

**INTERACTIVE EFFECTS OF POLY(ADP-RIBOSE)
POLYMERASE -1 AND DNA DEPENDENT PROTEIN KINASE
IN THE CELLULAR RESPONSES TO DNA DAMAGE**

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DECLARATION

This thesis records the work carried out at the University of Newcastle upon Tyne between October 1999 and July 2002 and is my own original work

ABSTRACT

DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase-1 (PARP-1) participate in non-homologous end joining and base excision repair respectively, and are key determinants of radio-resistance. The interactive effects of PARP-1 and DNA-PK in the cellular responses to DNA damage were investigated using novel specific inhibitors of DNA-PK (NU7026) and PARP-1 (AG14361) and cell lines proficient or deficient for DNA-PK or PARP-1.

Enzyme deficient cell lines were 4-fold more sensitive to ionizing radiation (IR) alone, and showed reduced potentially lethal damage recovery (PLDR), compared to their proficient counterparts. NU7026 potentiated IR cytotoxicity in exponentially growing DNA-PK proficient, but not deficient cells. Similarly AG14361 potentiated IR in PARP-1 +/+ but not PARP-1 -/- cells. When NU7026 and AG14361 were used in combination, their potentiating effects were additive. Both inhibitors reduced PLDR in the proficient cell lines. Furthermore, inhibitor combination completely abolished PLDR. Both inhibitors prevented IR-induced DNA double strand break repair, but only AG14361 prevented DNA single strand break repair

The enzyme activities were investigated using purified enzymes and permeabilised cells. In cell-free assays, PARP-1 activity was unaffected by the presence of DNA-PK, and *vice versa*, provided the enzyme substrates were present. DNA-PK inhibited PARP-1 when ATP was absent or NU7026 present. PARP-1 inhibited DNA-PK when NAD⁺ was absent or AG14361 present. Furthermore, PARP-1 inhibition increased with increasing ratio of DNA-PK to PARP-1, and *vice versa*. Similar results were obtained using the inhibitors in permeabilised cells. PARP-1 was inactive in the absence of histones, but activity was restored by the addition of DNA-PK. DNA-PK was inactive in the absence of its p53 peptide substrate unless PARP-1 was included.

These data suggest reciprocal regulation of PARP-1 and DNA-PK, and co-operation in DSB repair and survival. Thus, individually, or in combination, the DNA-PK and PARP-1 inhibitors act as potent radio-sensitisers.

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ABBREVIATIONS

3AB	3-aminobenzamide
AG14361	1-(4-dimethylaminoethylphenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6one
ATP	adenosine triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	base excision repair
cDNA	complementary DNA
DBD	DNA binding domain
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DNA-PKcs	DNA dependnet protein kinase catalytic subunit
DPM	disintegrations per minute
DSB	double strand break
dsDNA	double stranded DNA
EDTA	ethylenediaminetetraacetic acid
EMS	ethane methanesulfonate
FACS	fluorescent activated cell sorting
FCS	foetal calf serum
FEN	flap endonuclease
FSC	forward scatter
Gy	gray
HR	homologous recombination
HRP	horse radish peroxidase
IR	ionising radiation
KO	knockout
MEFs	mouse embryonic fibroblasts
MMS	methyl methanesulphate
MNNG	N-methyl-N'-nitro-nitroguanidine
MNU	N-methyl-N-nitrosurea
mRNA	messenger RNA

MTIC	5-(3-methy;-1-triazeno)imidazole-4-carboxamide
NAD ⁺	nicotinamide adenine dinucleotide
NER	nucleotide excision repair
NHEJ	non homologous end joining
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide mononucleotide adenyl trannsferase
NU1025	8-hydroxy-2-methylqunazolin-4(3H)-one
NU1085	2-(4-hydroxyphenyl)-benzimidazole-4-carboxamide
NU7026	(2-morpholin-4yl)-benzo[h]chromen-4-one)
PAGE	polyacrylamide gel electrophoresis
PARP-1	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PD128763	3,4-dihydro-5-methoxyisoquinolin-1-(2H)-one
PLDR	potentially lethal damage recovery
RNA	ribonucleic acid
SCE	sister chromatid exchange
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SSA	single strand annealing
SSB	single strand break
ssDNA	single stranded DNA
TCA	trichloroacetic acid
TM	temozolomide
TRF1	telomeric repeat binding factor
WT	wild type
XRCC1	x-ray cross complementing 1
XRCC3	x-ray cross complementing 3
XRCC4	x-ray cross complementing 4
XRCC5	x-ray cross complementing 5

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CHAPTER 1

INTRODUCTION

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Cancer is the most common cause of death in the UK and other developed countries, after heart disease, and it will affect around one in three people in the UK at some time in their lives. Alarming, one in four people will eventually die from cancer (Cancer Research Campaign, 1998). Cancer is a new growth that arises from abnormal and uncontrolled division of cells that may then go on to invade and destroy surrounding tissues (*i.e.* metastasise). More than 200 types of cancer have been identified (Fawcett and Drew, 1996). Cancers are classified according to the tissue and cell type from which they arise. For example, those arising from epithelial cells are defined as carcinomas whilst connective tissue or muscle tumours are termed sarcomas. Cancers which do not fall into either of these broad categories include leukaemias, which derive from hemopoietic cells and tumours of the nervous system. The likelihood of developing cancer varies with age and many cancers are a feature of the ageing process, resulting from an accumulation of mutations in genomic DNA. Accordingly, 65 % of all new cases of cancer are diagnosed in people over the age of 65.

1.1.1 Carcinogenesis

There is a strong genetic component in the development of cancer involving a multistage process of accumulation of genetic mutations in genes that control normal cellular growth and epigenetic alterations. At least four to seven 'hits' are thought to be required for cancer to develop. Colorectal cancers, for example, typically develop over decades and appear to acquire at least seven genetic events for completion (Kinzler and Vogelstein, 1996). Successive mutation enables a clone of cells to expand and gain selective advantage over surrounding cells and this phenomenon is termed clonal evolution.

Carcinogenesis is conventionally considered to comprise of two phases, namely initiation and promotion:-

- **Initiation** involves the irreversible mutation of cellular DNA, either spontaneous, ionising radiation (IR), virus or carcinogen induced, in such a way that the cell escapes the normal constraints of regulated growth.
- **Promotion** is the increased proliferative state that enhances the possibility of further genetic change. For example, mutation or amplification of proto-oncogenes and/or deactivation of tumour-suppressor genes, which result in the development of a fully malignant tumour.

Mutations in proto-oncogenes are known as ‘gain of function’ mutations and are generally activating mutations of cellular genes which have a dominantly acting role in the control of cell proliferation. A raised level of the nuclear oncogene c-myc has been reported in 60-70 % of carcinomas (Finley *et al.*, 1989). Mutations in tumour suppressor genes (TSGs) are, by contrast, ‘loss of function’ mutations. These may be identified as mutations that reduce or abolish the activity of genes that negatively regulate critical cellular functions, so that a loss of activity leads, for example, to enhanced cellular proliferation, blocked differentiation, or the suppression of cell death pathways. An example of a TSG that regulates the cell death pathway, termed apoptosis is p53. p53 is a common target for genetic alterations in cancer, with around 50 % of tumours possessing mutations in the p53 gene (Lutz and Nowakoska-Swirka, 2002).

1.2 Treatment

The ultimate goal of anticancer treatment is to achieve a cure. The ability to achieve a cure depends on the type of tumour and how far it has spread and, consequently, survival rates and the chances of recurrence differ according to the organ or tissues affected. Many cancer treatments do not achieve 100 % cure and therefore the aim is to achieve long-term survival. This recognises that it is virtually impossible to eradicate every single cancer cell in the body and that eventually the

condition may recur. Treatment effectiveness is therefore often assessed in terms of 5 or 10 year survival.

The principle approaches to the treatment of cancer are surgery, radiotherapy and chemotherapy. Where a tumour is accessible, surgery is usually the first line of action. Surgery aims to excise as much tumour as possible and therefore a wide margin of tissue, including normal tissue and lymph nodes, is usually removed in order to limit the risk of relapse. Unfortunately, it is often impossible to excise all tumour tissue which may eventually invade surrounding tissues and often tumour cells may have already spread to other parts of the body by the time of surgery. Consequently, radiotherapy is often used as an adjunct to surgery with the aim of suppressing the rate of cell division in any tumour cells that remain following surgery at the site of excision. Where cancer is advanced, surgery may be used to reduce tumour bulk or relieve obstruction as a means of palliative care.

Radiotherapy relies on the use of ionising radiation (IR) to directly damage the DNA of malignant cells (section 1.3.4.1). This form of treatment may be used either as the principle means of local control or as an adjunct to surgery as detailed above. It may be applied externally (beam therapy) or internally (brachy therapy) and the two approaches can be combined.

Surgery and/or radiotherapy are only effective in locally confined tumours and less than a quarter of those diagnosed with cancer will be cured solely by these means. Most patients will receive systemic chemotherapy at some time during their illness. In a small fraction of cancer patients, representing selected neoplasms, chemotherapy will result in a cure or prolonged remission. In most cases, however, drug therapy will produce only a regression of the disease and complications or relapse may eventually lead to death. In addition, most currently available anti-cancer drugs do not specifically recognise neoplastic cells but rather affect all proliferating cells. Consequently, treatment often lacks specificity and results in side effects which include gastrointestinal damage, hair loss and myelosuppression. As a result, anti-cancer drugs tend to have a steep dose response curve for both toxic and therapeutic effects.

Other forms of treatment include hormone therapy, gene therapy, immunotherapy, bone marrow transplant and peripheral blood stem cell transplants in conjunction with high dose chemotherapy. One approach which has received much publicity is the 'magic bullet' which employs toxin-linked monoclonal antibodies to target tumour cell antigens. In practice, however, monoclonal antibody therapy has been disappointing since the antibody is all too often recognised as foreign and destroyed by the actions of the immune system before any significant cell kill is achieved.

1.3 DNA damage

The number of ways that DNA can be damaged is extremely large since DNA is continuously subject to hydrolysis, oxidation and non-enzymic methylation damage, as well as environmentally and chemically induced lesions. The major causes of spontaneous DNA damage as well as that caused by IR and chemotherapeutic agents most commonly used in the treatment of cancer are reviewed below.

1.3.1 Endogenous damage

Base loss

The N-glycosyl base sugar bond linking DNA bases with deoxyribose is labile under physiological conditions. Within a typical mammalian cell, several thousand DNA purines and several hundred DNA pyrimidines are spontaneously lost per cell per day due to hydrolytic cleavage of the glycosyl bond. Loss of a purine or pyrimidine base creates an apurinic or apyrimidinic (AP) site also known as an abasic site.

Base modification

Deamination The primary amino groups of nucleic acid bases are unstable and may be converted to keto groups in a process termed deamination. Cytosine in DNA is deaminated to uracil and it is estimated that between 100 and 500 cytosine deamination events take place per human cell per day. Other deamination reactions

include conversion of adenine to hypoxanthine, guanine to xanthine and 5-methyl cytosine to thymine (Lindahl, 1993). Deamination of cytosine and 5-methyl cytosine is potentially mutagenic because if the damage remains uncorrected the resultant thymine or uracil can pair with an adenine instead of guanine at replication. Similarly, the deamination of adenine is a potentially mutagenic lesion because hypoxanthine forms a more stable base pair with cytosine rather than thymine.

Oxidation In the course of normal metabolism, cells are exposed to several types of reactive oxygen species (ROS) which include singlet oxygen, peroxide radicals, hydrogen peroxide and hydroxyl radicals (OH). ROS can modify DNA bases. A common product of thymine oxidation is a thymine glycol which has lost the 5,6 double bond and are non-coding bases. Hydroxyl radicals react with guanine to generate 8-hydroxyguanine, a potentially mutagenic lesion that shows preference for base pairing with adenine rather than cytosine. This latter reaction is estimated to occur at approximately the same rate as cytosine deamination, i.e. 100-500 events per day.

Methylation In addition to oxygen, living cells contain several other reactive molecules that have the potential to cause DNA damage. The most important of these is probably S-adenosylmethionine (SAM). SAM is a methyl group donor that is used as a co-factor in most cellular transmethylation reactions. SAM can react accidentally with DNA to produce alkylated bases which include 3-methyladenine and 7-methylguanine. The latter is relatively harmless as it does not alter the coding specificity of the base. However, 3-methyladenine blocks replication, and is therefore a cytotoxic DNA lesion.

1.3.2 Environmental damage

Ultraviolet light is absorbed by nucleic acid bases and the resulting deposition of energy can induce chemical changes. The most frequent photoproducts are the consequences of bond formation between adjacent pyrimidines within one strand, and, of these, the most frequent are cyclobutane pyrimidine dimers (CPDs). T-T CPDs are formed most readily, followed by T-C or C-T and C-C dimers are least abundant. CPDs cause extreme distortion of the DNA chain structure. The

mechanisms by which UV irradiation generates point mutations are only partially understood, however, the most frequently observed mutations are C to T transitions, with CC to TT double transitions also being commonly seen (Pfeifer, 1997).

1.3.3 Replication errors

Another major source of potential alterations in DNA is the generation of mismatches or small insertions or deletions during DNA replication, particularly in highly repetitive sequences. Although DNA polymerases are moderately accurate and most mistakes are immediately corrected by polymerase-associated 'proofreading' exonucleases, nevertheless, the replication machinery is not perfect.

1.3.4 Chemical damage

Many environmental chemicals including those substances encountered in daily living can damage DNA and act as carcinogens by modifying DNA bases, frequently by addition of an alkyl group (alkylation). Examples include, polycyclic aromatic hydrocarbons such as benzo(a)pyrene occurring in tobacco smoke, pitch, tar fumes and soot; aromatic amines which are used principally in the chemical and rubber industries.

The alkylating agents, dimethyl- and diethyl-nitrosamine, which require metabolic activation and are indirect acting, are found in trace amounts in many food products. In addition, nitrosamines are generated within the body by the action of nitrosating agents on ingested amines, or formed from diethylamine and methylnitrite in tobacco smoke (Beranek, 1990).

Radio- and chemo-therapy are effective treatments for cancer, yet paradoxically, they are also known to cause cancer since they induce genetic changes. With the ultimate goal of producing a cure, radio-and chemo-therapy aim to cause a cytotoxic lesion that will arrest tumour progression without affecting normal tissue. Often, the cytotoxic lesion is a DNA strand break. Strand breaks may be introduced into DNA by a variety of mechanisms which include direct damage and indirect damage by repair mechanisms which introduce breaks as part of the repair

process. The major lethal effect of ionising radiation (IR) is the creation of double stranded breaks (DSBs). Chemotherapeutic agents which damage DNA, producing strand breaks as part of their mechanism include alkylating agents, topoisomerase poisons, including etoposide, and the radiomimetic drug, bleomycin. The latter two agents have been used in the studies described in this thesis and are therefore reviewed below.

1.3.4.1 Radiotherapeutic agents

Ionising radiation (IR), which includes X rays, γ rays and high energy electrons, interacts with matter by transferring energy to the electrons in the irradiated sample. IR acts indiscriminately on all molecules in the irradiated area and DNA is the critical target of IR-induced cell death. The toxic effect of IR on cells is a result of a direct interaction of energy with DNA or indirectly through the formation of free radicals. These include the hydroxyl (OH) radical, the hydrated electron, the H atom, as well as secondary radicals that are produced as a consequence of reaction with small solute molecules within the cell.

The proportions of reactive species formed is dependent on the oxygen status of the cell, where under oxygenated conditions, the OH radical predominates. This radical can diffuse to the DNA and efficiently react with the sugar of any nucleotide resulting in base damage or disruption of a phosphodiester bond or ribose ring (by H abstraction, for example), ultimately giving rise to DNA strand breaks in the individual strands of the double helix. In addition, alkali-labile bonds result in the formation of apyrimidinic sites, base damage (For example, ring saturated thymines, thymine glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycysteine, phosphoglycols) and adducts such as formate and COO^- which cause protein cross links (Ward, 1988).

Double strand breaks (DSBs) are the major cytotoxic lesion produced by IR and it is estimated that a single unrepaired DSB is sufficient to cause cell death. DSBs can result from direct interaction of energy with the DNA or as repair intermediates through the conversion of multiply damaged sites (MDS) (described in more detail in section 5.1). IR is thought to produce about 40 double strand breaks (DSBs), 1000 single strand breaks (SSBs), 2000 base damages, 1500 sugar damages,

30 DNA-DNA cross-links, 150 DNA-protein crosslinks and 300 alkali-labile sites per Gray (Ward, 1990). Due to the wide spectrum of lesions introduced into the DNA, IR-induced DNA damage is repaired by multiple DNA repair pathways (see section 1.5.2).

Low LET radiation (gamma) predominantly causes indirect damage through OH radical intermediates. There are two main consequences of the reaction of hydroxyl radicals with the deoxyribose of DNA; Strand breaks leaving 5'- and 3'-phosphate termini; Strand break leaving 5'-phosphate and 3'-phosphoglycolate termini.

1.3.4.2 Chemotherapeutic agents

Alkylating agents

The alkylating agents, which include the nitrogen mustards, were the first used chemotherapeutic agents and are still widely used today. Indeed, the use of nitrogen mustard as an anticancer agent dates from 1946 when it was shown to induce regression of lymphomas (Chabner and Collins, 1990). Nitrogen mustards exert their cytotoxic effects through covalent reaction of alkyl groups (one or more saturated carbons) with cellular molecules thus forming covalent adducts with bases in the DNA. Alkylating agents are electrophilic agents that react with nucleophilic centres on DNA. The most common mechanism of action is thought to be the alkylation of DNA most frequently at the nucleophilic positions, N⁷ and O⁶ of guanine. The principal mutagenic lesion is the O⁶ methylguanine which mispairs with thymine during replication.

Monofunctional alkylating agents carry one reactive alkyl group that can lead to DNA SSBs during repair of such lesions. Bifunctional alkylating agents have two reactive alkyl groups and in addition to monofunctional lesions, can induce inter- or intra-strand DNA cross links with other bases. The cross-links are not easily repaired by DNA repair systems and are therefore readily cytotoxic.

Other agents such as the nitrosoureas and platinating agents (cisplatin, for example) have a similar mode of action to the nitrogen mustards, whereby reactive species from these agents (electrophilic) react with nucleophilic centres on DNA to form a variety of adducts (Chabner and Collins, 1990).

Bleomycin

Bleomycin is a radiomimetic drug because it produces similar DNA lesions in DNA compared to IR i.e. single and double strand breaks, through the generation of free radicals that cause scission of DNA through an oxidative process. The bleomycin molecule contains a metal binding region which forms a complex with Fe (II), thus allowing efficient oxygen binding and reduction and therefore giving rise to hydroxyl and superoxide radicals. Association of bleomycin with DNA occurs through a DNA binding domain directed to specific nucleotide sequences, 5'-GC-3' and 5'-GT-3', for example (Muraoka and Takita, 1988), and this enables the metal chelated portion of the molecule to generate free radicals in close proximity to DNA. The free radicals in turn produce DNA strand scission and base release.

