), and thus about the interpretation of the biological data (e.g. Hunting *et al.*, 1985; Moses *et al.*, 1990). Nevertheless, the literature spanning 15 years portrays a remarkable consistency regarding their effects. Thus, PADPRP inhibitors, at concentrations which are not cytotoxic *per se*, inhibit ADP-ribose polymer synthesis in intact cells (Rankin *et al.*, 1989). They retard the rejoining of DNA strand breaks and potentiate the cytotoxicity of a range of DNA-damaging agents (e.g. Durkacz *et al.*, 1980). PADPRP activation by DNA strand breaks causes cellular NAD depletion which is abrogated by PADPRP inhibitors (Durkacz *et al.*, 1980). The increased levels of DNA strand breaks obtained in the presence of PADPRP inhibitors has been assumed to be a consequence of inhibition of a late stage in the repair process (e.g. ligation, Creissen and Shall, 1982).

In more recent years, this scenario of events modulated by PADPRP function following DNA damage has been further substantiated by molecular, genetic and *in vitro* approaches to PADPRP function. Mutant cell lines which are deficient in PADPRP activity have been isolated by a number of different techniques (Maclaren *et al.*, 1990; Chatterjee *et al.*, 1991; Witmer *et al.*, 1994). These cell lines are typified by their hypersensitivity to monofunctional alkylating agents. Cell lines transfected with and overproducing the 'DNA binding domain' (DBD) of PADPRP, thus inhibiting endogenous PADPRP activation, are also hypersensitive to monofunctional alkylating agents, and are unable to carry out unscheduled DNA synthesis (Molinette *et al.*, 1993). Similar results have been obtained by reducing endogenous PADPRP synthesis by the use of antisense oligonucleotides to PADPRP (Smulson *et al.*, 1994). Finally, elegant *in vitro* experiments using crude cell extracts which can carry out DNA repair have established that PADPRP, in the absence of substrate, blocks DNA strand breaks snd prevents subsequent steps leading to religation of the DNA (Satoh and Lindhal, 1992; Satoh *et al.*, 1993).

PADPRP inhibitors have the potential to act as resistance modifiers when used in conjunction with radiation or chemotherapeutic agents. However, very little *in vivo* work

Correspondence: BW Durkacz
*Present address: CRC Laboratories, Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Rd., London W6 8RF, UK
Received 3 February 1995; revised 2 May 1995; accepted 12 May 1995

has been carried out to assess their capacity to increase the therapeutic index of anti-cancer drugs, mainly because benzamide and its derivatives lacked sufficient potency and were of low solubility. Recently, more potent PADPRP inhibitors have been identified (e.g. Suto *et al.*, 1991), but limited work has been carried out to establish their biological efficacy.

We have developed an evaluation system to compare candidate compounds with respect to their potency as PADPRP inhibitors and their effectiveness as chemopotentiators in intact cells. Two 'classical' inhibitors, benzamide (BZ) and 3AB, have been compared with 3,4-dihydro-5-methoxyisoquinolin-1(2*H*)-one (PD 128763), developed by Warner Lambert (Suto *et al.*, 1991), and 8-hydroxy-2-methylquinazolin-4(3*H*)-one (NU1025). NU1025 was synthesised in the Department of Chemistry, University of Newcastle upon Tyne (Griffin *et al.*, 1995), as part of an ongoing programme to design new PADPRP inhibitors. PD 128763 (100 mg kg^{-1}) has been shown to be a highly active radiosensitiser *in vivo*, causing > 50% reduction in tumour burden in mice bearing subcutaneous implants of SCC7 cells (Leopold and Sebolt-Leopold, 1992). The structures of the PADPRP inhibitors are shown in Figure 1 for comparison.

A chemotherapeutically relevant alkylating agent, temozolomide (TM, see Figure 1 for structure; Stevens *et al.*, 1987), which has shown promising results in phase I clinical trials (Newlands *et al.*, 1992), was used in these studies. TM breaks down in biological milieu to MTIC [5-(3-methyltriazen-1-yl)imidazole-4-carboxamide], and thence to the methyldiazonium ion, which directly methylates bases in DNA (Denny *et al.*, 1994).

In the body of work described here, we have used TM, in conjunction with the PADPRP inhibitors, to investigate the PADPRP-mediated repair and survival responses in murine leukaemia L1210 cells. The results demonstrate an excellent correlation between *in vitro* potency of the compounds as PADPRP inhibitors, and their ability to modulate cellular responses induced by DNA damage.

Materials and methods

Drugs and chemicals

3AB was obtained from Pfaltz and Bauer, Phase Separations, Deeside, UK; BZ from Sigma, St Louis, MO, USA; TM was a gift from MFG Stevens, Cancer Research Laboratories, University of Nottingham. The methodology for the synthesis of NU1025 is described elsewhere (RJ Griffin *et al.*, 1995). PD 128763 was a gift from WR Leopold, Parke-Davis Pharmaceutical Division, Warner Lambert, Ann Arbor, MI, USA. Stock solutions of 3AB and BZ were prepared by dissolving in complete medium and filter sterilising. NU1025 and PD 128763 were dissolved in dimethyl sulphoxide (DMSO) and added to cell culture at a final concentration of ≤ 1% DMSO. [^{32}P]NAD (1000 Ci $mmol^{-1}$), [methyl-^{3}H]TdR (41 Ci $mmol^{-1}$) and [2-^{14}C]TdR (52 mCi $mmol^{-1}$) were purchased from Amersham International (Amersham, UK).