Topoisomerase poisons

Topoisomerase I and II (topo I and II) are enzymes that regulate the strand breakage, rotation and rejoining of DNA to enable DNA transcription, replication, recombination and repair to occur.

Both enzymes catalyse the unlinking and relaxation of DNA strands by forming transient DNA breaks through the formation of a 'cleavable complex' thus allowing DNA to rotate around or traverse through these breaks. In the case of topoisomerase I, a SSB is formed that allows the DNA to unwind by one turn. Topoisomerase II forms a DSB, allowing relaxation of torsional strain by two turns.

Topo I poisons act by stabilising the cleavable complex thus giving rise to strand breaks. Topo I poisons are associated with single strand breaks whilst topo II poisons give rise to the formation of DSBs. Cytotoxic lesions can also appear through the processing of cleavable complexes by repair pathways thus leading to the formation

of frank DNA strand breaks. Alternatively, cytotoxic strand breaks appear due to an interaction between drug stabilised cleavable complexes and replication forks. Indeed, topo I poisons are particularly cytotoxic during S phase of the cell cycle. In this way, reversible Topo I DNA cleavable complexes are converted to irreversible DSBs.

1.4 Mechanisms of drug resistance

Anti-cancer agents and IR present a lethal threat to the cell and in some cases are able to achieve a cure. However, treatment of many types of cancer fails to achieve a cure because tumour cells are chemo- or radio-resistant. Chemo- and radio-resistance, the phenomenon whereby a cell can survive doses of a chemical or IR that would normally be cytostatic or cytotoxic is the main obstacle to the clinical success of chemotherapeutic agents and IR. Resistance may be intrinsic, i.e. when, despite no prior therapy, a tumour cell is refractory to treatment from the outset, or acquired, i.e. when an initially sensitive tumour cell becomes resistant. Drug resistance can arise as a result of various biochemical mechanisms. These mechanisms are reviewed below and in Figure 1.1. It should be noted that resistance to a particular drug could be achieved by more than one mechanism and in some cases, particularly those involving changes in drug transportation or detoxification, protection against more than one chemical (cross-resistance) is observed.

1.4.1 Drug delivery

Drug resistance may be caused by reduced drug delivery. The concentration of drug in the blood is of critical importance in the delivery of drug to the target tissue. The vascularisation of tumours is highly variable and poorly vascularised tumours may not receive adequate drug exposure. Another feature influencing drug delivery are the pharmacokinetics of the drug in plasma, which are dependent on factors such as chemical stability, metabolism, biliary and renal elimination. Alternatively, a reduction in the active drug concentration may result from drug sequestration due to increased intracellular drug binding.

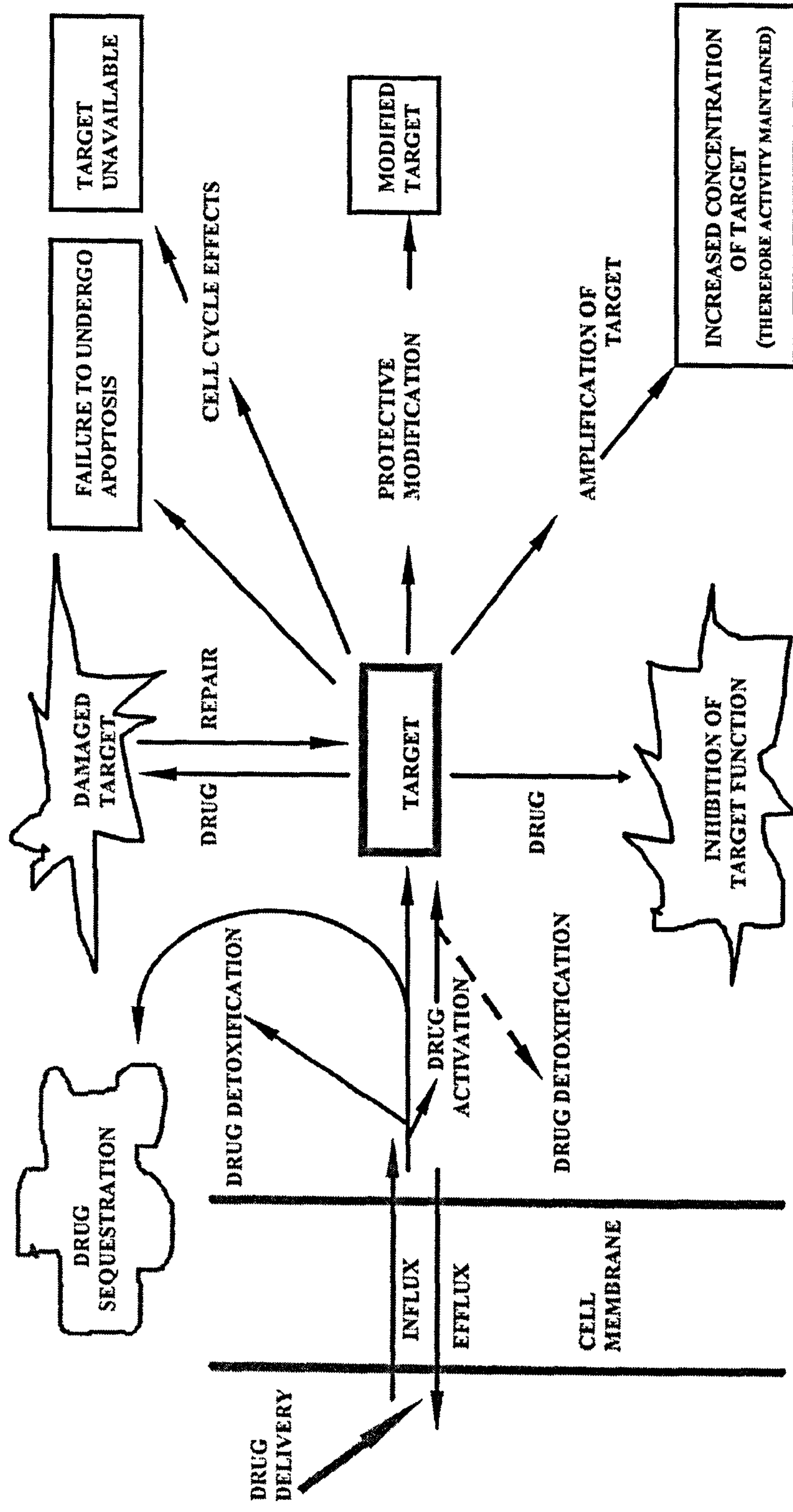


Figure 1.1 Mechanisms of Drug Resistance

1.4.2 Drug uptake/efflux

The defective cellular uptake of drugs constitutes a general mechanism of resistance. The contribution of decreased drug uptake to drug resistance varies according to the lipophilicity of the drug and on the physiochemical properties of the cell membrane and involves drugs that are absorbed passively or those requiring active transport. If the influx of the drug is by simple diffusion, the properties of the cell membrane may be altered. Carrier mediated transport is of importance in the uptake and resistance to several anticancer drugs, particularly those that are charged at physiological pH. Examples of carrier mediated transport of anticancer drugs include the uptake of nitrogen mustard via the choline transport system and methotrexate via the reduced folate carrier. Mutations affecting these transport systems have been implicated in resistance to anti-cancer drugs.

Multidrug resistance (MDR) describes the situation where exposure to one drug induces cross-resistance to a variety of agents of different chemical classes, to which the cell has never been exposed (Hochhauser and Harris, 1991). MDR occurs in a wide range of cell types and involves alterations to drug transport systems. A common example of MDR is P-glycoprotein which is a 179 kDa trans-membrane, energy-dependent efflux pump (Ling, 1997). Overexpression of P-glycoprotein causes increased drug efflux thus removing many anti-cancer drugs from the cell. P-glycoprotein and other similar pumps, including the multi-drug resistance associated protein (MRP) family, for example, are overexpressed in many tumour cell types.

1.4.3 Drug metabolism

Drug metabolising enzymes can act either to potentiate or reduce the toxicity of chemicals. Changes in both activation and de-activation pathways are important variables that can lead to drug resistance. Resistance to antimetabolite drugs is often associated with decreased activation. In contrast, resistance to 1-arabinofuranosylcytosine (araC) has been attributed to the increased deactivation of the drug by metabolising enzymes that serve to inactivate the drug, in this case, specific deaminases. In addition, resistance to alkylating agents can involve overexpression of glutathione S-transferases (Chabner and Collins, 1990).

1.4.4 Changes to target sites

Alteration of the structure of the drug target may reduce the affinity of the drug for the target, or reduce the activity of the target. This type of resistance is usually associated with drugs whose target is well defined. For example, mutations in the dihydrofolate reductase gene can result in resistance to methotrexate and mutated or down regulation of topo I can result in resistance to camptothecin. Similarly, over-expression of the target protein due to gene amplification and/ or increased transcription, reduced degradation, posttranslational modification, could increase the concentration of target sites. In this mechanism the structure of the target site is unchanged and resistance is solely due to an increase in target abundance. An example of this mechanism of resistance is the increase in dihydrofolate reductase protein concentration resulting from gene amplification observed in methotrexate resistant cell lines.

1.4.5 DNA repair as a resistance mechanism

Increased rates of repair of cellular DNA damage represents an important mechanism of resistance to chemo- and radiotherapy. A number of repair pathways have been described (section 1.5). In the context of this thesis, modulation of repair by the use of specific inhibitors has been evaluated as an attractive mechanism by which to modulate resistance to a number of anticancer agents.

1.5 DNA repair

As described in section 1.3, cells are continuously under assault from a wide array of DNA-damaging agents. These include reactive oxygen species generated as a consequence of oxidative metabolism, UV light, IR and radio-mimetic chemicals. The forms of damage are many, and range from base modifications, inter- and intra-cross-links, DNA SSB and DSBs.

The integrity of cellular DNA is of paramount importance for survival and therefore cells have evolved very efficient mechanisms for both DNA damage recognition and repair in order to ultimately maintain genomic stability and prevent

cancer. Following DNA damage, three main processes have been described; repair, excision and tolerance of the lesion (Friedberg, 1985). Those mechanisms responsible for the repair of DNA strand breaks include direct repair, excision repair and double strand break repair.

Key players in DNA damage recognition and signalling are considered to be the tumour suppressor protein p53, ataxia telangiectasia mutated (ATM), DNA dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase-1 (PARP-1) (Watters, 1999). The relative and joint roles of PARP-1 and DNA-PK in DNA damage repair and survival have been investigated in the studies described in this thesis. Evidence in the literature indicates that these enzymes are involved in base excision repair (BER) and double strand break repair (DSB repair), respectively, and therefore these mechanisms of repair are described in more detail below (sections 1.5.1 and 1.5.2).

Excision repair

This may be considered to be the most important repair mechanism. Briefly, it involves the recognition of damaged DNA, its removal either as free bases or as nucleotides followed by the gap being filled by synthesis of new DNA using the complementary strand as a template. Excision repair may be subdivided into the three main classes of base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). As PARP-1 has only been implicated in BER, only this pathway is described in more detail.

1.5.1 Base excision repair

The base excision repair (BER) pathway has evolved to protect cells against mutations induced by oxidative, alkylating and other DNA-damaging agents (McCullough *et al.*, 1999). BER is therefore one of the major pathways to repair base lesions, AP sites and SSBs. This pathway is a multi-step process involving the sequential action of several proteins (Figure 1.2). The key enzymes involved in this pathway are the DNA glycosylases. The glycosylases provide the only lesion specificity in the BER pathway with specific glycosylases for a specific type of

lesion (Seeberg *et al.*, 1995). For example, N-methylpurine-DNA glycosylase (MGP) removes N-methylpurines.

BER is initiated by the excision of any damaged bases by a glycosylase that cleaves the glycosidic bond between the base and the deoxyribose-phosphate backbone of DNA. The modified base is released and this results in the generation of an apyrimidinic or apurinic (AP) site that is then converted to a SSB through incision upstream of the AP site creating a 3'OH terminus adjacent to the AP site by an AP endonuclease or by the AP lyase action of bifunctional glycosylases. The resulting single base gap is then filled by extension of the 3'OH terminus, by a DNA polymerase which also removes the 5'phosphate terminus to permit ligation. The DNA strand is then sealed by a DNA ligase and either short-patch or long-patch processes.

Short-patch BER is responsible for the majority of repair and involves the removal of a damaged base by one of six DNA glycosylases that have been identified. The abasic site is recognised by APE-1, an endonuclease that incises 5' to the lesion leaving a normal 3'OH. One nucleotide is then added by DNA polymerase β which also excises the deoxyribose phosphate moiety using its deoxyribose phosphatase (dRPase) activity. This is followed by the rejoining of the DNA ends by DNA ligase III in partnership with the scaffold protein XRCC1. However, if a glycosylase/lyase is involved in the removal of the damaged base, the resulting 3'-phosphodeoxyribose group must be removed and replaced to leave a 3'OH for polymerase activity. A function for the recently identified polynucleotide kinase (PNK) in this process has been proposed (Whitehouse *et al.*, 2001).

Long-patch BER, a minor repair process, involves the incorporation of two to six nucleotides into the repair gap. This pathway is orchestrated by enzymes normally involved in DNA replication, including DNA polymerase β/δ , proliferating cell nuclear antigen (PCNA), which serves as a cofactor for both polymerases, flap endonuclease (FEN1), which excises the flap-like structure that is produced by DNA polymerase strand displacement and ligase 1.

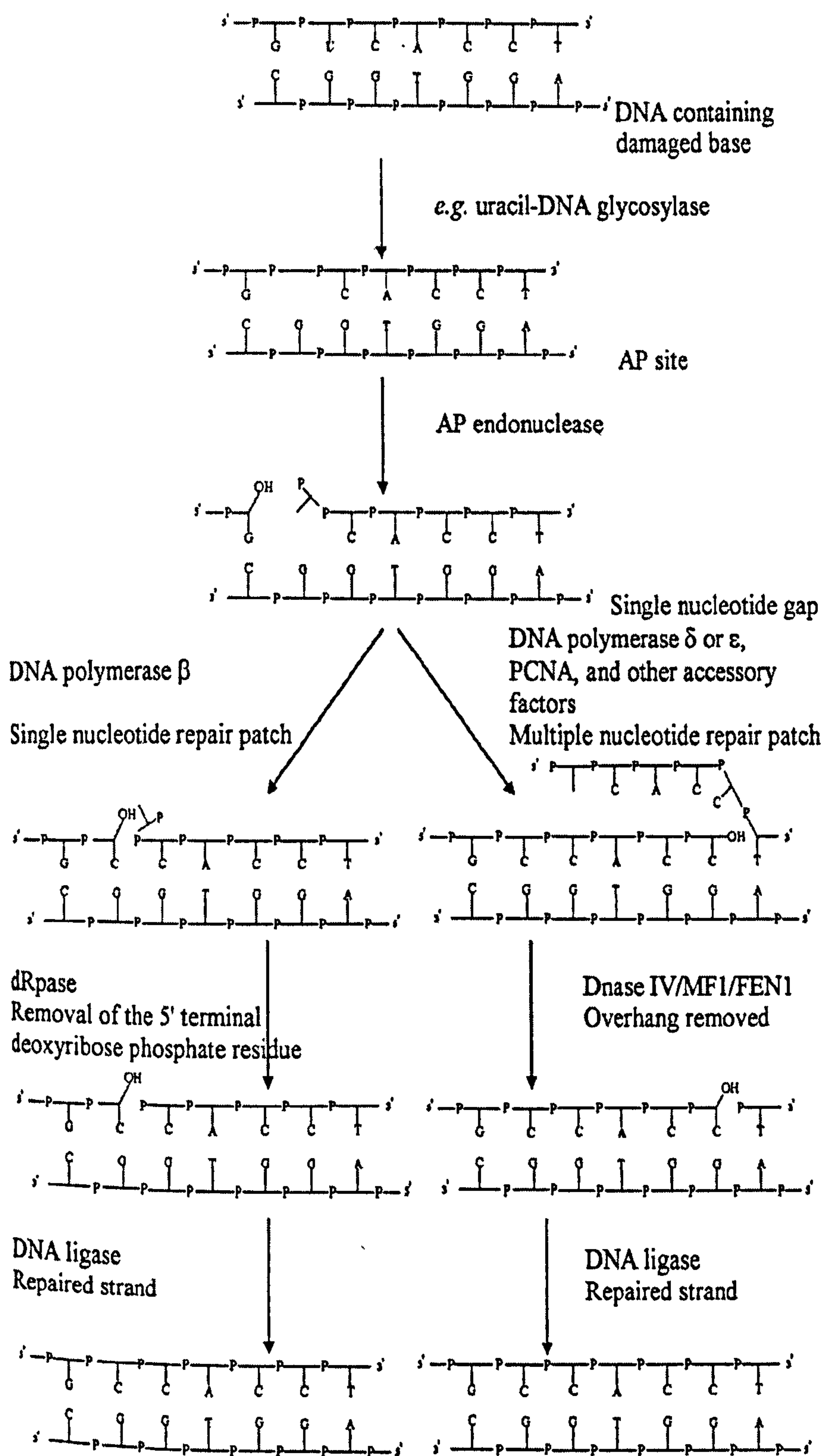


Figure 1.2 Model of Base Excision Repair
Adapted from Wood, 1996

1.5.2 Double strand break repair

DSBs arise from IR, free radicals, chemotherapeutic drugs and during replication of a SSB. A single DNA double strand break (DSB) may be sufficient to cause cell death and therefore repair of these breaks is an important process if a cell is to survive. To maintain the integrity of the genome and overcome the cytotoxic and mutagenic properties of DSBs, at least three pathways exist in eukaryotic organisms:-

(1) Non-homologous end joining (NHEJ), (2) homologous recombination (HR) and (3) single strand annealing (SSA). Although these three DSB repair mechanisms are evolutionarily conserved, their relative contributions differ between lower and higher eukaryotes. NHEJ is the predominant pathway for DSB repair in multicellular eukaryotes (Chu, 1997) since it requires little sequence homology and it has the advantage of being rapid. However, it is not necessarily error free.

1.5.2.1 Non-homologous end-joining

NHEJ is involved in repair and survival following exposure to IR, radiomimetic drugs and topoII poisons. This pathway occurs primarily in the G1 phase of the cell cycle and rejoins ends in the absence of significant homology. There are three processes; The first involves the precise joining of short overhanging, complementary ends, such as, those produced by site-specific endonucleases. This is a highly efficient process where most ends are successfully rejoined. However, when the ends are not complementary or when the continued presence of an endonuclease precludes precise re-ligation, there are two additional, but much less efficient, NHEJ processes. The first is independent of microhomology and involves the misalignment of overhanging ends. This occurs through the pairing of as few as one base pair (bp), followed by filling-in by a DNA polymerase and results in the ligation of ends with the insertion of a few bp. Alternatively, annealing of microhomologies of one to a few bp and removal of single stranded tails leads to the formation of deletions ranging from a few bp to several kb.

Many of the proteins important for NHEJ have been identified. At present, 5 proteins have been identified and these include the 3 components of the DNA-PK complex, DNA-PKcs, Ku70 and Ku80 (described in section 1.10.2). Repair is completed by DNA ligase IV and its associated protein XRCC4 (Lee *et al.*, 2003). A model for the basic NHEJ pathway is shown in Figure 1.3A. Although there have been many advances in the analysis of NHEJ, knowledge of additional factors implicated in this process is limited. Nevertheless, a role has been described for the recently identified cofactor, Artemis which is described in more detail in section 5.1 and reviewed in Jeggo and O'Neill, 2002.

1.5.2.2 Homologous recombination

The mechanism of DSB repair by homologous recombination (HR) is only partially understood, though many models have been proposed. What is known is that it takes advantage of a homologous sequence to repair the DNA DSB in order to ensure accurate rejoining. Whilst NHEJ is considered the major mechanism for DSB repair in mammals, HR has an important role in overcoming replication-specific breaks and as such occurs in the G2/M phases of the cell cycle. A model for the basic HR pathway is shown in Figure 1.3B.

Key HR proteins include RAD51, RAD52, RAD54, RAD55, RAD57 and RAD59. The RAD51 protein is a key member of this group and has a central role in HR. It mediates ATP-dependent homologous pairing and sister chromatid exchange (SCE) (Sonoda *et al.*, 1999). This strand exchange reaction is facilitated by RAD52 and RAD54, a DNA-dependent ATPase (Van Dyck *et al.*, 1999). Several other proteins have poorly defined roles in HR and these include the RAD51 related proteins (XRCC2 and XRCC3), products of the breast cancer genes (BRCA 1 and BRCA 2), replication protein A, p53, c-Abl and ATM (Bertrand, *et al.*, 1997; Mekeel *et al.*, 1997; Meyn *et al.*, 1993; Iftode *et al.*, 1999). In response to IR, distinct nuclear foci are formed in mammalian cells containing either RAD51 or RAD50 but not both (Haaf *et al.*, 1995; Zhong *et al.*, 1999) and these foci are associated with BRCA1 and BRCA2. The formation of RAD51 foci is dependent on the presence of RAD54 and XRCC3 (Bishop *et al.*, 1998). Despite an accumulation of evidence describing links between HR proteins and ATM, the involvement of ATM in DNA damage

recognition and repair remains unclear although ATM has been demonstrated to interact with H2AX through phosphorylation in some cell lines.

Individuals carrying germline mutations in BRCA1 or BRCA2 are at increased risk of developing breast cancer. At the cellular level, mutations in ATM result in increased IR sensitivity and inherited mutations of the ATM gene cause increased susceptibility to leukaemias. Defective ATM may also predispose to sporadic colon cancer and epidemiologic studies also indicate an increased incidence of breast cancer in the relatives of individuals with ataxia telangiectasia. (Shen *et al.*, 2000).

1.5.2.3 Single strand annealing (SSA)

Single strand annealing (SSA) is a form of HR that is initiated when a DSB is formed between two repeated sequences orientated in the same direction (Gottlich *et al.*, 1998). Briefly, single stranded regions are created adjacent to the breaks that extend to repeated sequences such that the complementary strands can anneal to each other. This is carried out through a series of steps; (1) generation of long single strands by helicase-mediated duplex unwinding (2) annealing of single strands at microhomology patches (3) removal of unpaired flap strands by endo-or exo-nucleases (4) DNA polymerase mediated fill in of gaps (5) sealing of remaining nicks by DNA ligase. A model for the basic SSA pathway is shown in Figure 1.3C.

Like HR, SSA requires a number of gene products, including, RAD52 and RAD59, however, RAD51 is not required for this process. SSA differs from HR in that it does not require a homologous chromosome and therefore, like NHEJ, occurs in the G1 phase of the cell cycle. Furthermore, DNA synthesis is 'conservative' i.e. within the newly synthesised stretch of DNA in the final repaired chromosome, both strands are newly synthesised and both template strands are restored to the template chromosome.

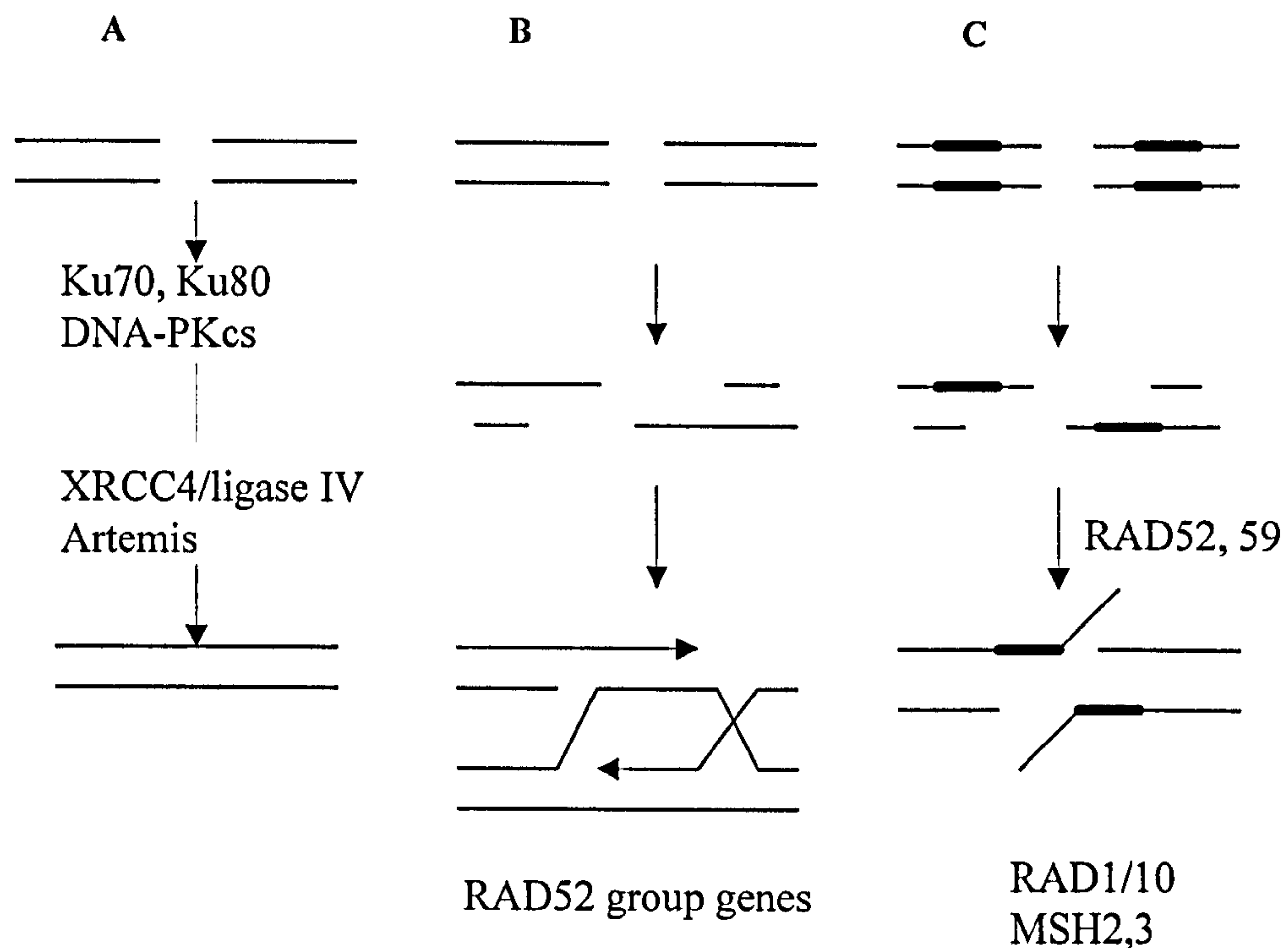


Figure 1.3 Schematic representation of eukaryotic DSB repair mechanisms

A, Non-homologous end joining which is based on the religation of the two ends, frequently involves the deletion and/or insertion of oligonucleotides; B, Homologous recombination involves the invasion of a homologous undamaged donor sequence by a 3' single strand end. Repair synthesis results in restoration of the original sequence of the damaged DNA; C, Single strand annealing pathway which involves exposure of regions of homology and repair is completed by the removal of non-homologous ends and ligation. As a consequence, a deletion is introduced into the DNA. Adapted from Pastnik *et al.*, 2001.

1.6 Poly(ADP-ribose) polymerase-1 (PARP-1)

1.6.1 General Introduction

The initial observation which led to the discovery of a nicotinamide adenine dinucleotide (NAD⁺) consuming enzyme was by Roitt in 1956 who showed that following treatment of tumours with alkylating agents, a depletion of intracellular NAD⁺ occurred (Roitt, 1956). Following further studies by research groups, the enzyme responsible for this NAD⁺ depletion was discovered:- Chambon *et al.*, 1963; Chambon *et al.*, 1966.

Chambon *et al.*, 1963, observed that the incorporation of radioactivity from [¹⁴C]-ATP into acid insoluble material by a nuclear preparation of hen liver was decreased 1000-fold when nicotinamide mononucleotide (NMN) was removed. In 1966, the group further demonstrated that a polymer of ADP-ribose was generated from ATP and NMN, with NAD⁺ as the intermediate precursor. A study by Nishizuka *et al.*, 1967, then showed that the ADP-ribose moiety of NAD⁺ was incorporated into ADP-ribose polymer with the concomitant release of nicotinamide, and that the enzyme responsible was localised exclusively to the nucleus. Initially, this enzyme was named ADP-ribose synthetase (ADPRS) or ADP-ribose transferase (ADPRT) but was later renamed poly(ADP-ribose) polymerase-1 and it is by this name that the enzyme will be hereafter referred to in this thesis.

Poly(ADP-ribose) polymerase-1 (PARP-1) [EC: 2.4.2.30] is a nuclear protein which is considered to have functions involved in numerous cellular processes including, differentiation, gene expression, recombination, transformation, chromatin structure formation and DNA repair (de Murcia *et al.*, 1991). PARP-1 is found in all mammalian cells and some other eukaryotes, but is absent in prokaryotes and several terminally differentiated cells, such as mature granulocytes, epidermal cells, intestinal epithelial cells and mature erythrocytes (Chatterjee and Berger, 1998). PARP-1 is ubiquitous, with approximately 1 enzyme per 10-20 nucleosomes per cell (Cleaver and Morgan, 1991) and it is estimated that there are approximately 1×10^6 molecules per cell in total (Chatterjee and Berger, 1998).

The catalytic activity of the enzyme has been shown to be dependent upon activation by DNA strand breaks (Benjamin and Gill, 1980). PARP-1 is continually present at high basal levels within the cell and therefore transcription of the enzyme remains unchanged in response to DNA damage (Bhatia *et al.*, 1990). However, the presence of DNA strand breaks, increases the activity of the enzyme by up to 500-fold (Menissier-de Murcia *et al.*, 1989). The enzyme catalyses the formation of ADP-ribose polymers from NAD⁺, by cleaving NAD⁺ at the N-glycosidic bond between the nicotinamide and ribose rings thus releasing nicotinamide as a by-product (Ueda and Hayaishi, 1985). Briefly, poly (ADP-ribose) (PAR) synthesis can be separated into 3 individual steps of initiation, elongation (the addition of successive ADP-ribose residues by ribose-ribose O-glycosidic linkages) and branching (characterised by ribose-ribose bonding between ADP-ribose units) (Alvarez-Gonzalez and Mendoza-Alvarez, 1995).

Poly(ADP-ribose) polymers can be over 200 residues long, at which point they form a complex structure that resembles nucleic acids. Accordingly, PAR has often been referred to as the 'third nucleic acid' within the literature. Once formed, the polymers are rapidly degraded by the enzyme poly(ADP-ribose) glycohydrolase (PARG), giving them a short half life of under one minute.

PARP-1 synthesises polymer and transfers it to acceptor proteins by covalent modification of glycine residues. Acceptors of PAR are mostly DNA-binding proteins which include nuclear proteins (heteromodification) such as histones, DNA and RNA polymerases, topoisomerases, transcription factors (D'amours *et al.*, 1999) and PARP-1 itself (automodification). Indeed, PARP-1 is the main acceptor in vivo and since both the polymer and DNA are strongly negatively charged, automodification of PARP-1 causes dissociation of the enzyme from DNA strands due to electrostatic repulsion (Ueda and Hayaishi, 1985). ADP-ribosylation results in altered functions of proteins (Althaus and Richter, 1987) although the precise role(s) of this post-translational modification remains unknown.

1.6.2 Structure

The PARP-1 gene has been mapped to chromosome 1q41-42 in humans (Cherney *et al.*, 1987). It is 43 Kb in length and contains 23 exons. PARP-1 is a protein of 1014 amino acids with a molecular weight of 113 kDa (Kurosaki *et al.*, 1987) (Table 1.1). Briefly, PARP-1 consists of three functional domains; an amino terminal DNA binding domain (DBD) containing two zinc finger motifs (Mazen *et al.*, 1989) and a nuclear location signal (NLS) which is required for transport of the enzyme to the nucleus (Schreiber *et al.*, 1992), a central automodification domain and a highly conserved catalytic domain at the carboxyl terminal Kameshita *et al.*, 1984; Lindahl *et al.*, 1995).

The elucidation of the PARP-1 structure into three functional domains was achieved by Kameshita *et al.*, 1984, using limited proteolysis with α -chymotrypsin and papain. Briefly, proteolysis with α -chymotrypsin cleaved the enzyme into two fragments of 54 kDa and 66 kDa, respectively. The latter fragment was then further cleaved using papain into two fragments of 46 kDa and 22 kDa thus indicating that PARP-1 contains three separable domains.

It was demonstrated by Zahradka *et al.*, 1984, that PARP-1 was inhibited by various chelating agents and therefore it was suggested that PARP-1 is a metalloenzyme. Following atomic absorption spectroscopy, the enzyme was shown to contain zinc and it was proposed that the presence of this metal ion might be involved in the interaction of PARP-1 with DNA since a variety of other DNA-dependent reactions require zinc. In addition, this provided an explanation for the requirement of DNA for PARP-1 activation. Mazen *et al.*, 1989, confirmed that each molecule of PARP-1 contains Zn(II) ions and demonstrated that PARP-1 binds DNA nicks in a zinc-dependent manner. Furthermore, the authors showed that the two metal binding sites correspond to two 'zinc fingers'. The two zinc fingers (FI and FII) are differentially required for the recognition of strand breaks (Ikejima, *et al.*, 1990). The second zinc finger (FII) detects predominantly single strand breaks (Gradwohl *et al.*, 1990). The first zinc finger (FI), on the other hand, recognises both single and double strand breaks. In addition, FI possibly contributes to increased enzyme activity following nick binding by FII since enzyme activity is abolished in

FI mutants (de Murcia *et al.*, 1994a). Thus both fingers are required for activation by single stranded nicks but only the first finger is required for activation by DSBs. Following the elucidation of the function of the zinc finger motifs, PARP-1 is sometimes referred to as a molecular nick sensor (de Murcia and Menissier de Murcia, 1994b).

The DNA binding domain

The N-terminal region of human PARP-1 is 42.5 kDa and contains a repeated amino acid sequence in which 35 amino acids are highly conserved (Chatterjee and Berger, 1998). The zinc finger motifs are contained within these sequences. In addition, this domain contains a caspase cleavage site.

The automodification domain

The automodification domain is 16 kDa and is located between amino acid residues 374 and 525. Since PARP-1 is the major acceptor of PAR, this domain contains 15 highly conserved amino acid residues thought to be the major autoribosylation sites (Uchida *et al.*, 1993). This domain also contains a BRCA1 C-terminal domain (BRCT) motif that is found in a number of proteins involved in the response to DNA damage. This motif mediates interactions between several DNA repair and cell cycle checkpoint proteins. In PARP-1, this motif is important for interactions with XRCC1, PARP-2, histones, DNA polymerase β and transcription factors (Bouchard *et al.*, 2003). In addition, this domain is involved in PARP-1 dimerization and other protein-protein interactions (Burkle *et al.*, 2002).

The catalytic domain

This domain is the NAD⁺ binding domain and is the most conserved region of the protein. It is located at the C-terminus and is 55.4 kDa, consisting of residues 526-1014. Residues 859-908 are designated the 'PARP signature' since this region contains a region of 50 amino acids that are conserved 100 % amongst invertebrates and 92 % across all species (de Murcia *et al.*, 1994a).

1.6.3 Activation by strand breaks

Benjamin and Gill, amongst others, were able to show that PARP-1 has an absolute requirement for double stranded DNA and that activation occurs as a result of nick binding (Benjamin and Gill, 1980). This work was supported by the findings of Kupper *et al.*, 1990, who showed that over expression of the DNA binding domain markedly reduced the activity of the enzyme in a dominant negative fashion, by competing with holoPARP-1 for binding at DNA nicks.

A study by de Murcia *et al.*, in 1994 investigated what PARP-1 recognises in nicked DNA that ultimately leads to its activation. A study which employed a 39 bp DNA duplex containing a single stranded break in its centre, revealed that upon the introduction of a break to only one strand of the DNA duplex, flexibility was conferred at the site of the breaks, which under electron microscopy, appeared as a distinctive 'V' conformation (de Murcia *et al.*, 1994b). The authors observed that PARP-1 specifically bound to the centre of the 'V' and from this it was inferred that this may be a specific structure required to stimulate PARP-1 activity. In addition, this research group found that PARP-1 protected symmetrically +/- 7 nucleotides either side of the nick and this is referred to as the PARP-1 'footprint'. Caution should be taken when considering this data, however, as isolated DNA in the absence of associated histones was used and this may be different to chromosomal DNA.

PARP-1 exhibits a preference for various types of strand interruptions. In an elegant study, Benjamin and Gill, 1980, showed that closed circular DNA was ineffective in activating PARP-1, whilst greatest stimulation could be seen with double strand breaks with blunt ends followed by those with 3' overhangs, 5' overhangs and lastly by single strand breaks (Benjamin and Gill, 1980; Hengartner *et al.*, 1991). Contradictory evidence has recently been given by D'Silva *et al.*, 1999, who found that whilst PARP-1 had greatest affinity for blunt ends, they were the weakest activators of PARP-1. The authors propose the possibility that the use of purified PARP-1 and an absence of magnesium in the assays performed by Benjamin and Gill in 1980 may explain the different results.

1.6.4 Functions for PARP-1 in DNA repair

The high basal levels of PARP-1 suggest PARP-1 is involved in the immediate, emergency response to DNA damage. Furthermore, the rapid yet transient synthesis of ADP-ribose polymers in response to DNA damage, which imposes a high energy cost to the cell due to the resulting consumption of NAD^+ and ATP, coupled with the fact that PARP-1 is well conserved across species suggests that PARP-1 may play a fundamental role in the DNA damage response network.