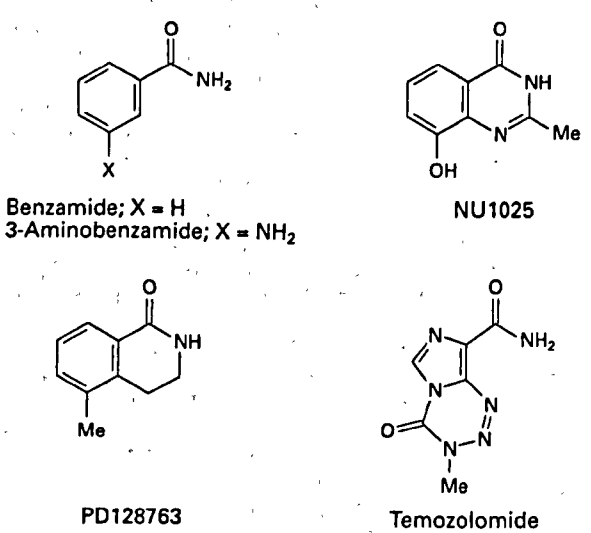


Figure 1 Structures of PADPRP inhibitors and temozolomide.

Cell culture, growth inhibition and clonogenic survival assays

The murine leukaemia L1210 cell line was maintained as a suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine (2 mM) and antibiotics (penicillin, 100 U ml^{-1}; streptomycin, 100 μg ml^{-1}). Hepes and sodium bicarbonate were added at final concentrations of 18 mM and 11 mM respectively. Cell densities were routinely maintained between 1×10^4 and 8×10^5 ml^{-1}.

Growth inhibition experiments were used to assess the cytostatic effects of the compounds. Cells were seeded at 1×10^4 ml^{-1} in triplicate in 24-well multidishes. After 24 h drugs were added in the combinations and at the concentrations specified in the figure legends. At this time one set of replicates was counted using a Coulter counter (N_0). After 48 h the remaining samples were counted (N_t). The percentage growth inhibition of drug-treated samples was estimated as $N_t - N_0$ (drug treated)/$N_t - N_0$ (control) × 100. In drug combination experiments, in which evidence of synergistic effects on cell growth or clonogenicity (see below) was being sought, the single, fixed concentration drug sample was taken as the control value (N_t control) in the above equation.

Clonogenic survival assays were used to assess the cytotoxicity of the compounds. They were performed as previously described (Sebolt-Leopold and Scavone, 1992), except that colonies were counted by eye on a gridded light box. The drug treatment protocols are described in the figure legends, and were carried out in suspension culture before plating the cells in agar, in the absence of drugs, to estimate survival. Survival and growth inhibition curves show the mean of three independent experiments ± s.e. Where error bars are not displayed in the figures, it is because they are obscured by the symbols.

PADPRP assays

PADPRP activity was measured in a permeabilised cell assay. L1210 cells were rendered permeable to exogenous [^{32}P]NAD by exposure to hypotonic buffer and cold shock, as described by Halldorsson *et al.* (1978). In order to reveal total available enzyme activity, a palindromic dodecanucleotide, which forms a short double-stranded hairpin loop with a blunt end demonstrated to activate PADPRP (Grube *et al.*, 1991), was included in the assay at a concentration of 20 μg ml^{-1}. Following incubation of the permeabilised cells with [^{32}P]NAD, incorporation of ^{32}P into acid precipitable counts was estimated. The results are expressed as percentage activity of the drug-treated relative to the control samples, and are the mean of quadruplicate samples ± s.e.

NAD assays

Cellular NAD levels were determined by a modification of the method of Nisselbaum and Green (1969). Cells were treated with drugs at the concentrations and for the times specified in the figure legends. Approximately 5×10^6 cells per sample were harvested at 4°C, washed once with ice-cold phosphate-buffered saline (PBS) and repelleted. The pellet was resuspended in 1.0 ml of 50% (v/v) ethanol and sonicated for 20 s. An aliquot was removed for protein estimation (Bradford, 1976), and then the suspension was centrifuged for 2 min in a microfuge. The supernatant liquid was used for NAD assays as described. Results are expressed as pmol NAD mg^{-1} protein, and represent the average of three independent samples ± s.e.

DNA strand break assays

DNA strand-break levels were assessed using the technique of Kohn *et al.* (1981). Cells were prelabelled with 0.4 μCi ml^{-1} [^{14}C]TdR for 24 h, followed by a 6 h chase in non-radioactive medium. Cells were then exposed to drugs at the concentrations and for the times specified in the figure legends. Internal standards were similarly labelled with 1 μCi ml^{-1} [^{3}H]TdR, exposed to 300 cGy, then loaded on the same filters as the drug treated samples, and eluted at pH 12.2. To summarise the data obtained, the results were expressed using the 'relative elution' (RE) formula of Fornace and Little (1977). RE represents the amount of DNA from the treated samples retained on the filter as a ratio of control (untreated). It is calculated using (log RR_{sample}) − (log $RR_{control}$), where RR (relative retention) is the fraction of sample DNA retained on the filter when 50% of the internal standard DNA has eluted. Points represent six replicates from three individual experiments ± s.e.

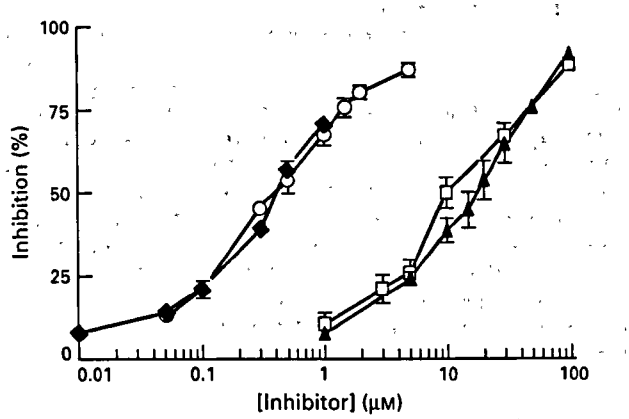


Figure 2 Effect of inhibitors on PADPRP activity in a permeabilised cell assay. Results are expressed as percentage inhibition of enzyme activity in the presence of increasing concentrations of inhibitors. (◆) PD 128763; (○) NU1025; (□) BZ; (▲) 3AB.