Durkacz *et al.*, 1980, presented the initial evidence to indicate the involvement of PARP-1 in the cellular recovery from DNA damage. This group demonstrated that inhibitors of PARP-1, such as 3-aminobenzamide (3-AB), were able to potentiate the cytotoxicity of alkylating agents and retard DNA strand break rejoining. Furthermore, intracellular levels of the PARP substrate, NAD^+ have been shown to be acutely lowered by agents that generate DNA damage (Roitt, 1956; Satoh *et al.*, 1993). Several functions for PARP-1 in DNA repair, and in maintaining the integrity of the genome have been proposed and these are described below.

PARP-1 function may be mediated by the synthesis of the poly (ADP-ribose) polymers that confer a negative charge on modified proteins although the precise physiological role of polymerisation in response to DNA damage is unclear. The attachment of long negatively charged polymers causes electrostatic repulsion of the enzyme from the negatively charged DNA. In addition, covalent poly (ADP-ribosylation) of DNA binding enzymes will likely alter their physiological and biochemical properties by reducing their affinity for DNA and presumably increasing their affinity for positively charged cellular macromolecules. A proposed mechanism of action for PARP-1 is shown in Figure 1.4.

1.6.4.1 Histone shuttling model

The formation of poly(ADP-ribose) polymer at the sites of DNA damage may serve to loosen chromatin by temporarily extracting the histones in a process termed histone shuttling (Realini and Althaus, 1992; Althaus *et al.*, 1993; Althaus *et al.*, 1994). In this model, positively charged histones associate with negatively charged ADP-ribose polymers, either by direct covalent modification of H1 by PARP-1 or by

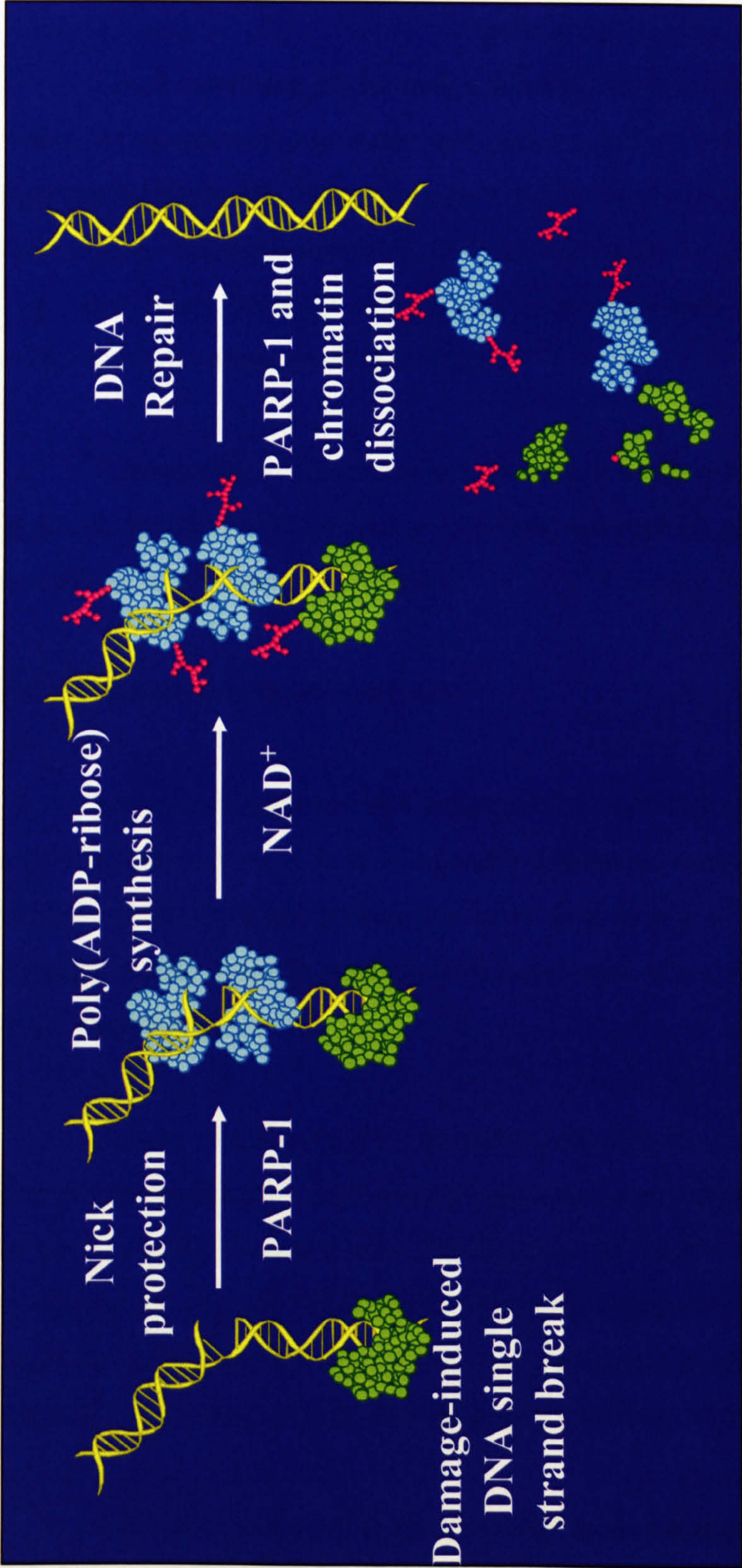


Figure 1.4 Mechanism of Action of PARP-1

Two PARP-1 molecules bind adjacent to the DNA nick (blue), and catalyse the synthesis of negatively charged polymers of ADP-ribose (pink). This creates an area of negative charge in the vicinity of the break which may not only cause the electrostatic repulsion of PARP-1 and nuclear proteins (including histones, green), from the negatively charged DNA, thus increasing the accessibility of DNA of repair enzymes but could also signal to repair enzymes and other critical cellular proteins.

attraction to the automodified PARP-1. This, in turn, causes repulsion from negatively charged DNA thus facilitating the displacement of histones from DNA and hence relaxation of chromatin.

Local disruption of chromatin structure may be critical for repair in areas where chromatin is highly condensed, and secondly, by altering steric congestion imposed by chromatin, this gives rise to a more open chromatin structure rendering the damaged site more accessible to repair enzymes. Indeed, many repair factors are large complexes, DNA-PK being a good example, whose functions may be otherwise hindered by the presence of chromatin.

Reassembly of chromatin structure occurs following degradation of the polymers by poly(ADP-ribose) glycohydrolase (PARG) and the subsequent reassociation of histones with DNA.

1.6.4.2 Antirecombinational factor

Satoh and Lindahl, 1992, proposed that the binding of PARP-1 to DNA strand breaks may serve to prevent inappropriate recombination events which could lead to chromosome aberrations and spurious initiation of transcription at sites of damage. PARP-1 could serve this function in two ways that are not necessarily mutually exclusive:-

Firstly, PARP-1 might bind and prevent the access of exonucleases that could generate recombination intermediates until repair enzymes are recruited to the sites of DNA damage. Alternatively, the formation of a dense concentration of negative charges at the site of a break could impede the approach of any neighbouring duplexes of DNA which might otherwise participate in spurious recombination reactions, and repel other molecules from the vicinity of the breaks.

An involvement of PARP-1 in DNA recombination is supported by the observation that PARP-1 *-/-* cells have elevated sister chromatid exchanges (Schreiber *et al.*, 1995) and that the PARP-1 inhibitor 3-aminobenzamide (3-AB) is a potent inducer of sister chromatid exchanges (Morgan *et al.*, 1986). Moreover,

Morrison *et al.*, 1997, demonstrated that whilst severe combined immunodeficiency (SCID) mice deficient in DNA-PK activity were unable to perform V(D)J recombination, SCID-PARP-1 knock out mice were able to. Thus the absence of PARP-1 appears to rescue the V(D)J recombination defect seen in SCID mice and these data are therefore consistent with the proposal that PARP-1 serves to protect DNA ends from illegitimate recombination reactions. In addition, PARP-1 has also been found to be a component of a multiprotein complex which includes numatrin, B23 and nucleolin. This complex has been shown to be capable of some DNA recombination activities (Borggreffe *et al.*, 1998).

1.6.4.3 Signalling model

Several authors have suggested the possible contribution of PARP-1 to DNA damage signalling. The activation of PARP-1 and synthesis of PAR are immediate responses to DNA damage and therefore PARP-1 may play a role in nick detection and stimulate repair by signalling the presence of these breaks through PAR formation to downstream effectors involved in the repair of DNA lesions (Lindahl *et al.*, 1995). Indeed, recent evidence suggests that PARP-1 modulates p53 function (Malanga *et al.*, 1998) and stimulates DNA-PK activity (Ruscetti *et al.*, 1998). In addition, evidence for PARP-1 and polymer association with both of these proteins has been demonstrated. As PARP-1 is one of the first factors to recognise and bind to lesions in DNA, it is ideally positioned to directly recruit repair machinery, such as DNA-PK, for example. One such role for PARP-1 is its proposed role in BER. PARP-1 has been suggested to recruit factors involved in BER pathway whose activities are regulated by poly(ADP-ribosylation).

1.6.4.4 The role of PARP-1 in base excision repair

Studies using DNA damaged by different DNA damaging agents have pinpointed a role for PARP-1 in base excision repair (BER) but not in nucleotide excision repair (NER). For example, the most potent activators of PARP-1 are those that induce DNA damage repaired by the BER pathway, including the monofunctional alkylating agents and IR. Other agents such as UV irradiation, which predominantly cause lesions that are repaired by NER, are not as potent at activating PARP-1 (de Murcia *et al.*, 1994a) and any stimulation of PARP-1 activity by UV irradiation was shown by Satoh and Lindahl, 1992, to be due to the repair of minor pyridine hydrate lesions which are processed by BER. Furthermore, the repair of UV damage is not inhibited by PARP-1 inhibitors (Cleaver *et al.*, 1983) and this is reviewed in section 1.7.

The involvement of PARP-1 in BER was disputed until a direct link between PARP-1 and other proteins involved in this pathway was discovered. It has been suggested that PARP-1 may be a member of a multiprotein complex consisting of PARP-1, XRCC1, DNA ligase III and DNA polymerase β (Menissier de Murcia *et al.*, 1997) and therefore PARP-1 may be involved in recruiting components of the BER complex to the damaged site. An association of PARP-1 with DNA polymerase β has been described by Dantzer *et al.*, 2000. XRCC1 (X-ray repair cross complementing 1), a protein that acts as a molecular scaffold, interacts with all components of the complex individually. Accordingly, an association of PARP-1 with XRCC1, has been demonstrated (Caldecott *et al.*, 1996; Masson *et al.*, 1998). Specifically, XRCC1 interacts with PARP-1 via its central region which contains a BRCT domain and is also associated with ligase III via a second BRCT domain. Similarly, PARP-1 strengthens its interaction with XRCC1 via its own BRCT domain located within the automodification domain of the protein or through its N-terminal zinc finger domain. Importantly, XRCC1 can be ADP-ribosylated *in vitro* and therefore PARP-1 may modulate its ability to interact with other components of the complex and by doing so, PARP-1 could regulate the function of the BER complex (D'amours *et al.*, 1999).

Overexpression of XRCC1 reduces PAR synthesis in response to hydrogen-peroxide generated DNA strand breaks. This suggests that XRCC1 negatively regulates PARP-1 activity following DNA damage. Furthermore, by preventing autoribosylation of PARP-1 and therefore dissociation of PARP-1 from the damaged site, this could be a mechanism by which XRCC1 indirectly participates in the protection of DNA ends produced during DNA damage and repair. Moreover, when in excess over PARP-1, ligase III can inhibit PARP-1 activity in vitro, presumably through competition for nick binding since ligase III has been demonstrated to exhibit nick binding activity (Caldecott *et al.*, 1996).

Alternatively, it has been suggested that PARP-1 may play an essential structural role in which it serves to stabilise the two broken ends thus enabling ligation to take place (Lindahl *et al.*, 1995).

1.7 Development of PARP-1 inhibitors

Much of the evidence for the function of PARP-1 has been derived from studies using inhibitors of PARP-1 (reviewed in sections 1.7.1 and 4.1.1.1). The majority of PARP-1 inhibitors act as competitive inhibitors by competing with the substrate NAD^+ for the nicotinamide binding domain of the enzyme. Early inhibitors included the nicotinamides (Fujimura *et al.*, 1967), methylxanthines, aromatic amides, benzamides (Shall, 1975; Purnell and Whish, 1980) and the pyrimidine derivative thymidine. A number of problems are associated with these compounds as inhibitors of PARP-1 activity. First, lack of specificity for PARP-1, a key criterion for in vivo activity, has been demonstrated by many of the early compounds (Griffin *et al.*, 1995a). Second, low potency and thirdly, poor solubility in aqueous solution have been demonstrated (Griffin *et al.*, 1995b). All problems present as major obstacles to the potential use of these agents to probe the mechanism of PARP-1 function let alone enable their use in a clinical situation. Moreover, many of the early inhibitors have the potential to influence a number of other cellular pathways.

Nicotinamide

Nicotinamide was the first compound to be identified as an inhibitor of PARP-1 (Fujimura *et al.*, 1967). Briefly, it was established by Fujimura *et al.* that a five-fold reduction in the incorporation of [^{32}P]-NAD $^{+}$ into acid insoluble material could be achieved with 10 mM nicotinamide. Unfortunately, nicotinamide has the potential to influence a number of cellular pathways since NAD $^{+}$ is utilised as a cofactor in a variety of metabolic reactions thus limiting any potential for clinical use. Indeed, nicotinamide is a substrate for phosphoribosyl transferase and nicotinamide N-methyl transferase deaminase (Banasik, 1992). In addition, it inhibits mono(ADP-ribosyl) transferase, microsomal NADase and adenosine 3'5'cyclic monophosphate phosphodiesterase (Shimoyama, 1994).

Benzamide

Benzamide (BZ) is an analogue of nicotinamide and its use as an inhibitor of PARP-1 was first demonstrated by Shall *et al.*, in 1975. Benzamide and its 3-substituted analogues were first developed by Purnell and Whish, 1980, and most studies have utilised benzamides to provide an insight into the function of PARP-1 in DNA repair. At millimolar concentrations, these inhibitors show increased specificity and potency compared to the nicotinamides since they do not act as substrates for other NAD $^{+}$ utilising enzymes. The use of benzamide as an inhibitor of PARP-1 is hindered by its poor aqueous solubility and although some of the 3-substituted analogues, for example, 3-aminobenzamide (3-AB), were shown to have enhanced solubility in water, through the introduction of polar substituents onto the ring of benzamide, studies by various research groups have suggested that these compounds exhibit effects on purine biosynthesis and de novo nucleotide biosynthesis (Cleaver, 1984; Hunting *et al.*, 1985).

With the aim of developing PARP-1 inhibitors with greater selectivity, structure activity relationships (SARs) have been elucidated and a variety of structural requirements for a PARP-1 inhibitor have been identified (Sestili *et al.*, 1990). These are illustrated in Figure 1.5.

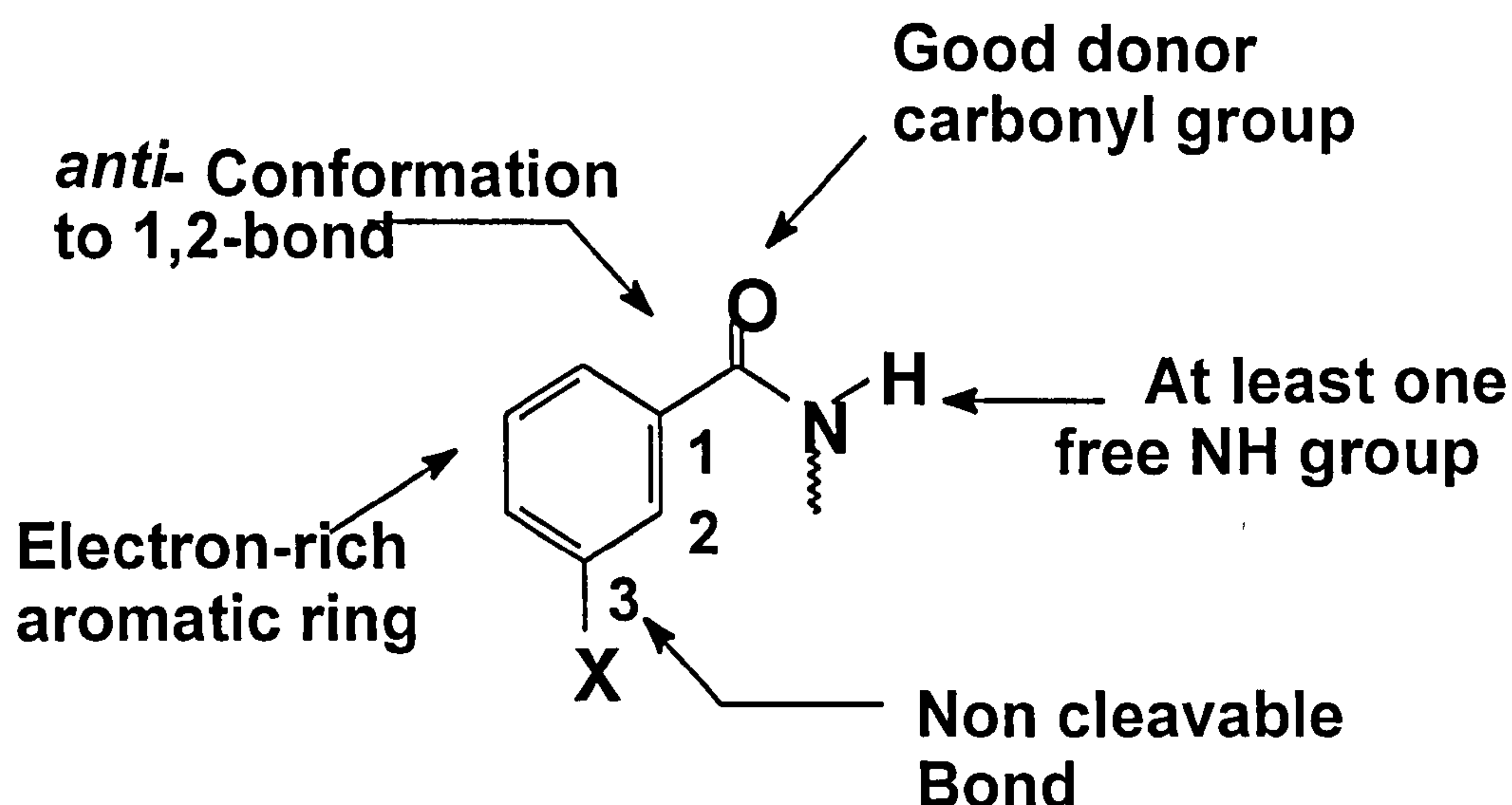


Figure 1.5 Structural requirements for a poly(ADP-ribose) polymerase-1 (PARP-1) inhibitor

A number of structural features are required for optimum PARP-1 inhibition;

Planarity and Aromaticity An unsubstituted aromatic or polyaromatic heterocyclic system is required as saturation of the ring system results in a loss of activity.

A carboxamide group A carboxamide group is required as part of a second ring structure or attached to the aromatic ring system. It has been shown by Cantoni *et al.*, 1987, that substitution of the C=O with C=S results in a reduction in activity. Furthermore, Sims *et al.*, 1982, showed that the replacement of the amide with a carboxylic acid or ketone renders the compound inactive. In addition, the *anti* conformation of the amide is more favourable than the *syn* conformation and this confers inhibitory activity. Indeed, derivatives with unrestricted carboxamides are less active (Suto *et al.*, 1991).

Amide proton At least one amide NH is required for putative hydrogen bonding to PARP-1.

A non-cleavable bond Introduction of a non-cleavable bond at the position corresponding to the 3-position of benzamide increases activity and makes the structure distinct from nicotinamide.

Following these observations, Suto *et al.*, 1991, developed a series of new potent inhibitors; the 5' substituted dihydroquinolinones, of which 3,4-dihydro-5-methoxyisoquinolin-1(2H)-one (PD128763) was the lead compound. These inhibitors showed improved potency and were as much as 50 –fold more potent than 3-AB (Suto *et al.*, 1991; Sebolt-Leobold and Scavone, 1992).

In addition, the University of Newcastle Drug Development Group have developed novel inhibitors of PARP-1 for use as chemo and radiopotentiators, including the quinazolinones, which include, 8-hydroxy-2-methyl-quinazolin-4-[3H]one (NU1025) and the benzamidazoles which include 2-(4-hydroxyphenyl)benzamidazole-4-carboxamide (NU1085) and 2-methylbenzamidazole-4-carboxamide (NU1064). These compounds have been used in a variety of cell culture systems, including human tumour cells, to exploit the potentiation of a range of anticancer agents as described in more detail in section 4.1.1.1. However, like earlier inhibitors of PARP-1, these compounds exhibit poor solubility in aqueous solution. More recently, the tricyclic indoles which have enhanced solubility and potency have been developed through a collaboration with Agouron Pharmaceuticals. These include the inhibitor used in the studies reported here, AG14361 (1-(4-dimethylaminoethylphenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one), which has a $K_i < 5\text{nM}$ and is described in detail in section 3.1.2. Small molecule inhibitors of PARP-1 are shown in Figure 1.6.

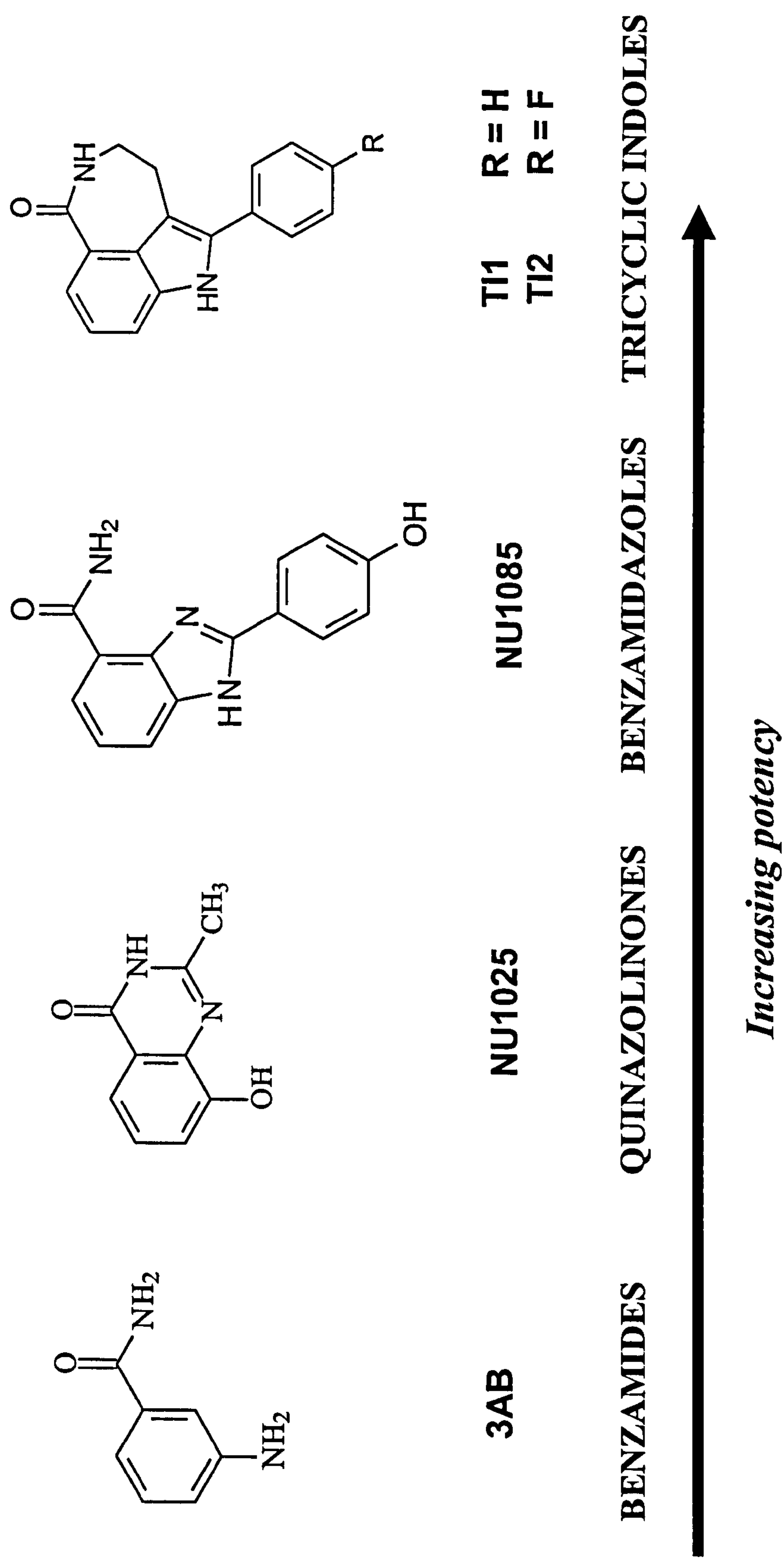


Figure 1.6 Small molecule inhibitors of PARP-1

1.7.1 Biological functions of PARP-1 as determined by inhibitors of PARP-1

Studies have shown that chemical inhibitors of PARP-1 can effectively abolish the DNA damage-induced depletion of NAD^+ and render cells hypersensitive to DNA damaging agents thus suggesting a role for PARP-1 in repair. There is now good evidence for the role of PARP-1 in BER (section 1.6.4.4). Many studies have implicated PARP-1 in the repair of IR and alkylating agent damage (reviewed by Shall *et al.*, 1984). Furthermore, as described in section 4.1.1.1, the most potent activators of PARP-1 are the monofunctional alkylating agents and IR whose damage is repaired by the BER pathway. Conversely, PARP-1 inhibitors do not potentiate the cytotoxicity of damaging agents that are repaired by NER, for example, 3-AB did not inhibit the repair of UV irradiation damage or potentiate the cytotoxic effects of UV in human or hamster cells (Ben-Hur, 1985; Cleaver, 1983).

Although these data indicate that PARP-1 plays a pivotal role in DNA repair, the limited specificity of the early PARP-1 inhibitors raises questions about the validity of these results. Nevertheless, the proposal that they act primarily through inhibition of PARP-1 is vindicated by genetic studies in PARP-1 $-/-$ mice and trans-dominant inhibition which have demonstrated similar heightened sensitivity to alkylating agents and IR (section 1.8). Moreover, the overexpression of the DNA binding domain, that mimics the mechanism of the inhibitors, results in a specific increase in the half life of DNA lesions that are usually repaired by the BER pathway.

1.8 Methods of PARP-1 depletion

1.8.1 In vitro systems

Elegant studies by Satoh and Lindahl, 1992, using a human cell-free system, showed that inhibited PARP-1 can have very different effects from PARP-1 deficiency. Briefly, these authors studied human cell-free extracts (containing no DNA) supplemented with plasmid DNA containing SSBs. The breaks were repaired in the presence of NAD^+ but not in the absence of NAD^+ or in the presence of 3AB, indicating that PARP-1 was a participatory factor in DNA repair. However, specific

depletion of PARP-1 from extracts by chromatography on DS DNA-cellulose resulted in extracts that repaired DNA just as efficiently as previously, but no longer exhibited NAD⁺ dependence.

The above results indicate that in the absence of its substrate NAD⁺, PARP-1 interferes with BER presumably because bound PARP-1 molecules block the access of repair enzymes. In this model, release of PARP-1 following automodification, or removal of PARP-1 from the reaction mixture, enables DNA repair by allowing the access of other repair enzymes. These experiments clearly suggest that inhibited PARP-1 could interfere with DNA repair by preventing automodification and subsequent removal of PARP-1 from DNA breaks, whereas in a situation with no PARP-1 such as in cells from knockout mice no such interference would be apparent.

1.8.2 Antisense strategies

Smulson and co-workers described the characterisation of cell transfectants with hormone-inducible expression of PARP-1 antisense RNA. Using the mouse mammary tumour virus (MMTV) promoter upstream of the full length, antisense-orientated human PARP-1 cDNA, an expression vector was constructed. The group then transfected HeLa cells with this PARP-1 construct and RNA expression was induced by dexamethosone treatment. Following 48-72 h, an 80 % depletion of PARP-1 activity was achieved with a corresponding 90 % reduction in PARP-1 protein (Stevnsner *et al.*, 1994; Ding *et al.*, 1992; Ding and Smulson, 1994). PARP-1 levels returned to normal 16 h after the removal of dexamethosone. Similarly, Gaken *et al.*, 1996, reported on the use of PARP-1 antisense DNA oligonucleotides which target the PARP-1 mRNA translation initiation site. The authors observed a 74 % decrease in PARP-1 expression after 72 h exposure, in medium, of intact L1210 cells.

In the studies described by Smulson and co-workers, cells depleted of PARP-1 lost their ability to efficiently rejoin DNA strand breaks at early time points following induction of DNA damage by treatment with the alkylating agent methyl-methanesulphonate (MMS). Furthermore, they were 10-fold more sensitive to MMS and were shown to have a slightly prolonged doubling time. Conversely, the cells

were not deficient in the repair of UV-induced pyrimidine dimers. In support of the findings with inhibitors, these data therefore suggest that decreased PARP-1 function leads to compromised repair and that PARP-1 participates in BER but not NER (Ding *et al.*, 1992).

1.8.3 Overexpression of the DNA binding domain

The PARP-1 DNA binding domain (DBD) binds to DNA ends and is unable to undergo automodification and hence dissociation from the DNA due to the absence of both the automodification and catalytic domains (Smulson *et al.*, 1998). This irreversible binding has previously been shown to reduce PARP-1 activity due to a competition for strand breaks with full length PARP-1 protein thereby preventing PARP-1 activation and polymer formation at the site of a break, in an analogous manner to PARP-1 inhibitors (Kupper *et al.*, 1990). Such transdominant inhibition of PARP-1 in Chinese hamster cells overexpressing the PARP-1 DBD under the control of the hormone inducible MMTV promoter resulted in a 90 % reduction in PAR formation following IR (Kupper *et al.*, 1990). These cells were also sensitised to the cytotoxic effects of IR and MNNG. These data are consistent with the findings of Schreiber *et al.*, (1995) who reported that HeLa cells, which constitutively overexpress the DBD, were sensitised to alkylating agents and IR but not UV, as would be expected for a protein involved in BER. In addition, these cells demonstrated higher frequencies of both spontaneous and MNNG induced SCE, consistent with a role for PARP-1 as an anti-recombination factor.

Overexpression of the DBD has also been shown to block unscheduled DNA synthesis (i.e. DNA repair) in human fibroblasts, induced by MNNG (that occurs during BER) but not that induced by UV irradiation (that occurs during NER), presumably by blocking the access of repair enzymes to the nicked DNA (Molinete *et al.*, 1993). This again supports a role for PARP-1 in BER.

1.8.4 Cell lines

Cell lines deficient in PARP-1 have been established by mutagenesis. Chatterjee and co-workers, produced a series of cell mutants deficient in PARP-1 (Chatterjee *et al.*, 1987). The treatment strategy used to obtain these mutants was based on their observation that high levels of DNA damage caused maximal stimulation of PARP-1 activity, resulting in depletion of cellular NAD⁺ and cell death as a consequence of ATP depletion (Berger, 1985). Chinese hamster V79 cells were exposed to low concentrations of MNNG to induce mutations, and surviving clones were then grown in nicotinamide-free medium to reduce their NAD⁺ levels. These cells were then treated with high concentrations of MNNG, an approach that generated cell mutants with 37-82 % of the PARP-1 activity of parental cells. Consistent with compromised repair, these mutants were found to have prolonged doubling times and an increased frequency of SCE exchange compared to parental cell lines. However, it should be noted that it is possible that the resulting phenotype could be due to other genetic abnormalities as a result of repeated drug treatment (secondary effect of the mutagen). Therefore, Stamato *et al.*, isolated a series of PARP-1 deficient mutants by exposing CHO cells to a low dose of ethyl-methanesulphonate (EMS) for 16 h, followed by a 72 h incubation in drug free medium to allow recovery and expression of the mutant phenotype. The treated cells were then plated out to form colonies, replica plated and directly assayed for PARP-1 activity. This study isolated two colonies, PADR-1 and PADR-2 which had 50 % less PARP-1 activity and were 20 % more sensitive to the DNA damaging agents EMS, DMS and MMS than control cells. However, the mutants had no increased sensitivity to IR.

1.8.5 Mouse deficient models for PARP-1

To ultimately elucidate the biological function of PARP-1, in vivo, a number of PARP-1 knockout mouse models have been generated independently using homologous recombination. The first PARP-1 knockout mouse was generated by Wang *et al.*, in 1995 by interruption of part of the 2nd exon and 2nd intron of the PARP-1 gene. A second PARP-1 knockout mouse was created by Menissier de Murcia *et al.*, in 1997, by interruption of Exon 4 in the PARP-1 gene (section 3.1.1)

and a third set of PARP-1 deficient mice, disrupted in Exon 1 were developed by the Sugimura group (Matsutani *et al.*, 1999 a;b).

PARP-1 KO mice are fertile and healthy. Initial studies by Wang *et al.*, 1995, suggested that their cells, derived from these mice, were not hypersensitive to alkylating agents or IR and therefore did not support the notion that PARP-1 contributed to DNA repair. Indeed, the only defects in these PARP-1 KO mice were those of epidermal hyperplasia and spontaneous skin lesions. Thus, initially, it appeared that poly(ADP-ribose) was not necessary for either BER or NER.

Studies with the other strains of PARP-1 KO mice, however, contradict some of these earlier results in that these mice are acutely sensitive to both N-methyl-N-nitrosourea (MNU) and IR. When MNU was injected, all PARP-1 deficient mice died within 4 weeks whilst less than half of the PARP-1 proficient mice died in this time. Similarly, when exposed to 8 Gy of whole body irradiation, all PARP-1 *-/-* mice died within 9 days. In contrast, none of the wild type mice had died within this time although by 15 days half had died (Menissier de Murcia *et al.*, 1997).

Cells derived from these PARP-1 deficient mice have also been demonstrated to be acutely sensitive to methylmethanesulfonate (MMS) and exhibited delayed DNA repair as measured by the comet assay, indicating a defect in the BER pathway (Trucco *et al.*, 1998). These authors demonstrated that following exposure to MMS, PARP-1 *-/-* MEFs displayed a severely decreased growth rate, had reduced viability and showed G2/M accumulation and an increased frequency of micronuclei an indication of chromosomal damage, compared to wild-type MEFs. Consistent with the findings of Menissier de murcia *et al.*, 1997, Matsutani *et al.*, demonstrated that cells derived from their mice were hypersensitive to IR and the alkylating agent MMS (Matsutani *et al.*, 1999 a;b).

The initial results presented by Wang *et al.*, in 1995, were based on the capacity of cells to repair and accurately transcribe alkylated plasmids. These plasmids could be repaired by an alternative pathway involving methyltransferases and therefore the finding that the PARP-1 *-/-* cells were able to carry out normal repair is unreliable. More recently, subsequent studies by Wang *et al.*, 1997, reported

that their PARP-1 $-/-$ mice are very sensitive to alkylating agents (MNNG, for example) and IR thus supporting the findings of de Murcia *et al.*, (1997). Moreover, in a parallel experiment, the authors demonstrated no difference in the repair of UV damage between the PARP-1 deficient cells and proficient cells, supportive of a role for PARP-1 in BER and not NER. These findings are also consistent with data from the chemical inhibitors, where, in particular, repair following UV was unaffected. In support of the findings of de Murcia *et al.*, the same group have also demonstrated, that when exposed to 8 Gy whole body irradiation, PARP-1 $-/-$ mice were hypersensitive and died within 10 days. In contrast, all of the PARP-1 $+/+$ mice survived.

In summary, all three independently derived PARP-1 knockout animals show hypersensitivity to both alkylating agents and IR, consistent with earlier observations with inhibitors, antisense strategies and trans dominant inhibition and this is indicative of a role for PARP-1 in BER.

Conclusive evidence for a role of PARP in BER has been given by Dantzer *et al.*, 1999, who demonstrated that following treatment with MMS, mouse embryonic fibroblasts (MEFs) derived from PARP-1 $-/-$ mice have a reduced capacity to repair a single abasic site present on a circular duplex molecule. In mammalian cells, two distinct subpathways of BER that differ in the type of DNA polymerase required, have been reported (see section 1.5.1). Briefly, the short patch pathway involves DNA polymerase β , XRCC1 and DNA ligase III, while the long patch pathway requires DNA polymerase ϵ or δ , PCNA, Dnase IV (FEN1) and DNA ligase I. These authors demonstrated that short patch repair is half as efficient in PARP-1 $-/-$ cells whilst long patch repair is almost completely abolished. In both pathways, the defect is in the polymerisation step. The major role for PARP-1 therefore appears to be in long patch repair. This is a surprising finding since an intimate association has been found between PARP-1 and XRCC1, ligase III and polymerase β , all of which are required for short patch repair. Both PARP-1 and FEN-1 bind to the 'hinge' of flaps of repair intermediates and therefore PARP-1 may be necessary to recruit FEN-1.

A second phenotype that has been observed in all PARP-1 knockout animals is an increase in sister chromatid exchange (SCE). Indeed, de Murcia *et al.*, observed a 5-fold increase in SCEs both before and after DNA damage (Menissier de Murcia *et al.*, 1997). Moreover, where measured, cells with reduced PARP-1 activity had an increased frequency of SCE. The ability of PARP-1 to rapidly bind DNA strand breaks may influence events such as homologous and non-homologous recombination. In support of this, evidence has shown that PARP-1 inhibition also results in an increased frequency of SCE (Morgan *et al.*, 1986). These findings support a role for PARP-1 as an antirecombinational factor.