Results

PADPRP assays

The relative potencies of the four compounds studied as inhibitors of PADPRP are shown in Figure 2, in which percentage PADPRP inhibition is plotted against compound concentration. The IC_{50} values of PD 128763 and NU1025 in this *in vitro* assay were 0.36 ± 0.01 and 0.44 ± 0.13 μM respectively. 3AB and BZ were more than an order of mag-

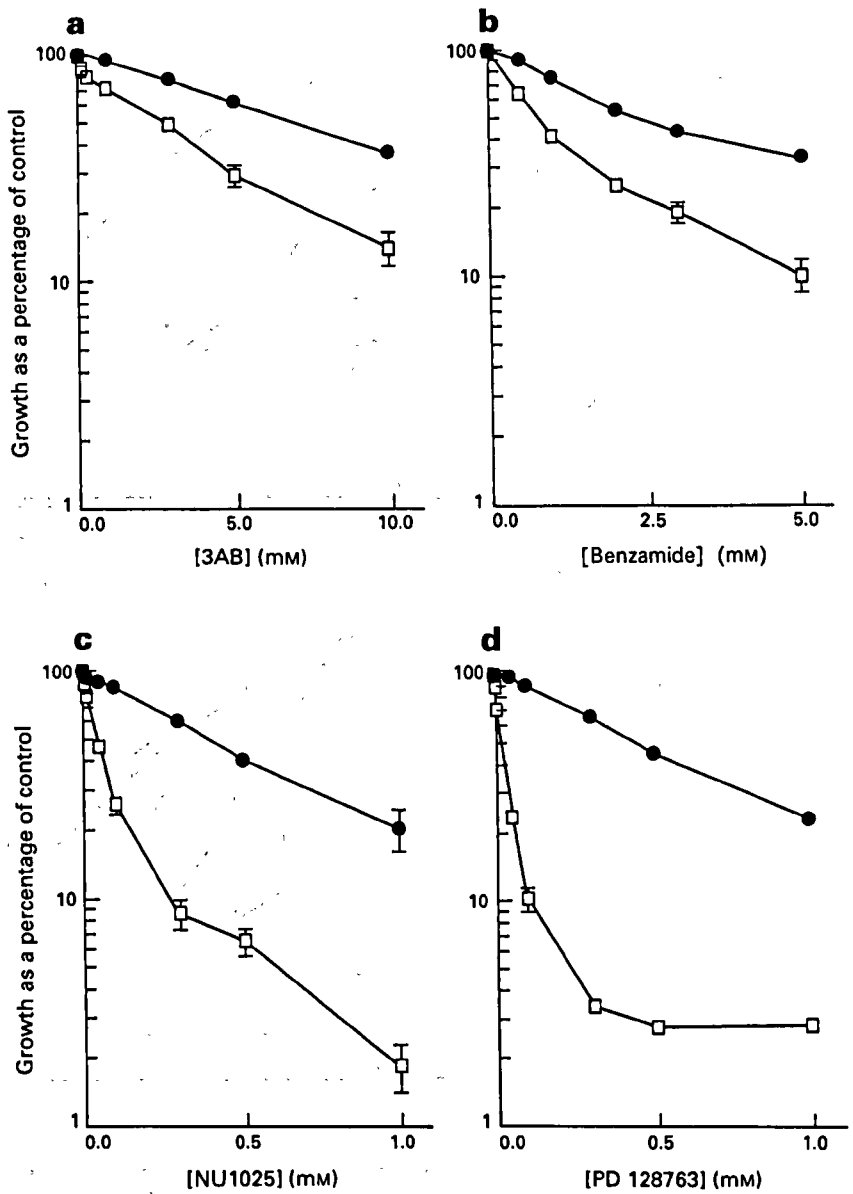


Figure 3 The effect of increasing concentrations of PADPRP inhibitors alone (●) or in conjunction with a fixed (100 μM) concentration of TM (□) on cell growth. (a) 3AB. (b) BZ (c) NU1025. (d) PD 128763.

nitude less potent, with IC_{50} values of 19.1 ± 5.9 μM and 13.7 ± 6.9 μM. This approximately 50-fold decrease in the IC_{50} value of PD 128763 compared with 3AB is in excellent agreement with the data of Suto *et al.* (1991).

Growth inhibition assays

The cytostatic effects of PADPRP inhibitors used alone or in conjunction with a fixed concentration (100 μM) of TM were investigated (Figure 3). Exposure of cells to TM alone caused inhibition of cell growth, with an IC_{50} value of 361 ± 25 μM (results not shown). Co-exposure of cells to 100 μM TM with increasing concentrations of PADPRP inhibitors caused a synergistic increase in growth inhibition (Figure 3). Note that for these experiments, the growth of cells in 100 μM TM, which itself reduced growth by about 26%, has been normalised to 100% (see Materials and methods). The IC_{50} values for the inhibitors alone or in conjunction with 100 μM TM are summarised in Table I.

Ten to 20-fold higher concentrations of PD 128763 and NU1025 alone were required to inhibit cell growth than were required when the compounds were used in conjunction with 100 μM TM. For example, the IC_{50} of NU1025 alone was 0.41 mM, and this was reduced to 0.04 mM in the presence of TM. In comparison, only 2- to 3-fold differences were obtained with 3AB and BZ, where there was considerable overlap between the growth-inhibitory effects of the compounds *per se*, and their effects in conjunction with TM. The potency of the compounds as PADPRP inhibitors reflected their effectiveness as inhibitors of cell growth, although this does not constitute proof that PADPRP function is essential for cell growth.

Table I Comparison of the IC_{50} values of the PADPRP inhibitors alone or in conjunction with 100 μM TM estimated from the growth inhibition experiments

Inhibitor	*IC_{50} (mM) ± s.e. inhibitor alone*	*IC_{50} (mM) ± s.e. inhibitor + 100 μM TM*
3-Aminobenzamide	6.7 ± 0.2	2.5 ± 0.1
Benzamide	2.5 ± 0.3	0.84 ± 0.12
NU1025	0.41 ± 0.06	0.04 ± 0.003
PD 128763	0.45 ± 0.01	0.023 ± 0.002

The IC_{50} values were derived from the smooth curve analysis of GraphPad Inplot, San Diego, CA, USA software and were averaged from at least three independent experiments ± s.e.