All studies have shown that aberrant or reduced PARP-1 activity increased the sensitivity of cells to DNA damaging agents, probably due to an inhibition of DNA repair.

1.9 PARP Family

Until recently, PARP activity and ADP-ribose polymer formation, was assumed to result from the function of a single enzyme. In 1998, Shieh *et al.*, observed that PARP-1 deficient cells had some residual PARP activity. In addition, this group showed that the ADP-ribose polymers formed in these cells were indistinguishable from those formed in PARP-1 +/+ cells, as measured, firstly by binding to boronate resin which has selective affinity for ADP-ribose polymers and, secondly, treatment with snake venom phosphodiesterase and alkaline phosphatase revealed the unique ribosyl-ribosyl linkages of ADP-ribose polymers. Finally, the polymers were digested by recombinant poly(ADP-ribose) glycohydrolase (PARG), a highly specific enzyme for ADP-ribose polymers. This study, together with a study by Smith *et al.*, 1998, which identified a further PARP enzyme, termed tankyrase, demonstrated the existence of at least two structurally different PARP enzymes and opened up the possibility of multiple forms of PARP in mammalian cells.

Since the initial observation by Shieh *et al.*, (1998), a number of groups have attempted to identify the enzyme(s) responsible for this activity. At the time of writing this thesis, the de Murcia group have identified a total of 18 novel PARP cDNAs by sequence homology to PARP-1 and 7 PARP proteins have been

characterised. These enzymes exhibit differences in domain structure, sub-cellular localisation, tissue distribution and ability to bind DNA and these are described in more detail below. In addition, they are summarised in Table 1.1.

1.9.1 PARP-2

Three recent publications have reported the isolation of partial (Berghammer *et al.*, 1999; Johansson *et al.*, 1999) and full length (Ame *et al.*, 1999) cDNAs from mouse and human encoding a protein, with considerable homology to the catalytic domain of PARP-1. This protein, termed PARP-2, is the closest homologue to PARP-1 and is a 62 kDa protein. It contains a nuclear localisation signal (NLS) and is activated by DNA strand breaks although its DNA binding domain is markedly different from PARP-1. Furthermore, the automodification domain is absent. Nevertheless, PARP-2 is capable of autoribosylation. This enzyme is probably responsible for the polymer formation in PARP-1 KO mice, first described by Shieh *et al.*, in 1998. The genomic sequence of the PARP-2 promoter indicates that the expression pattern of this gene is different from that of PARP-1, indicating independent regulation and complementary roles for the two proteins.

Schreiber *et al.*, 2002, developed mice deficient in PARP-2 by gene disruption. This group demonstrated that following treatment with the alkylating agent, N-nitroso-N-methyl-urea (MNNU), PARP-2 $-/-$ cells show slow DNA rejoining kinetics similar to those seen in PARP-1 $-/-$ cells. A role for this enzyme in BER has therefore been suggested. In support of this proposal, PARP-2 also interacts with the 3 major proteins involved in BER, XRCC1, polymerase β and ligase III. Furthermore, XRCC1 negatively regulates PARP-2 activity as well as PARP-1 and PARP-1 and PARP-2 heterodimerise. PARP-1 and PARP-2 may act together with XRCC1 in a DNA repair complex in the BER process (Schreiber *et al.*, 2002). In addition, PARP-1 and PARP-2, may compensate for the absence of each other.

1.9.2 PARP-3

PARP-3 is the smallest PARP characterised so far and is only 60 kDa (Johansson *et al.*, 1999). It lacks both the DNA binding domain and the automodification domain, resembling sPARP, an alternative form of PARP-1 that is not involved in DNA strand break signalling (Sallmann *et al.*, 2000). The function and activity of PARP-3 remain unknown to date although it may play a role in centrosomal duplication.

1.9.3 PARP-4 (V-PARP)

V-PARP is another protein that contains a domain homologous (28 %) to the catalytic domain of PARP-1 (Jean *et al.*, 1999). V-PARP is one of the three proteins present in vaults that are large ribonucleoprotein complexes located primarily in the cytoplasm and which, as yet, are of no known function although they have been suggested to have a role in cellular transport (Jean *et al.*, 1999). Moreover, up-regulation of vault proteins has been detected in multi-drug resistant cancer cell lines (Schroeijers *et al.*, 2000). V-PARP is a large PARP protein. It is 193 kDa in size and contains a BRCT domain and several putative NLS sequences. In addition, V-PARP contains a domain that is known to interact with the major vault protein (MVP). Accordingly, V-PARP has been shown to associate with and ADP-ribosylate MVP. This ADP-ribosylation may be involved in the regulation of vault conformation and consequently in the transport of molecules. However, not all V-PARP is associated with vaults and has been shown to localise to the mitotic spindle thus suggesting a role in maintaining genomic integrity. Interestingly, V-PARP does not require DNA for activity.

1.9.4 PARP-5 and 6 (Tankyrase 1 and 2)

Tankyrase-1 or PARP-5 is a 142 kDa protein that was initially identified through its interaction with human telomeres (Smith *et al.*, 1998). It is a homologue of PARP-1, however, it lacks both the automodification and DNA binding domain and like V-PARP, it does not require DNA for activity. PARP-5 binds to and ADP-ribosylates both itself and telomeric repeat binding factor-1 (TRF-1), a protein that is

associated with telomeres, both in vitro and in vivo (Griffith *et al.*, 1999). Tankyrase-1 therefore acts as a positive regulator of telomere length, since ADP-ribosylation of TRF-1 causes its release from telomeres thus allowing access to telomerase, a specialised reverse transcriptase that restores the telomeric sequences lost during cell division (Smith and de Lange, 2000).

Tankyrase-2 (PARP-6) is a 130 kDa protein that shares 85 % amino acid sequence homology with tankyrase-1, however, the function of this is unknown although evidence suggests that it has a different role from tankyrase-1. Overexpression of tankyrase-1 induces a progressive telomere elongation in human cells (Kaminker *et al.*, 2001) whilst, in contrast, overexpression of tankyrase-2 results in rapid cell death by necrosis (Smith and de Lange 2000).

Name	Location	Chromosome	No of a.a	MW (kDa)
PARP-1	Nucleus	1q41-1q42	1014	113
PARP-2	Nucleus	14q11.2	570	64
PARP-3	Centrosome	3p21	533	60
PARP-4 (V-PARP)	Vaults	13q11	1724	193
PARP-5 (Tankyrase-1)	Cytoplasm and Nucleus	8p22-p23	1327	142
PARP-6 (Tankyrase-2)	Golgi	10q23.2	1165	130

Table 1.1 PARP family members.
 Comparison of the cellular localisation, chromosome position and molecular weights of the known PARP family members.

1.10 DNA dependent protein kinase (DNA –PK)

1.10.1 General Introduction

The phenomenon of DNA- dependent protein phosphorylation was first described approximately 18 years ago in cell extracts (Walker *et al.*, 1985). Work carried out by Jackson *et al.*, in 1990 observed that Sp1 protein present in human HeLa cell nuclear extracts became phosphorylated when the extract was incubated under conditions suitable for in vitro transcription. Furthermore, during the characterisation of this phosphorylation, it was discovered that it required DNA to be present in the reaction and that phosphorylation was most effective if the DNA contained binding sites for Sp1. In support of the hypothesis that the kinase required DNA for activity, the authors observed that the addition of non-specific competitor DNA led to reduced Sp1 phosphorylation. The kinase responsible for this phosphorylation is termed DNA-dependent protein kinase (DNA-PK) and three groups simultaneously published on the existence of this enzyme (Jackson *et al.*, 1990; Carter *et al.*, 1990; Lees-Miller *et al.*, 1990).

DNA-PK is a serine threonine kinase (phosphorylates serine or threonine preceding a glutamine) which is found in the nucleus of a wide range of eukaryotic cells and which may have functions involved in regulating gene expression, replication, cell cycle progression and DNA repair (Lees-Miller and Anderson, 1991). The enzyme belongs to a recently discovered family of large, phosphatidylinositol-3 kinase (PI3-K)-like proteins that include ATM, FRAP and ATR and function in DNA repair and cell cycle regulation. The kinase domain of DNA-PK has been shown to share sequence homology with the PI 3-kinase family that includes the above members (Hartley *et al.*, 1995). A more detailed description of this family is given in section 1.13.

Like PARP-1, DNA-PK is not an inducible gene product, rather it is constitutively expressed in the cell at high basal levels during normal cellular metabolism (Muller *et al.*, 1998). It is estimated that a similar number of DNA-PK molecules are present per human nucleus as the enzyme PARP-1, ie. approximately $1 \times 10^5 - 1 \times 10^6$ molecules per nucleus (Lindahl *et al.*, 1995; Jackson, 1997). It has

also been suggested that the expression of DNA-PK may be cell type and tissue specific (Moll *et al.*, 1999).

In addition to PARP-1, DNA-PK is one of the few known cellular enzymes whose activity is regulated directly by DNA. Upon activation, DNA-PK phosphorylates various DNA-binding proteins and is most efficient at phosphorylating proteins bound to the same DNA molecule. In vitro substrates include histones, topoisomerases and various transcription factors (Anderson, 1993) although an in vivo target remains to be identified. DNA-PK activity is autoregulatory and in the absence of a substrate, autophosphorylation results in the dissociation of DNA-PK from DNA (Chan and Lees-Miller, 1996).

1.10.2 Structure

DNA dependent protein kinase is a trimeric nuclear complex that consists of a large protein kinase and a Ku heterodimer. Following cloning of the human gene for DNA-PK, the catalytic subunit was shown to be a nuclear protein of 465 kDa, designated DNAPKcs (Hartley *et al.*, 1995). The gene for the catalytic subunit has been mapped to chromosome 8q 11 and is estimated to contain around 100 exons and be around 180 kbp in length (Sipley *et al.*, 1995). The carboxyl terminal of DNA-PKcs (approx 500 residues) comprises the catalytic domain.

Chiu *et al.*, 1998, utilised cryoelectron microscopy imaging of DNA-PKcs to demonstrate that DNA-PKcs has an open symmetric structure with a gap separating a 'crown shaped' top from a rounded bottom. The hollow nature of the DNA-PKcs interior suggests that it may interact with DNA via internalisation of double stranded ends. More recently, Leuther *et al.*, 1999, demonstrated using electron crystallography that DNA-PKcs possess an open channel and enclosed cavity with 3 large openings large enough to accommodate single stranded ends. Protein-DNA cross linking studies have revealed that DNA-PKcs makes intimate contacts with the DNA, suggesting that DNA might directly induce conformational alterations in the protein itself (Gottlieb and Jackson, 1993).

Human Ku is composed of two tightly associated polypeptides of approximately 69 and 83 kDa, designated Ku70 and Ku80, respectively. Ku70 and 80 are biochemically distinct, and in yeast, they only share approximately 38 % homology over a region in the C terminal half of the protein that contains part of the DNA binding domain. The human Ku70 gene maps to chromosome 22q13 and the Ku80 gene maps to 2q33 (Cai *et al.*, 1994). The subunits of Ku protein are tightly associated and do not separate during protein purification. The carboxyl terminal region of approximately 150 amino acid residues in both Ku70 and Ku80 are essential for Ku dimerization. The Ku heterodimer interacts with DNA in a two step mechanism, where it first recognises DNA ends and then translocates to internal sites (Paillard and Strauss, 1991; Zhang and Yaneva, 1992). This allows binding of multiple Ku molecules to a single DNA fragment. Ku binds DNA via a preformed channel (Walker *et al.*, 2001). However, the precise residues that mediate the interactions between the Ku heterodimer and DNA remain to be identified although it appears that Ku70 makes the most intimate interactions (Dynan and Yoo, 1998). Moreover, neither subunit alone can bind DNA effectively.

The Ku heterodimer regulates DNA-PK activity (Gottlieb and Jackson, 1993) by targeting the catalytic subunit to DNA and stimulating activity through direct protein-protein interaction. More recently, it has been determined that it is the extreme C-terminus of Ku80, specifically the final 12 amino acids, that contains a protein-protein interaction motif that mediates a highly specific interaction with DNA-PKcs (Gell and Jackson, 1999). This is consistent with the absence of an analogous Ku80 C- terminal tail in *S. cerevisiae* which also lacks a homologous DNA-PKcs protein. Notably, Ku is unable to bind DNA-PKcs in the absence of DNA (Suwa *et al.*, 1994), suggesting DNA binding induces a conformational change that allows interaction with DNA-PKcs. In some conditions (Yaneva *et al.*, 1997; Hammersten and Chu, 1998) DNA-PKcs can bind double stranded DNA ends and be activated in the absence of Ku, however, optimal DNA-PK activity is only exhibited when Ku is present suggesting that Ku is required for stabilising the binding of DNA by DNA-PKcs *in vivo*.

The regulation of DNA-PK occurs not only through binding to DNA, but also by interaction with other proteins. One clear example is binding to the high affinity DNA binding protein, C1D, with which DNA-PKcs interacts through a putative leucine zipper motif in the absence of DNA (Yavuzer *et al.*, 1998). The physiological consequence of this activation is not clear but C1D is a component of the nuclear matrix and is activated by IR.

1.10.3 Activation by DNA strand breaks

Ku binds strongly to DNA ends, stem-loop and bubble structures, and to transitions between double stranded DNA and two single strands.

Like PARP-1, DNA-PK is unusual in that it is active only when bound to DNA (Gottlieb and Jackson, 1994). DNA-PK is activated by binding to DNA ends, nicks and gaps (Gottlieb and Jackson, 1993) and by linear double stranded DNA but not by closed, fully duplexed, circular DNA or by single stranded DNA, by DNA/RNA heteroduplexes or by RNA (Carter and Anderson, 1991). There is no preference for a particular type of strand break and therefore DNA-PK is activated by blunt, 5' overhanging or 3' overhanging DNA breaks with equal efficiency.

1.10.4 Models of DNA-PK in DNA DSB repair

The binding to and activation of DNA-PK by DNA DSBs suggested a role for this enzyme in DNA repair. Investigations into the DNA substrates utilised by DNA-PK, together with analyses of mutant cell lines (described in detail in section 1.12.2) have revealed the DNA-PK complex has a major role in NHEJ. The most advocated model is that Ku binds DNA DSBs specifically via a preformed channel and then recruits DNA-PKcs which is then activated. The central role of Ku protein in the activation of DNA-PK was first demonstrated in two independent studies which showed that Ku protein and DNA-PKcs can be separated biochemically, that activity can be restored by mixing Ku and DNA-PKcs fractions and that the Ku protein physically recruits DNA-PKcs to DNA (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993). This recruitment can be demonstrated by the formation of distinctive complexes on an electrophoretic mobility shift assay (EMSA).

Several roles for DNA-PK in repair have been proposed and these are described below. The assembly of the DNA-PK complex at the site of a DNA double strand break and possible functions in repair are shown in Figure 1.7.

1.10.4.1 Protection factor

Like PARP-1, DNA-PK may function in detecting the presence of a DSB and in protecting it from the action of harmful nucleases and other proteins which could interfere with the assembly and functioning of DSB repair apparatus at the site of damage. In support of the former, DNA-PK deficient cell lines have been shown to accumulate deletions in their DNA (Jackson, 1996). Therefore, like PARP-1, DNA-PK may serve to prevent aberrant recombination events that could interfere with repair.

DNA-PK could carry out this role simply by binding and blocking access to the damaged site until other repair factors can be recruited. Alternatively, DNA-PK may phosphorylate proteins that may affect or effect DNA repair. Such proteins might include transcription factors that have the potential to interfere with the repair apparatus by eliciting spurious transcription at the site of a break. To this end, of the many proteins which DNA-PK can phosphorylate in vitro, DNA-PK is known to phosphorylate the transcription factors Sp-1, c-jun and p53 (Anderson, 1993). Furthermore, DNA-PK is a potent inhibitor of transcription by RNA polymerase I which mediates transcription of large ribosomal RNA precursors (Kuhn *et al.*, 1995).

Finally, the kinase function may serve a regulatory role in which it ensures the disassembly of repair components from the site of damage following DNA repair. Indeed, autophosphorylation leads to the dissociation of DNA-PKcs from DNA-bound Ku and inactivation of kinase activity.

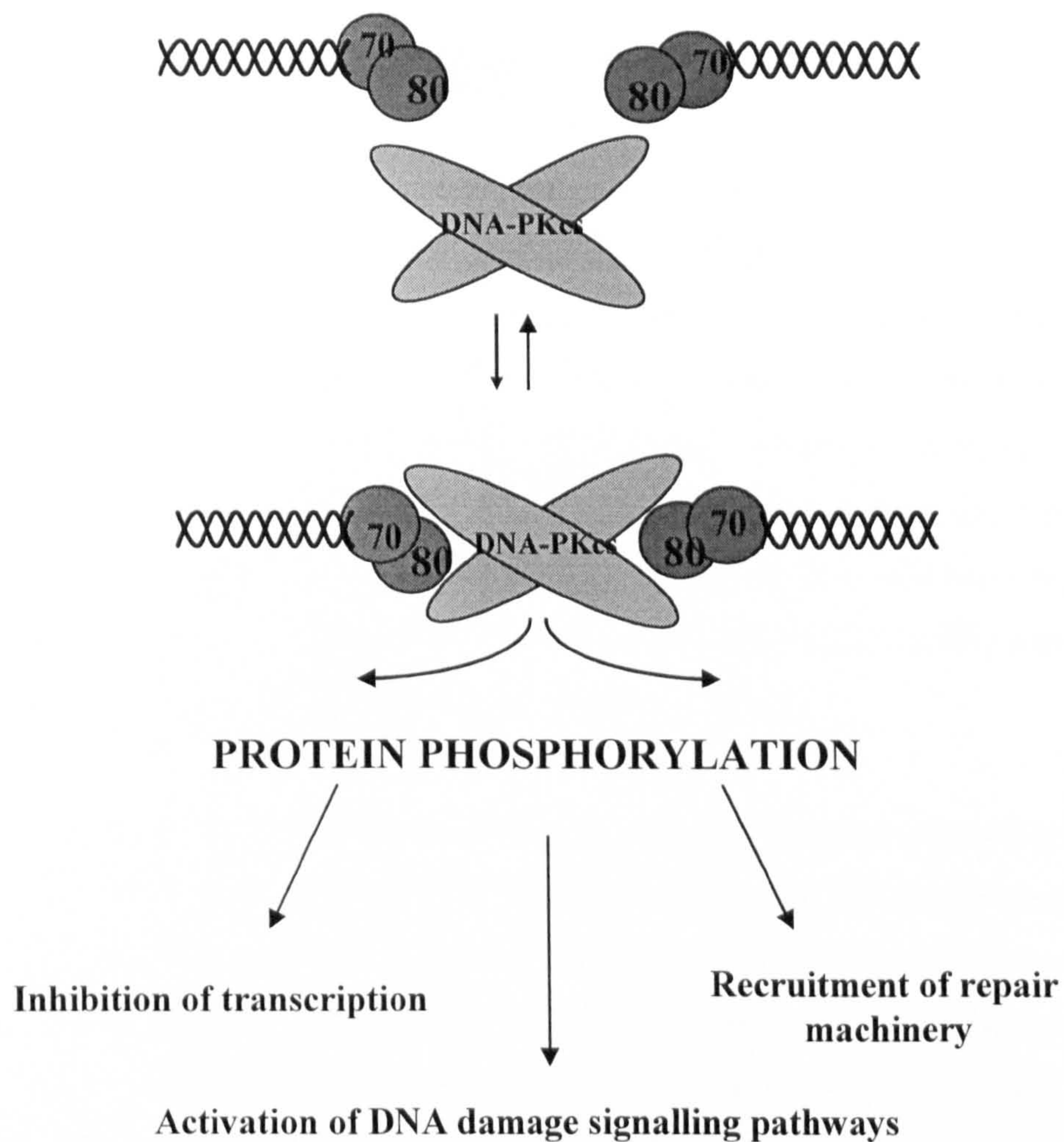


Figure 1.7 Organisation of the DNA-PK complex at the site of a break and possible functions in repair

The Ku heterodimer binds to DNA DSBs initially and this is followed by the binding of DNA-PKcs to the Ku-DNA complex. DNA-PK is then activated to phosphorylate a number of protein targets. DNA-PK may have a number of functions in DNA DSB repair and these include inhibition of transcription at sites of DNA damage and recruitment and / or activation of repair machinery. DNA-PK also undergoes automodification, leading to its dissociation from DNA

1.10.4.2 Recruitment and/or signalling model

DNA-PK may recruit and/or regulate essential components of the DNA repair apparatus through protein-protein interactions or phosphorylation. Thus, like PARP-1, DNA-PK may function in a not yet understood 'DNA damage surveillance network' in which DNA-PK serves as the primary damage sensor important for the essential process of DNA strand break recognition. Consistent with its proposed role as a primary DNA damage sensor and not as an inducible downstream effector of DNA damage signalling, DNA-PK, like PARP-1, is present at relatively high levels (up to 1 % of HeLa cell nuclear protein) that is not inducible but rather a pre-formed protein that is activated by DNA breaks (Lee *et al.*, 1997).