Clonogenic survival assays

It was necessary to establish that growth inhibition actually reflected cytotoxicity. Clonogenic survival assays were performed, where cells were exposed to increasing concentra-

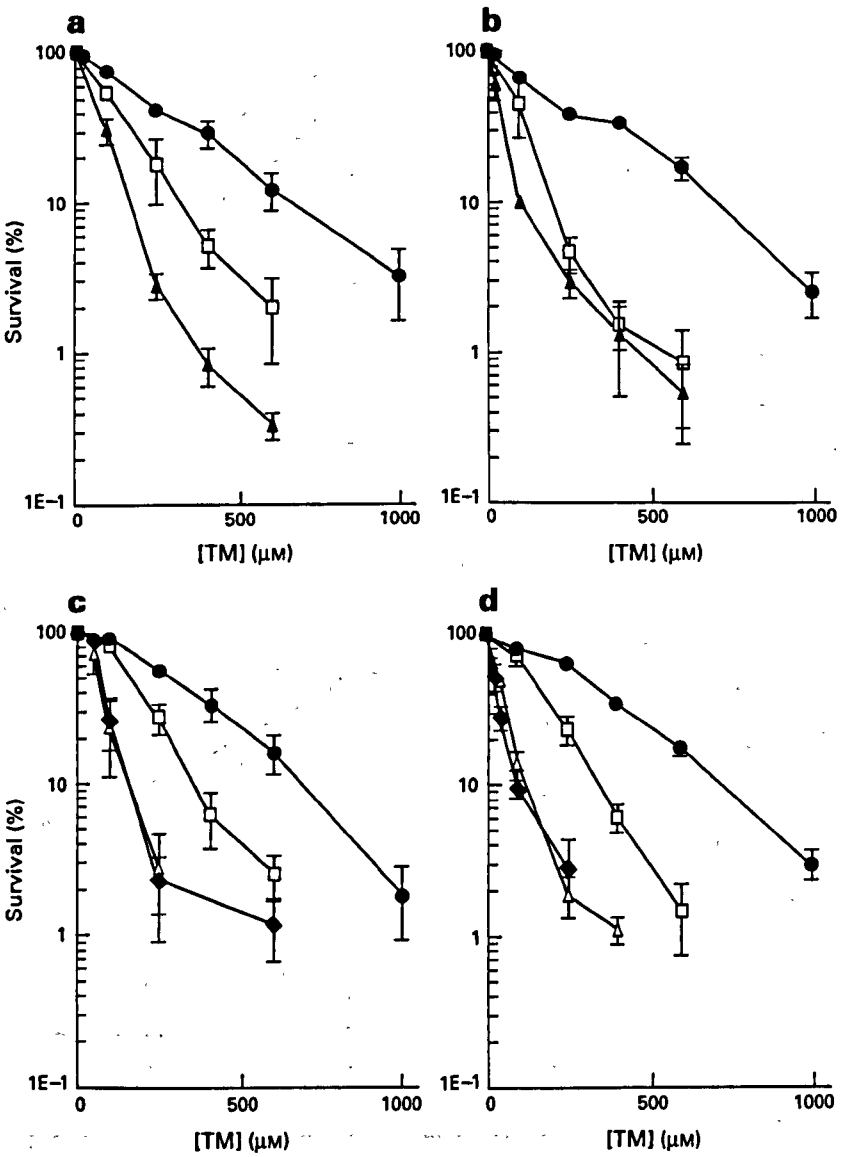


Figure 4 The effect of a 16 h exposure of cells to increasing concentrations of TM, in the presence or absence of fixed concentrations of PADPRP inhibitors, on clonogenic survival (a) ●, Control; □, + 1 mM 3AB; ▲, + 5 mM 3AB. (b) ●, control; □, + 1 mM BZ; ▲, + 3 mM BZ. (c), ●, control; □, + 10 μM NU1025; △, + 50 μM NU1025; ◆, + 100 μM NU1025. (d) ●, control; □, + 10 μM PD 128763; △, + 50 μM PD 128763; ◆, + 100 μM PD 128763.

tions of TM for 16 h, either alone or in the presence of fixed concentrations of PADPRP inhibitors, before plating for survivors in the absence of drugs. The survival curves are presented in Figure 4, and the DEF_{10} values given in Table II. (DEF_{10} is the ratio of the concentration of TM that reduces survival to 10% divided by the concentration of TM that reduces survival to 10% in the presence of a fixed concentration of PADPRP inhibitor). It can be seen that there was a reasonable correlation between growth inhibitory and cytotoxic effects for TM alone with an IC_{50} value of 361 μM ± 25 μM and a LD_{50} value of 251 ± 13 μM, respectively, despite the differing exposure times (48 h for growth inhibition and 16 h for cytotoxicity). TM has a half-life in culture of about 40 min (Tsang *et al.*, 1991), and therefore will exert its full effects well before the minimum duration of exposure of either experiment. All compounds potentiated the cytotoxicity of TM, but PD 128763 and NU1025 produced about the same DEF_{10} values at approximately 100-fold and approximately 60-fold lower concentrations than 3AB and BZ respectively (Table II). For example, 50 μM NU1025 and 5 mM 3AB gave equivalent DEF_{10} values of approximately 4. For both PD 128763 and NU1025, maximal potentiation of cytotoxicity was obtained by concentrations of 50–100 μM, and was significant at doses as low as 10 μM.

Table II Comparison of the DEF_{10} values obtained for a range of concentrations of the PADPRP inhibitors derived from the clonogenic assays shown in Figure 4

Inhibitor	*Concentration*	*DEF_{10}*[a]
3-Aminobenzamide	1 mM	2.4 ± 0.3
	5 mM	4.1 ± 0.4
Benzamide	1 mM	4.0 ± 0.7
	3 mM	6.9 ± 0.2
NU1025	10 μM	2.0 ± 0.2
	50 μM	4.0 ± 0.5
	100 μM	5.1 ± 0.7
PD128763	10 μM	2.0 ± 0.1
	50 μM	6.0 ± 0.5
	100 μM	7.1 ± 0.4

Def_{10} values were calculated using the smooth curve analysis described in Table I. Each value represents the average ± s.e. derived from the averaged 10% survival for TM alone (675 ± 31 μM from 22 independent survival curves) divided by individual 10% survival values from at least three independent survival curves performed in the presence of a fixed concentration of inhibitor.