Alternatively, or in addition, DNA-PK might function in signalling the presence of DNA damage through the initiation of a signal transduction pathway by phosphorylation of relevant substrate(s) thus alerting the cell to the presence of DSBs. The interactions of DNA-PK with signalling proteins and its function in activating components of the repair system have yet to be fully elucidated. DNA-PK has been suggested to act as an upstream regulator of p53. Moreover, DNA-PK has recently been shown to play a role in triggering induction of the p53 pathway following exposure to IR (Woo *et al.*, 1998; Lees-Miller *et al.*, 1992). However, various studies have yielded conflicting results and these are discussed in more detail in section 1.11.2.

The final ligation step of NHEJ is mediated, at least in part, by ligase IV and its associated protein XRCC4. Studies in cells mutated for XRCC4 have shown that these proteins are essential for NHEJ (Lee *et al.*, 1997). This is similar to the requirement for PARP-1 in the BER pathway, in which the complex of XRCC1 and DNA ligase III is used (section 1.6.4.4). XRCC4 is a substrate for DNA-PK in vitro and the XRCC4/ligase IV complex can interact directly or indirectly with DNA-PK in crude nuclear extracts (Critchlow *et al.*, 1997; Leber *et al.*, 1998). Thus DNA-PK may signal to and recruit or activate this complex.

Another complex that might be recruited and/or activated by DNA-PK is that containing the Rad50, Mre11 NBS1 complex whose nuclease activities may be critical in 'tidying up' damaged DNA termini prior to ligation.

1.10.4.3 Structural role

More direct roles for DNA-PK in DNA repair have also been suggested. Since DNA-PK associates directly with DNA, this suggests a proximal role for the enzyme. DNA-PK could act as a structural framework to which enzymes bind (Hartley *et al.*, 1995). A function for DNA-PK as an alignment factor in which it serves to hold the two chromosome ends together in a manner that will facilitate ligation of the two broken ends has been proposed (Jeggo *et al.*, 1995). DNA-PK deficient cell lines have an increased level of chromosomal rearrangements following treatment with IR when compared to wild type cells (Disney *et al.*, 1992).

There is currently no consensus of opinion on whether the modulation of repair by DNA-PK is dependent on the catalytic activity of the enzyme, or, alternatively, on its physical presence (or both). Although the lack of clear functional substrates for DNA-PK during DSB rejoining has led to the proposition that DNA-PK acts primarily as a scaffold to hold the ends in alignment for repair to occur, an important study by Kurimasa *et al.*, 1999, has demonstrated that the kinase activity of DNA-PKcs is needed for NHEJ. This group complemented mutant DNA-Pkcs deficient cell lines with cDNA for DNA-PKcs and showed that it could restore the DSB repair defect of these cells. When the cells were complemented with cDNA incorporating deletions and site directed mutations important for kinase activity, the DSB repair defect of these cells was not complemented. Thus expression of protein was not sufficient for NHEJ but additionally required kinase activity.

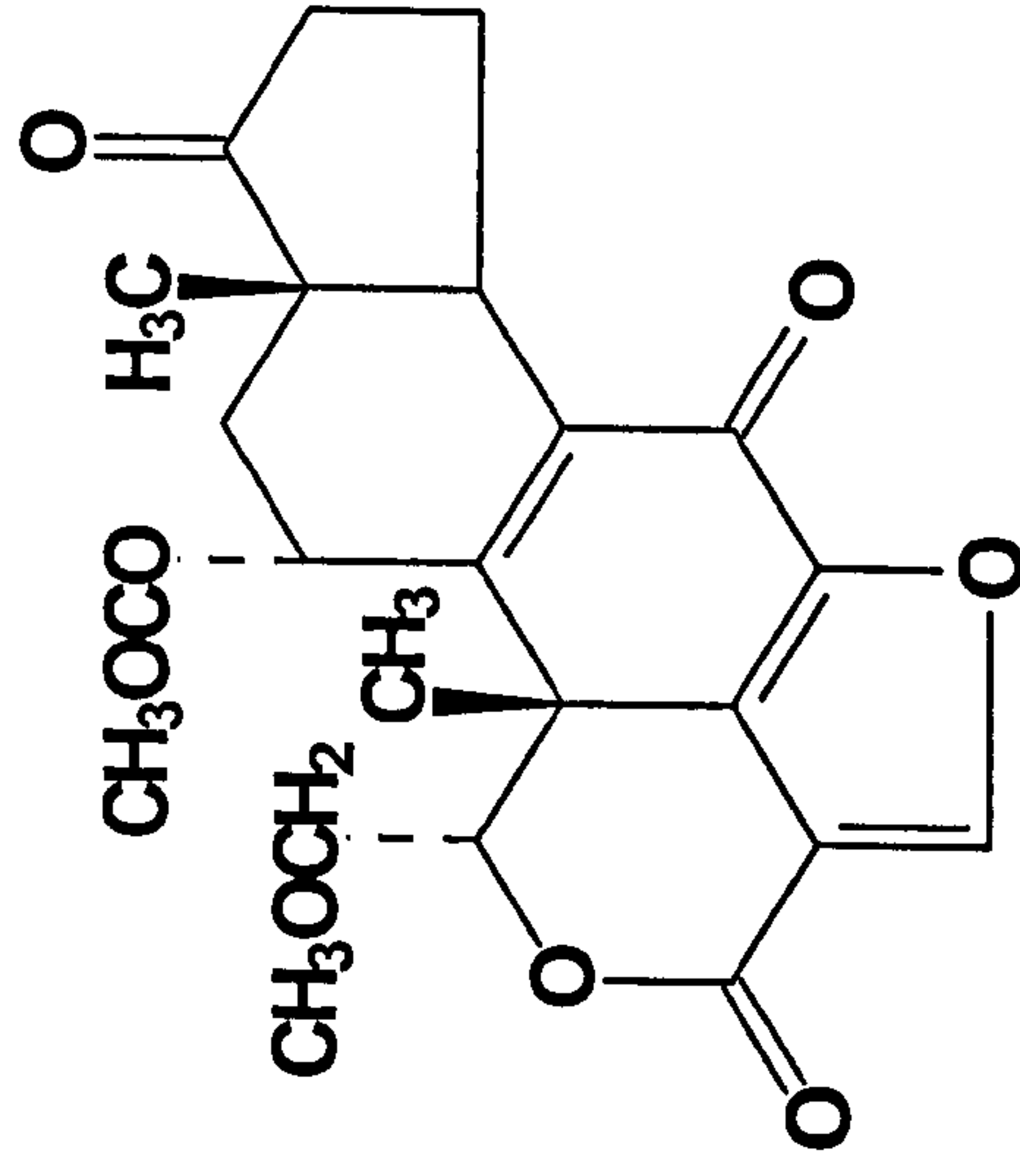
1.11 Development of DNA-PK inhibitors

To date, only inhibitors of the phosphorylation activity of DNA-PK have been explored. Wortmannin, a sterol-like fungal metabolite, shown previously to be a potent inhibitor of PI3-kinase was one of the first identified inhibitors demonstrated to be capable of inhibiting DNA-PK, *in vitro*, on the basis of similarity between the kinase domains of the two proteins. Wortmannin has been shown to bind directly to DNA-PKcs, *in vivo*, (Izzard *et al.*, 1999). Wortmannin acts as a non-competitive irreversible inhibitor with respect to ATP (Izzard *et al.*, 1999).

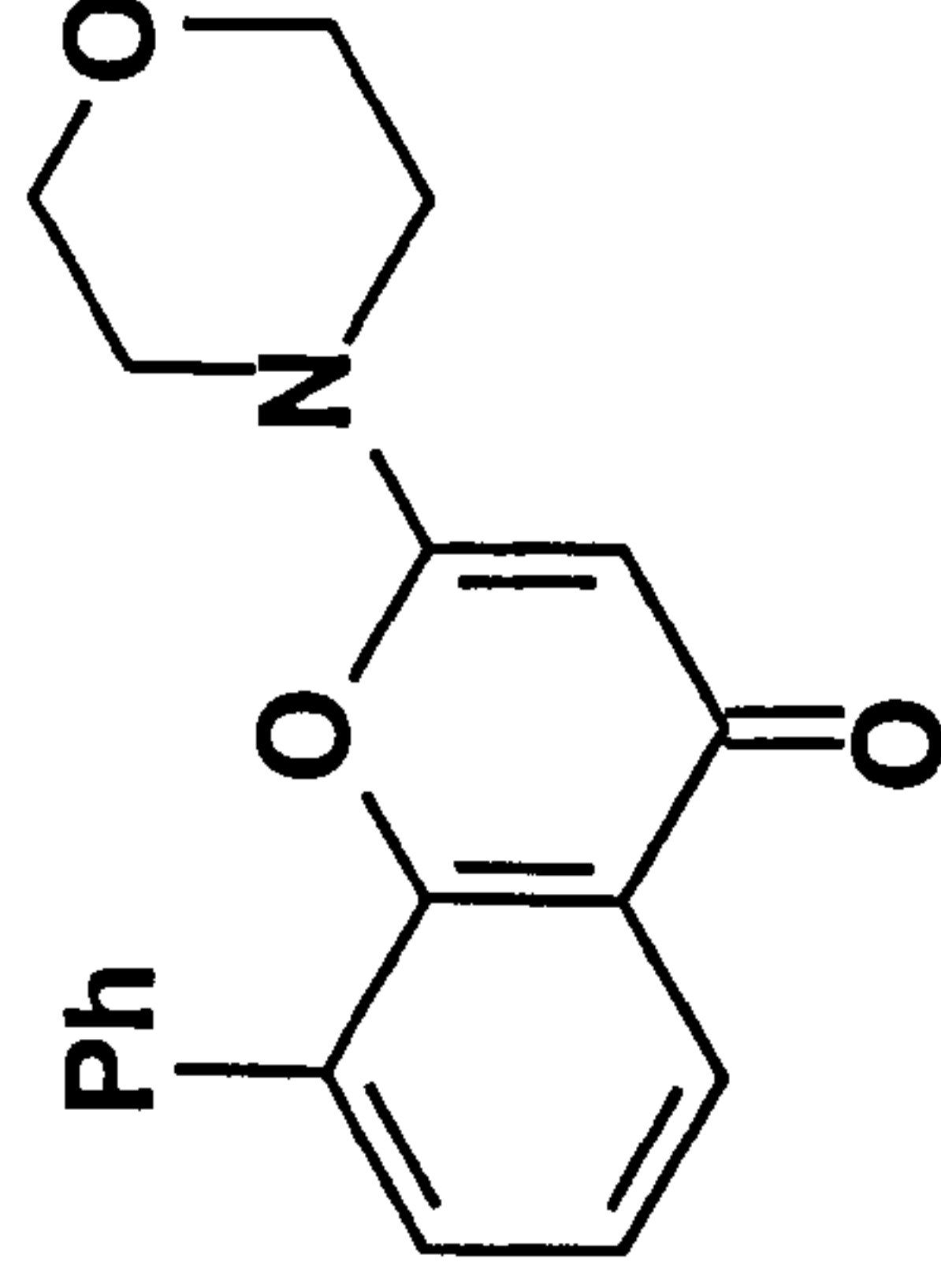
The action of wortmannin is not entirely specific for DNA-PK, however, and it has been demonstrated that cells deficient in DNA-PK may be sensitised by wortmannin (Rosenzweig *et al.*, 1997) and therefore this implies that other kinases involved in DSB repair may be inhibited by wortmannin. Indeed, wortmannin has been shown to inhibit the product of the AT gene, ATM (Hartley *et al.*, 1995; Sarkaria *et al.*, 1998). Furthermore, wortmannin is unstable in serum-containing media (Price and Yourmell, 1996) and therefore this is a major limiting factor that may affect the use of this compound in a clinical situation.

The PI3-kinase inhibitors, LY294002 (2-(4-morpholynyl)-8-phenyl-4H-1-benzopyran-4-one) and OK-1035 (3-cyano-5-(4-pyridyl)-6-hydrazonomethyl-2-pyridone) have been identified (Vlahos *et al.*, 1994; Take *et al.*, 1995), both of which are inhibitors of DNA-PK kinase activity. Both compounds are chemically unrelated to wortmannin and are reversible competitive inhibitors. LY294002 is a synthetic compound that was designed as a PI3-kinase inhibitor based on the flavonoid quercetin. Although the reported IC₅₀ is much higher than that of wortmannin, LY294002 is widely used in cell biology as a specific PI3-kinase inhibitor because it is much more stable in solution than wortmannin.

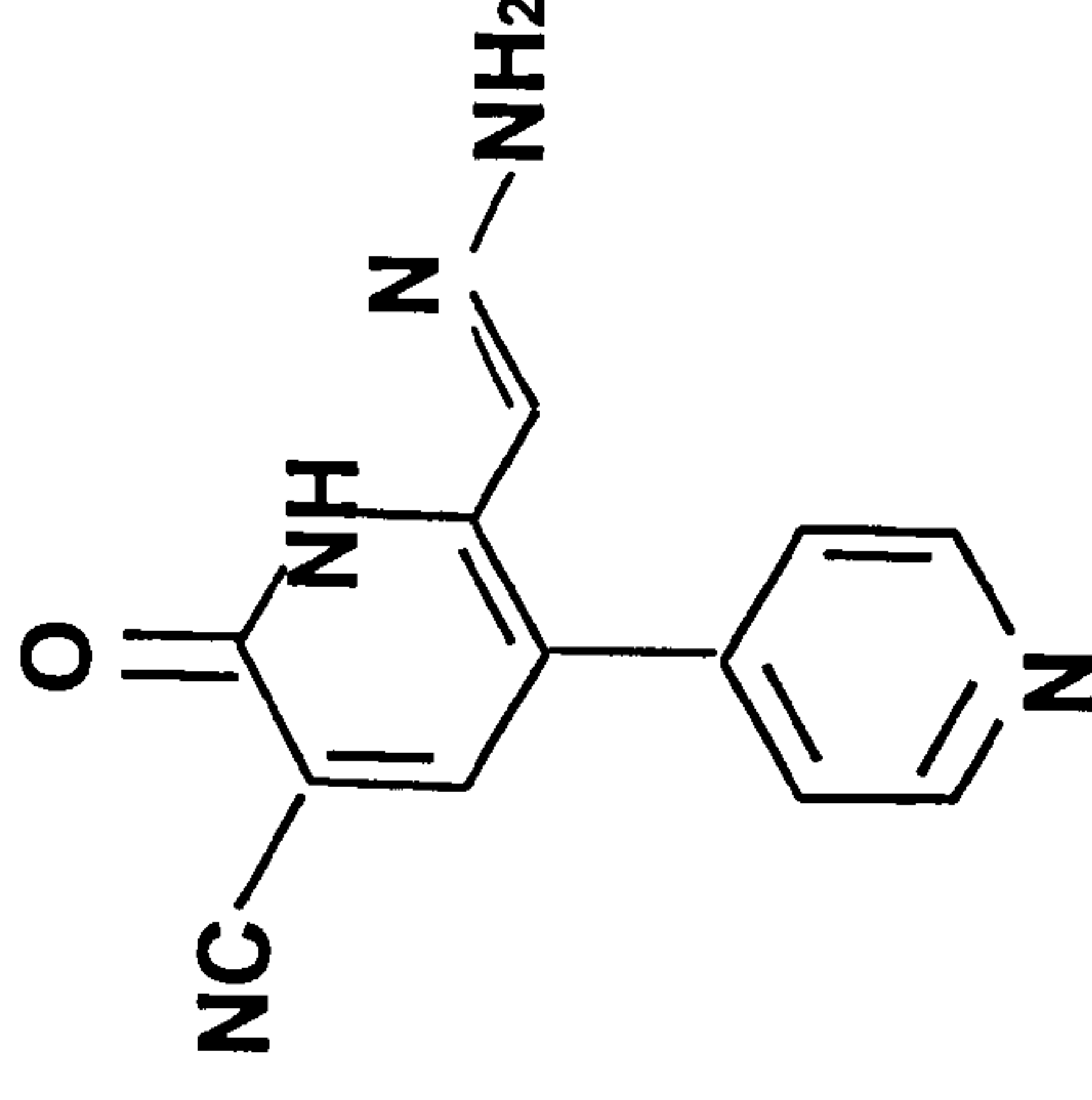
OK-1035, in particular, shows promise as a more selective inhibitor of DNA-PK since it has been shown to inhibit DNA-PK with greater selectivity than for PI-3 kinase. (Take *et al.*, 1995). However, this has been disputed by Stockley *et al.*, 2001, who have demonstrated an IC₅₀ that is approximately 12-fold higher than that reported previously. Small molecule inhibitors of DNA-PK are shown in Figure 1.8.