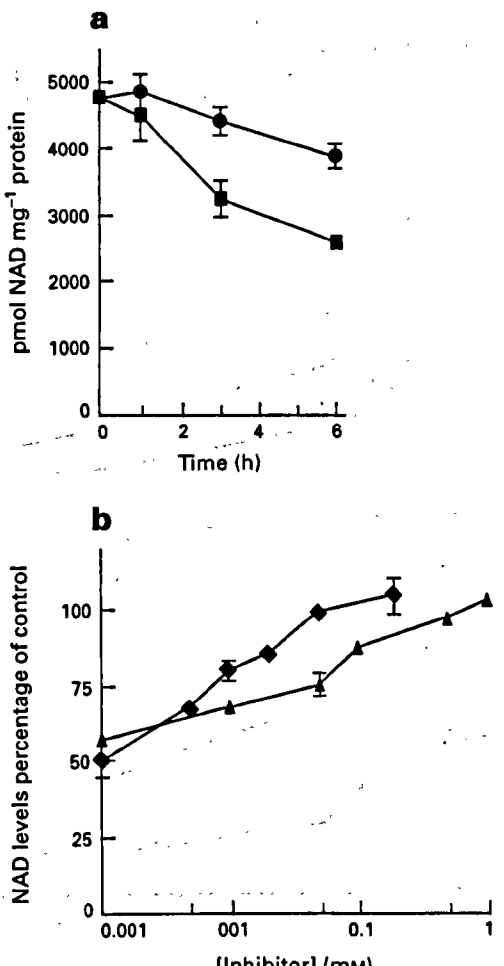


Figure 5 The effect of TM and the PADPRP inhibitors on the cellular NAD levels. (**a**) The effects of two fixed concentrations of TM was followed with time ●, 1 mM; ■, 2 mM. (**b**) The effect of increasing concentrations of PADPRP inhibitors on NAD levels in cells treated with 2 mM TM for 4 h incubation: ▲, 3AB; ◆, PD 128763.

The cytotoxic effects of the compounds alone were also investigated. The LD_{50} values for a 24 h exposure were 14 ± 1.0 mM (3AB); 6.0 ± 1.5 mM (BZ); 1.6 ± 0.1 mM (NU1025) and 0.99 ± 0.18 mM (PD 128763) (results not shown). The LD_{50} values differed by ⩽3-fold from the IC_{50} values, and again reflected their potency as PADPRP inhibitors. In agreement with the growth inhibition data there was a ⩾10-fold difference between the concentrations of PD 128763 and NU1025 required to produce maximal potentiation of TM cytotoxicity and the concentrations required to produce cytotoxicity *per se*.

NAD assays

Changes in NAD levels are a convenient, albeit indirect, assessment of PADPRP activation in TM-treated cells. Figure 5a shows a time-dependent depletion of NAD levels following treatment with 1 or 2 mM TM. Evidence that the NAD depletion is mediated by PADPRP activation is shown in Figure 5b. A 4 h incubation with 2 mM TM caused a 50% decrease in cellular NAD levels, and this was abrogated in a concentration-dependent manner by PD 128763 and 3AB. Note that 10 μM PD 128763 sufficed to prevent approximately 50% of NAD drop, and that NAD depletion was completely prevented by 100 μM. These data correlate with the concentration ranges of PD 128763 required to effect potentiation of cytotoxicity in the clonogenic survival experiments. In contrast, at least an order of magnitude higher concentrations of 3AB were required to exert the same effects on NAD levels in TM-treated cells.

DNA strand break assays

The effect of the PADPRP inhibitors on DNA strand break levels in TM-treated cells was monitored by alkaline elution. A 1 h treatment with TM resulted in a concentration-dependent increase in the rate of elution (results not shown). Changes in DNA strand break levels were detectable at levels of TM as low as 150 μM, which reduced survival by about 30%. All the compounds were tested for their ability to produce strand breaks when used alone. A 24 h incubation of cells with 1 mM PD 128763 or NU1025 and 20 mM 3AB or BZ had no effect on DNA strand-break levels compared with untreated cells (results not shown).

Co-incubation of a fixed concentration of TM (150 μM) with increasing concentrations of all PADPRP inhibitors for 1 h caused a progressive increase in the rate of elution compared with TM alone. A specimen elution profile for the effect of increasing concentrations of NU1025 on TM-induced DNA strand break levels is shown in Figure 6. The results for all four compounds have been summarised by plotting RE values *vs* inhibitor concentration, and are shown in Figure 7. Note that the RE values for TM + inhibitor-treated cells have been calculated using TM alone controls, and not untreated cells. For all the compounds, the RE value increased linearly with increasing concentration. However, RE values started increasing for PD 128763 and NU1025 at about 100 μM, whereas concentrations above 3 mM and 5 mM were required to increase significantly the RE values for BZ

and 3AB respectively. Again, the potency of the compounds in the DNA strand break assay demonstrated an excellent correlation with *in vitro* PADPRP inhibitory potency.

Finally, the temporal kinetics of TM-induced DNA strand-break formation and religation was analysed, and the results are presented in Figure 8. In cells treated with 200 μM TM, DNA strand-break levels increased rapidly up to 4 h and declined thereafter. By 24 h DNA strand break levels had returned to almost control levels. Both 3AB (5 mM) and NU1025 (300 μM) increased net levels of DNA strand breaks over the entire time period. The time interval during which DNA strand break levels were highest (approximately 2–4 h) correlated with the reported timing of the peak levels of MTIC obtained in culture medium following addition of TM (Tsang *et al.*, 1991). This implies that the breakdown of MTIC to the methyl diazonium ion, which directly alkylates DNA, is relatively rapid compared to the decomposition of TM to MTIC in culture medium. It should be emphasised that these data do not differentiate between enhanced incision or reduced ligation as a causative mechanism for the net increase in DNA strand break levels observed in inhibitor-treated cells.

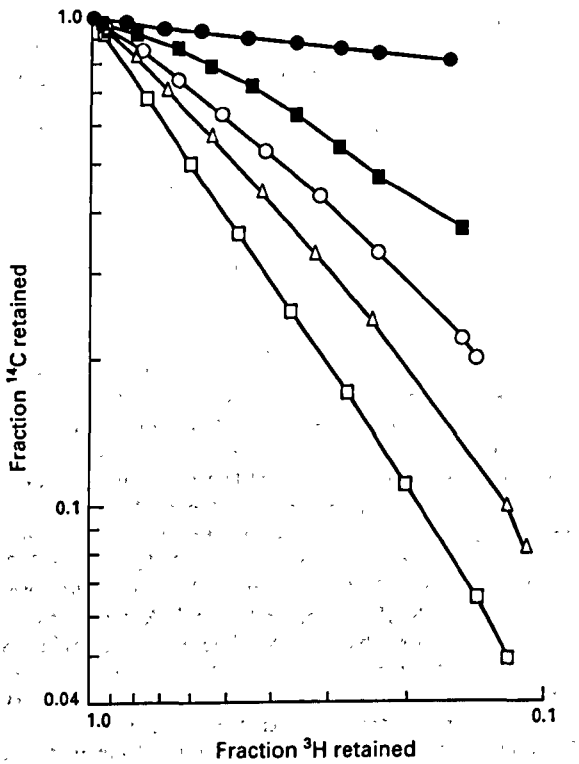


Figure 6 DNA strand-break levels assessed by alkaline elution. The effect of co-incubation with increasing concentrations of NU1025 in cells treated with a fixed concentration (150 μM) of TM for 1 h. ●, Control (untreated); ■, TM alone; ○, TM + 0.3 mM NU1025; △, TM + 0.5 mM NU1025; □, TM + 1.0 mM NU1025.

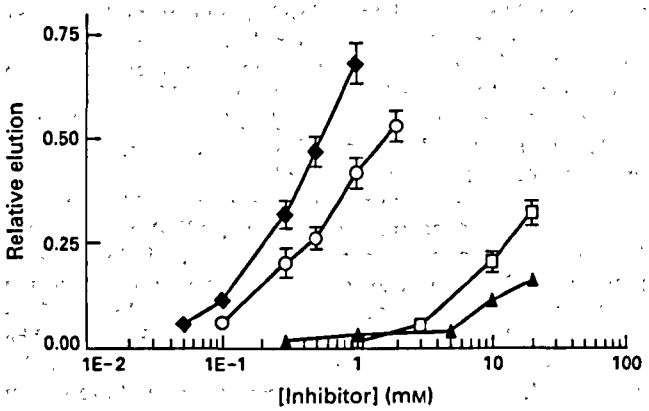


Figure 7 The effect of increasing concentrations of PADPRP inhibitors on DNA strand-break frequency in cells treated with 150 μM TM for 1 h. In this case RE values have been calculated as a ratio of the RR values for PADPRP inhibitor-treated cells over the RR value of TM-treated cells. RE values have been plotted against increasing inhibitor concentration. ◆, PD 128763; ○, NU1025; □, BZ; ▲, 3AB.

Discussion

This is the first report of a comprehensive and quantitative analysis comparing the effects of a range of PADPRP inhibitors on PADPRP activity and on the biological end points associated with the cellular responses to DNA damage. Suto *et al.* (1991) and Sebolt-Leopold and Scavone (1992) demonstrated that PD 128763 potentiated the cytotoxicity of ionising radiation, the monofunctional alkylating agent, streptozotocin, and also 2-nitroimidazole. However, PD 128763 was only used at a concentration of 500 μM in their experiments, thereby potentially underestimating the potency of this compound in intact cells. Here we have established that maximal potentiation of TM is obtained at a 10-fold lower concentration (50 μM), and is significant at concentrations as low as 10 μM. However, we cannot rule out the possibility that the concentration-dependent effects of PADPRP inhibitors may vary with different DNA-damaging agents, and in different cell lines.

In phase I trials, following a dose of TM (200 mg m^{-2}), peak plasma levels of approximately 50 μM were achieved by 2 h (Newlands *et al.*, 1992). These levels are of the same order of magnitude as the concentration (≥ 100 μM) used in our experiments, in which potentiation of cytotoxicity by PADPRP inhibitors was observed.

The new compounds are about 50-fold more potent as PADPRP inhibitors than 3AB and BZ, and this differential is

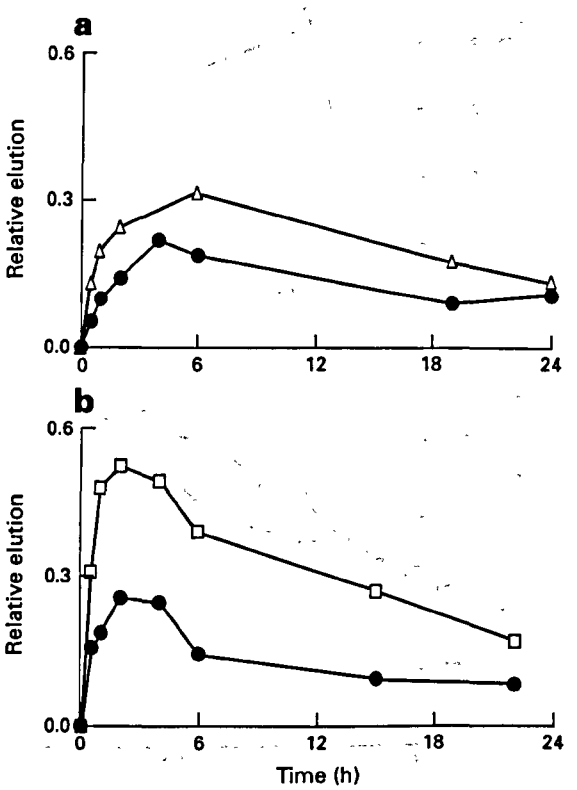


Figure 8 The effect of 200 μM TM ± PADPRP inhibitors on DNA strand-break levels over a 24 h time course. RE values have been plotted against time (a) ●, 200 μM TM alone; △, 200 μM TM + 5 mM 3AB. (b) ●, 200 μM TM alone; □, 200 μM TM + 300 μM NU1025. Single data points are shown from representative experiments, where TM ± PADPRP inhibitor treatments were carried out in parallel.

maintained in intact cells when the cellular responses to DNA damage known to be modulated by PADPRP function (i.e. cytotoxicity and DNA repair) are investigated. Furthermore, there is more than an order of magnitude difference in the concentration of PD 128763 and NU1025 (10–50 μM) required to potentiate TM cytotoxicity compared with the concentrations of the compounds alone required to exert cytotoxic effects (≥ 1 mM). In comparison, considerable overlap is evident in the concentrations of 3AB and BZ required to exert these two effects. Thus, there is a bigger gap between the synergistic enhancement of cytotoxicity and the independent toxicity of the new inhibitors compared with 3AB and BZ, indicative of improved specificity.

It has not escaped our notice that the data presented here suggest a dissociation between the effect of PADPRP inhibitors on cell survival and their effect on DNA strand break repair. For example, significant potentiation of TM cytotoxicity is obtained with PD 128763 at 10 μM, and has reached its maximum by 50 μM (see Figure 4). In contrast, PD 128763 does not affect TM-induced DNA strand break levels significantly until concentrations of ≥ 100 μM are reached (Figure 7). A plausible explanation for these observations is that PADPRP mediates not only DNA repair processes, but also independently modulates DNA damage-inducible responses involved in cell survival (e.g. specific gene transcription, p53 stabilisation; Kastan *et al.*, 1991). The difference in the concentrations of inhibitors required would reflect the degree of inhibition of PADPRP necessary to modulate these responses. A more detailed analysis of these observations is currently under way.

In conclusion, PD 128763 and NU1025 can potentiate the cytotoxicity of clinically relevant concentrations of TM, and at micromolar compared with millimolar concentrations required for BZ and 3AB. These are important considerations if the use of these compounds as potentiators of drug-induced cytotoxicity is to be extrapolated to the clinic. These data provide the groundwork for initiating *in vivo* studies to establish the ability of PADPRP inhibitors, used in conjunction with chemotherapeutic agents, to enhance tumour regression.

Abbreviations

3AB, 3-aminobenzamide; BZ, benzamide; IC_{50}, concentration which reduces growth/activity by 50%; DMSO, dimethyl sulphoxide; DBD, DNA-binding domain; DEF_{10}, dose enhancement factor at 10% survival; LD_{50}, dose which reduces survival by 50% (lethal dose); MTIC, 5-(3-methyl-triazen-l-yl) imidazole-4-carboxamide; PBS, phosphate-buffered saline; PADPRP, poly(ADP-ribose) polymerase; RE, relative elution; RR, relative retention; TM, temozolomide; s.e., standard error.

Acknowledgements

We would like to express our gratitude to Professor MFG Stevens, University of Nottingham, for the provision of temozolomide, and to Dr WR Leopold, Warner Lambert, for the provision of PD 128763. Our thanks to Professor DR Newell, Cancer Research Unit, University of Newcastle upon Tyne, for invaluable discussion and advice. Our thanks also to Karen Bowman for performing some of the PADPRP assays.

References

ALTHAUS FR, HOFFERER L, KLECZKOWSKA HE, MALANAGA M, NAEGELI H, PANZETER P AND REALINI C. (1993). Histone shuttle driven by the automodification of poly(ADP-ribose) polymerase. *Environ. Mol. Mutagen.*, **22**, 278–282.

BANASIK M, KOMURA H, SHIMOYAMA M AND UEDA K. (1992). Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl) transferase. *J. Biol. Chem.*, **267**, 1569–1575.

BOULIKAS T. (1991). Relation between carcinogenesis, chromatin structure and poly(ADP-ribosylation) (review). *Anticancer Res.*, **11**, 489–528.

BRADFORD MM. (1976). A rapid and sensitive method for the quantitiation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CHATTERJEE S, CHENG MF, BERGER SJ AND BERGER NA. (1991). Alkylating agent hypersensitivity in poly(adenosine diphosphate-ribose) polymerase deficient cell lines. *Cancer Commun.*, **3**, 71–75.

CREISSEN D AND SHALL S. (1982). Regulation of ligase activity by poly(ADP-ribose). *Nature*, **296**, 271–272.

DE MURCIA G AND MÉNISSIER DE MURCIA J. (1994). Poly(ADP-ribose) polymerase: a molecular nick-sensor. *Trends Biol. Sci.*, **19**, 172–176.

DENNY BJ, WHEELHOUSE RT, STEVENS MFG, TSANG LH AND SLACK JA. (1994). NMR and molecular modeling investigation of the mechanism of activation of the antitumor drug temozolomide and its interaction with DNA. *Biochemistry*, **33**, 9045–9051.

DURKACZ BW, OMIDIJI O, GRAY DA AND SHALL S. (1980). $(ADP\text{-}ribose)_n$ synthesis participates in DNA excision repair. *Nature*, **283**, 593–596.

FERRO AM, HIGGINS NP AND OLIVERA BM. (1983). Poly(ADP-ribosylation) of a DNA topoisomerase. *J. Biol. Chem.*, **258**, 6000–6003.

FORNACE JR AJ AND LITTLE JB. (1977). DNA crosslinking induced by X-rays and chemical agents. *Biochim. Biophys. Acta*, **477**, 343–355.

GRIFFIN RJ, PEMBERTON LC, RHODES D, BLEASDALE C, BOWMAN K, CALVERT AH, CURTIN NJ, DURKACZ BW, NEWELL DR, PORTEOUS JK AND GOLDING BT. (1995). Novel potent inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). Anticancer Drug Design (in press).

GRUBE K, KÜPPER JH AND BÜRKLE A. (1991). Direct stimulation of poly(ADP-ribose) polymerase in permeabilised cells by double-stranded DNA oligomers. *Anal. Biochem.*, **193**, 236–239.

HALLDORSSON H, GRAY DA AND SHALL S. (1978). Poly(ADP-ribose) polymerase activity in nucleotide permeable cells. *Febs Lett*, **85**, 349–352.

HUNTING DJ, GOWANS BJ AND HENDERSON JF. (1985). Specificity of inhibitors of poly(ADP-ribose) synthesis. Effects of nucleotide metabolism in cultured cells. *Mol. Pharmacol.*, **28**, 200–206.

KASTAN MB, ONYEKWERE O, SIDRANSKY D, VOGELSTEIN B AND CRAIG RW. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.

KOHN KW, EWIG RAG, ERICKSON LC AND ZWELLING LA. (1981). Measurement of strand breaks and crosslinks by alkaline elution. In: *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, part B, Friedberg EC and Hanawalt PC (eds). pp. 379–401. Marcel Dekker: New York.

LEOPOLD WR AND SEBOLT-LEOPOLD JS. (1992). Chemical approaches to improve radiotherapy. In: *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development*, Valeriote FA, Corbett TH anmd Baker LH (eds), chapter 9. Kluwer Academic Publishers: Boston.

MACLAREN RA, WITMER MV, RICHARDSON E AND STAMATO TD (1990). Isolation of Chinese hamster ovary cells with reduced poly(ADP-ribose) polymerase activity. *Mutat. Res.*, **231**, 265–274.

MOLINETTE M, VERMEULEN W, BÜRKLE A, MÉNISSIER-DE MURCIA J, KÜPPER JH, HOEIJMAKERS JHJ AND DE MURCIA G. (1993). Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. *EMBO J.*, **12**, 2109–2117.

MOSES K, WILLMORE E AND DURKACZ BW. (1990). Correlation of enhanced 6-mercaptopurine cytotoxicity with increased phosphoribosylpyrophosphate levels in Chinese hamster ovary cells treated with 3-aminobenzamide. *Cancer Res.*, **50**, 1992–1996.

NEWLANDS ES, BLACKLEDGE GRP, SLACK JA, RUSTIN GJS, SMITH DB, STUART NSA, QUARTERMAN CP, HOFFMAN R, STEVENS MFG, BRAMPTON MH AND GIBSON AC. (1992). Phase I trial of temozolomide (CCRG 81045: M & B 39831: NSC 362856). *Br. J. Cancer*, **65**, 287–291.

NISSELBAUM JS AND GREEN S. (1969). A simple ultramicro method for the determination of pyridine nucleotides in tissue. *Anal. Biochem.*, **27**, 212–217.

PURNELL MR AND WHISH WJD. (1980). Novel inhibitors of poly(ADP-ribose) synthetase. *Biochem. J.*, **185**, 775–777.

RANKIN PW, JACOBSON EL, BENJAMIN RC, MOSS J AND JACOBSON MK. (1989). Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. *J. Biol. Chem.*, **264**, 4312–4317.

SATOH MS AND LINDAHL T. (1992). Role of poly(ADP-ribose) formation in DNA repair. *Nature*, **356**, 356–358.

SATOH MS, POIRIER GG AND LINDAHL T. (1993). NAD^+-dependent repair of damaged DNA by human cell extracts. *J. Biol. Chem.*, **268**, 5480–5487.

SEBOLT-LEOPOLD JS AND SCAVONE SV. (1992). Enhancement of alkylating agent activity *in vitro* by PD 128763, a potent poly(ADP-ribose) synthetase inhibitor. *Int. J. Radiat. Oncol. Biol. Phys.*, **22**, 619–621.

SMULSON M, ISTOCK N, DING R AND CHERNEY B. (1994). Deletion mutants of poly(ADP-ribose) polymerase support a model of cyclic association and dissociation of enzyme from DNA ends during DNA repair. *Biochemistry*, **33**, 6186–6191.

STEVENS MFG, HICKMAN JA, LANGDON SP, CHUBB D, VICKERS L, STONE R, BAIG G, GODDARD C, GIBSON NW, SLACK JA, NEWTON C, LUNT E, FIZAMES C AND LAVELLE F. (1987). Antitumour activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (CCRG81045; M & B 39831), a novel drug with potential as an alternative to decarbazine. *Cancer Res.*, **47**, 5846–5852.

SUTO MJ, TURNER WR, ARUNDEL-SUTO CM, WERBEL LM AND SEBOLT-LEOPOLD JS. (1991). Dihydroisoquinolinones: the design and synthesis of a new series of potent inhibitors of poly(ADP-ribose) polymerase. *Anti-cancer Drug Design*, **7**, 101–107.

TSANG LLH, QUARTERMAN CP, GESCHER A AND SLACK JA. (1991). Caomparison of the cytotoxicity in vitro of temozolomide and decarbazine, prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. *Cancer Chemother. Pharmacol.*, **27**, 342–346.

WITMER MV, ABOUL-ELA N, JACOBSON ML AND STAMATO TD. (1994). Increased sensitivity to DNA-alkylating agents in CHO mutants with decreased poly(ADP-ribose) polymerase activity. *Mutat. Res. DNA Repair*, **314**, 249–260.

YOSHIHARA K, ITAYA A, TANAKA Y, OHASHI Y, ITO K, TEAOKA H, TSUKADA K, MATSUKAGE A AND KAMIYA T. (1985). Inhibition of DNA polymerase α, DNA polymerase β, terminal deoxynucleotidyltransferase and DNA ligase by poly(ADP-ribosyl)ation in vitro. *Biochem. Biophys. Res. Commun.*, **128**, 61–67.