WORTMANNIN



LY294002



OK1035

Figure 1.8 Small molecule inhibitors of DNA-PK

Further inhibitor development is currently underway by the Drug Development Group at Newcastle University to identify potent and specific DNA-PK inhibitors. Despite the similarity of the ATP binding sites among protein kinases, it has been possible to exploit differences in the modes of ATP interaction in order to develop protein kinase-specific inhibitors. The inhibitor used in the studies described in this thesis, NU7026 (2-morpholin-4-yl)-benzo[h] chromen-4-one), is a potent, novel and specific inhibitor of DNA-PK and is described in more detail in section 3.1.2.

1.12 Methods of DNA-PK depletion

1.12.1 Molecular strategies

Marangoni *et al.*, 2000, utilised a dominant negative approach to deplete cells of DNA-PK activity. Briefly, this group created a construct containing a 32 kDa fragment encoding the C-terminal region of Ku80. Expression of this construct in CHOK1 cells, resulted in decreased Ku-dependent DNA binding activity, a diminished capacity to repair DNA DSBs (as measured by pulse field gel electrophoresis) and decreased survival following exposure to IR. In a similar study, Kasten *et al.*, 1999, demonstrated that overexpression of Ku70/Ku80 in rat cells resulted in a 20 % reduction in DNA-PK activity. In addition, the cells had a reduced capacity for DNA DSB repair and were hypersensitive to IR. More recently, Kim *et al.*, 2002, created a peptide of the C terminus of Ku80 which selectively targeted and disrupted the interaction between the Ku complex and DNA-PKcs. Cells transfected to express this peptide were less efficient in their capacity to repair DSBs. All of these studies are therefore consistent with a role for DNA-PK in DSB repair and survival.

More recently, a number of groups have utilised antisense strategies as a means to deplete cells of DNA-PK activity. Sak *et al.*, 2002, constructed antisense oligodeoxynucleotides (AS-ODNs) targeting various regions of DNA-PKcs mRNA. When expressed in non-small cell lung cancer (NSCLC) cell lines, the kinase activity of DNA-PKcs was specifically inhibited and this corresponded with a marked inhibition of DNA DSB rejoining and an increase in radiosensitivity. Blenkov *et al.*,

2002, generated AS-ODNs which down regulated Ku80 mRNA in the isogenic DNA-PK proficient MO59K and DNA-PK deficient MO59J cells. There was a marked decrease in the survival of the MO59K cells but no change in the MO59J cells, following exposure to IR, bleomycin and etoposide. No sensitisation of either cell line was demonstrated when exposed to chlorambucil or cisplatin which create cross links. These data are consistent with a role for DNA-PK in DSB repair. A further study by Omori *et al.*, 2002, utilised antisense to Ku70 in human squamous cell lung carcinoma and demonstrated that the cells were more radio-and chemo-sensitive.

1.12.2 Cell lines deficient in the components of DNA-PK

The involvement of DNA-PK in DSB repair first became evident from analyses involving a specific series of mutant rodent cell lines (Jeggo, 1990). Mammalian cell mutants were isolated, many of which were derived from the Chinese Hamster Ovary CHOK1 cell line (Reviewed in Jeggo *et al.*, 1995; Zdzienicka, 1995 and references therein). They displayed sensitivity to a range of DNA damaging agents. At least 11 complementation groups have been described (Jeggo *et al.*, 1991; Thacker and Wilkinson, 1991).

A subset of these mutants were found to be hypersensitive to IR and radio-mimetic agents with little or no cross sensitivity to other types of DNA damaging agents. Further studies showed them to be defective in the repair of chromosomal DNA DSBs (for review see Zdzienicka, 1995 and references therein). Furthermore, these cells were also shown to be deficient in the resolution of DNA DSBs that arise during V(D)J recombination, the site specific recombination process that helps generate the mature genes for immunoglobulin and T-cell receptor proteins in the developing mammalian immune system (reviewed in Lewis, 1994). Analyses of V(D)J recombination in wild type and mutant cells has revealed that this reaction proceeds via a cut and ligation process that involves DNA DSBs as intermediates. Moreover, providing a clear link to DSB repair processes, it was shown that it is the ligation and not the cutting step of V(D)J recombination that is impaired in IR-sensitive cell mutants. The common phenotype of these cells suggests that these mutants may be involved in a common pathway of DSB repair.

Subsequent cell fusion studies allowed these DNA DSB repair-deficient mutant cells to be assigned into 3 distinct complementation groups, termed IR4, IR5 and IR7. The human genes complementing them were pre-assigned the XRCC nomenclature (X-ray Cross Complementing). IR 5 mutants were shown to lack a DNA binding activity that corresponded to Ku. In contrast, IR7 mutants contained normal Ku-dependent end binding activity (Getts and Stomato, 1994; Rathmell and Chu, 1994). Ku80 and DNA-PKcs are the products of the XRCC5 and XRCC7 genes as demonstrated by their ability to complement the radiosensitivity defects of IR5 and IR7 mutants, respectively (Taccioli *et al.*, 1994a; Boubnov *et al.*, 1995; Smider *et al.*, 1994; Blunt *et al.*, 1995; Kirchengesner *et al.*, 1995; Peterson *et al.*, 1995). Importantly, this finding provided a clear link between the DNA-PK complex and DSB repair. None of the rodent cell lines were defective in Ku70. However, targeted disruption of the gene for Ku70 in mouse cells also renders cells hypersensitive to IR and has allowed such cells to be designated IR6 and the gene for Ku70 to be designated XRCC6 (Gu *et al.*, 1997). IR4 is complemented by XRCC4 which is a nuclear phosphoprotein of 334 amino acids and a substrate for DNA-PK (Critchlow *et al.*, 1997; Leber *et al.*, 1998). Strikingly, homozygous deletion of XRCC4 confers embryonic lethality. The proposed functions of this protein in DNA DSB repair is described in more detail in section 5.1.

Taken together, these studies have demonstrated unequivocally that both DNA-PKcs and Ku play important roles in DNA DSB repair. NHEJ is a largely error prone process since it does not use extensive homologies to repair the damage (section 1.5.2.1). Characteristically, group 4 and 7 mutants accumulate deletions in their DNA thus providing a link between these proteins and NHEJ.

Despite the existence of a variety of rodent cell lines containing mutations in the proteins of NHEJ pathways and thus defective in DSB repair, only one glioma-derived cell line of human origin (MO59J) has been described thus far (Allalunis-turner *et al.*, 1993). MO59J cells which are defective in DNA-PKcs expression and defective in DSB repair (Dibiase *et al.*, 2000) are therefore group 7 mutants. MO59K cells, proficient in DNA-PK activity, are derived from a different area of the same tumour and therefore have a common genetic background but cannot strictly be termed isogenic.

1.12.3 Knockout mouse models for components of DNA-PK

The first mutant mouse model for analysing DNA-PK function was the spontaneously arising severe combined immunodeficiency (SCID) mouse (Bosma and Carroll, 1991). Biochemical and genetic studies revealed a loss of DNA-PKcs function in SCID cells derived from the mice (Blunt *et al.*, 1995). This results from a mutation in the DNA-PKcs gene that converts Tyr-4046 codon to a stop codon thus creating a truncated protein that is missing the last 83 amino acids of the kinase domain. Although this mutated protein is highly unstable, it is still present at detectable levels, raising the possibility that SCID cells retain residual DNA-PKcs function (Danska *et al.*, 1996). Consistent with a role for DNA-PK in DSB repair, SCID mice and cells derived from them are hypersensitive to IR and display defects in rejoining of IR-induced DSBs.

Recently, knockout mice have been generated that are null for DNA-PKcs (Gao *et al.*, 1998) or ablated for its kinase domain (Taccioli *et al.*, 1998). Mice have also been produced that are defective in Ku70 or Ku80 (Zhu *et al.*, 1996; Gu *et al.*, 1997). There are common phenotypes among these knock out mice and these include 2-5-fold increased sensitivity to IR, and a reduced ability to repair DSBs. In addition to these defects, mice with targeted disruptions for Ku70 and 80 are small and have prolonged doubling times and premature cell senescence.

1.13 The PI3-K family of enzymes

The purification of DNA-PKcs, coupled with the determination of the sequences of several peptides derived from it, enabled a partial cDNA clone for DNA-PKcs to be isolated. The kinase domain of DNA-PKcs, located at the C-terminus, is very different from those of most serine/threonine kinases, instead it falls into the phosphatidylinositol –3-kinase family of enzymes (PI3-K). Some members of the PI 3-kinase family function in signal transduction by phosphorylating inositol phospholipids (Kappeller and Cantley, 1994). A well characterised example is the mammalian PI 3-kinase, which phosphorylates phosphatidylinositol (PI) in response to a variety of stimuli, including the activation of various growth factor receptors.

Despite being in the 'PI 3-kinase family', however, DNA-PKcs is not a lipid kinase but is a protein kinase. Several of the family members, including PI 3-kinase, itself are able to phosphorylate both lipid and protein targets and therefore the PI 3-kinase family can be subdivided into two distinct subgroups. The first contains mammalian PI 3-kinase and other lipid kinases, the other contains DNA-PKcs and a series of other large (> 250 kDa) proteins which are involved in controlling cell cycle progressions and/or functioning in DNA repair and DNA damage signalling termed the PI 3K-related kinases (PIKK). These proteins share considerable homology in the kinase domain at their 3' terminus. Outside the kinase domain, however, DNA-PKcs has little or no similarity. Other PI-3K related kinases include the ATM protein, ATR and FRAP.

1.13.1 ATM

ATM (ataxia-telangiectasia gene product) was identified in 1995, based on its role in a rare, inherited disorder, ataxia telangiectasia (AT). ATM is one of the best characterised DNA-PKcs-related proteins (Rotman and Shiloh, 1998). The gene is located on 11q22.3-q23 and is ubiquitously expressed and codes for a 370 kDa protein of 3056 amino acids. Although its cellular localisation is variable, it is primarily localised to the nucleus of proliferating cells.

Cells from AT patients, in which the ATM gene is mutated, display reduced survival and an inability to activate cell cycle checkpoints following IR exposure. Although it is possible that ATM plays direct roles in DNA repair, the available evidence indicates that its primary role is in DNA damage signalling. Most normal proliferating cells temporarily arrest cell cycle progression at the G1/S or G2/M cell cycle 'checkpoints' in response to DNA damage. Arrest in G1 or S-phase prevents replication of damaged DNA. Arrest in G2 prevents mitosis until DNA damage is repaired. A-T cells are deficient in the IR-induced G1/S, S and G2/M cell cycle checkpoints due, at least in part, to defective induction of the transcription factor p53 in response to IR (Siliciano *et al.*, 1997; Barlow *et al.*, 1996)

1.13.2 ATR

ATR (human AT-related) is the family member most closely related to ATM. The gene is located on 3q22-q24 and codes for a 301 kDa protein. This protein is required for the G2/M checkpoint in response to DNA damage and inhibition of replication (Cimprich *et al.*, 1996; Cliby *et al.*, 1998; Wright *et al.*, 1998). It is also involved in DNA damage signalling. Purified ATR from human cell extracts displays a DNA-stimulated protein kinase activity that targets the N-terminal region of p53. Since this region of p53 has been shown to function in transcription and in controlling p53 protein stability, this indicates that ATR acts upstream of p53 in DNA damage-induced signalling pathway.

Like ATM, ATR controls BRCA1 phosphorylation in response to IR. ATR forms distinct nuclear foci at the sites of stalled replication forks (Tibbetts *et al.*, 2000). Protein kinase mutants of human ATR display increased sensitivity to both IR and UV demonstrating that ATR may be a component of repair (Wright *et al.*, 1998).

1.13.3 FRAP

FRAP, also known as the mammalian target of rapamycin, (mTOR) is a 450 kDa protein which has been shown to function in the regulation of protein translation in response to mitogenic signals, thus allowing progression from G1 to S phase of the cell cycle (Kuruvilla and Schreiber, 1999). In addition, FRAP has been proposed to be a regulator of cell growth (Schmelzle and Hall, 2000).

In summary, the activation of these proteins by various forms of DNA damage might trigger phosphorylation cascades that impinge on the transcription, cell cycle and apoptotic machineries. Given that DNA-PKcs, ATM and ATR have all been shown to function in p53-dependent DNA damage signalling, this supports models in which DNA-PKcs, ATM and ATR function in distinct yet overlapping DNA damage detection pathways.

1.14 P53

The tumour suppressor protein p53 is a key regulator of the cell cycle, it is also vital for the maintenance and stability of the genome. It is the most common target for genetic alterations in cancer, with around 50 % of tumours possessing mutations in the p53 gene. P53 accumulates and is activated in response to DNA damage and subsequently functions as a transcription factor, stimulating transcription of various genes. These genes produce proteins that are involved predominantly in cell cycle arrest or the apoptotic pathway, depending upon the extent of damage. The use of mouse model systems (Attardi and Jacks, 1999) and various mutant cell lines has led to a clearer elucidation of p53 function.

1.14.1 PARP-1 and p53

As described in section 1.6.4.3, PARP-1 has been implicated in signalling to several molecules, perhaps, most significantly, p53. The effects of PARP-1 on p53 induction and activity have been studied using both PARP-1 inhibitors such as 3-aminobenzamide (3-AB) and PARP-1 deficient mice and cells derived from these. Different studies investigating the role of PARP-1 in p53 regulation have yielded conflicting results. For example, a number of studies indicate that PARP-1 contributes to the accumulation of p53 following DNA damage whilst other studies have implicated a role for PARP-1 in determining the duration but not the magnitude of the p53 response following DNA damage.

The carboxyl terminal fragment of PARP-1 possesses a consensus sequence which has been shown to facilitate protein-protein interactions with p53. Similarly, p53 possesses an amino acid consensus sequence that non-covalently binds PARP-1 bound ADP-ribose polymers in a highly specific manner (Agarwal *et al.*, 1997). PARP-1 has been shown to physically interact with p53. Indeed, p53 contains 3 sequence motifs that bind PAR polymers, two of which are located in the sequence specific DNA binding domain (Malanga *et al.*, 1998). Malanga *et al.*, (1998) showed in vitro binding of PAR to p53. This binding involves domains within p53 and was subsequently shown to impair the DNA binding and transcriptional activation functions of the protein probably by blocking protein-DNA interactions.

Alternatively, negatively charged PAR could cause electrostatic repulsion between p53 and DNA. However, no poly(ADP-ribosyl)ation of wild type p53 can be detected in cells (Kumari *et al.*, 1998; Wesierska-Gadek *et al.*, 1996).

PARP-1 inhibition has been found to suppress both the accumulation of p53 and G1/S phase arrest in MEFs in response to IR (Matsutani *et al.*, 1995). Specifically, following exposure to IR in the presence of 3-AB, there was a 40 % decrease in p53 activity and, corresponding with the suppression of p53-induced transactivation, MDM2 expression was considerably diminished. Reduced accumulation of p53 following DNA damage has also been demonstrated in cell lines derived from PARP-1 deficient mice and cells selected for low PARP-1 levels (Whitacre *et al.*, 1995). Simbulan-Rosenthal *et al.*, 1998, showed that partial inhibition of PARP-1 expression (>90 %) in a cell line expressing PARP-1 antisense delays the p53 response to DNA damage but the magnitude of the response was unaffected.

Moreover, Agarwal *et al.*, 1997, demonstrated that there was a reduction in the basal level of p53 in PARP-1 null cells in both the presence and absence of IR. However, in contrast to the studies described above, the authors showed that following IR, downstream events such as activation of p21 and G1 checkpoint were unaffected in these cells thus indicating that PARP-1 has no effect on p53 activity. Menissier de Murcia *et al.*, 1997, reported that induction of p53 can be observed to some extent in PARP-1 ^{-/-} animals treated with high doses of DNA damaging agents. Moreover, work by Paul Jowsey (unpublished results, personal communication) has shown that following exposure to IR, UV or the alkylating agent temozolomide, inhibition of PARP-1 by the PARP-1 inhibitor used in the studies described in this thesis, AG14361, or loss of PARP-1, failed to show any effect on the ability of p53 to act as a transcriptional transactivator of either p21 or MDM2. These observations have led to the proposal by Agarwal *et al.*, 1997, that DNA damage-induced accumulation of p53 is likely to occur through both PARP-1-dependent and independent pathways but that the actual activation of p53 is probably controlled by a PARP-1 independent pathway.

In conclusion, several groups have reported defective or no p53 induction in PARP-1 deficient cells following DNA damage, whereas others have reported normal or elevated p53 induction. The differences are likely to be a result of different cell lines and DNA damaging agents that the different groups have used. These data suggest that PARP-1 is required for efficient activity and accumulation of p53 but not essential since PARP-1 deficient cells retain p53 activity. Interpretation of data regarding the role of PARP-1 in p53 induction either by comparison of proficient and deficient cells or the use of PARP-1 inhibitors is complicated by the fact that loss of PARP-1 hinders DNA repair thus providing a greater and prolonged p53 stimulus.

1.14.2 DNA-PK and p53

The biochemical functions of p53 may be regulated by reversible serine phosphorylation. Salles-Passador *et al.*, proposed a link between p53 and DNA-PK in mediating the responses to IR (Salles Passador *et al.*, 1999).

DNA-PK has been shown to phosphorylate serines 15 and 37 in vitro (Lees-Miller *et al.*, 1992). This has been reported to destabilise interactions between p53 and MDM2 (Shieh *et al.*, 1997), a protein that negatively regulates p53 in vivo, by targeting it for ubiquitin-mediated proteolysis (for review see Alarcon-Vargos and Ronai, 2002 and references therein). This leads to p53 stabilisation and hence to p53-dependent downstream events. However, initial analysis of cells defective in components of the DNA-PK complex have shown that they retain intact DNA damage checkpoints and are capable of mediating both p53 induction and stabilisation in response to IR (Bogue *et al.*, 1996; Fried *et al.*, 1996; Guidos *et al.*, 1996; Huang *et al.*, 1996; Candeias *et al.*, 1997; Rathmell *et al.*, 1997; Shieh *et al.*, 1997). For example, Jiminez *et al.*, 1999, showed that the p53 response is fully functional in primary MEFs lacking DNA-PK, with normal response of p53 accumulation, phosphorylation of p53 at residue serine 15, nuclear localisation and binding to DNA of p53. Furthermore, the upregulation of p53-target genes and cell cycle arrest occurred normally. These data therefore imply that other pathways, distinct from DNA-PK, can phosphorylate p53 at serine 15 in DNA-PK deficient cells and that DNA-PK activity is not essential for cells to mount a p53-dependent response to DNA damage.

The DNA-PK related proteins ATM and ATR are capable of phosphorylating p53 on serine 15 in vitro (Banin *et al.*, 1998). Thus, ATM, ATR and possibly DNA-PK, might signal different but partially overlapping types of DNA damage to a common p53 pathway. In contrast, however, recent work using DNA-PKcs defective cell lines have indicated that DNA-PK activation in response to DNA damage (IR), is necessary, but not sufficient for the activation of sequence specific DNA binding by p53 through an, as yet, unknown mechanism (Woo *et al.*, 1998).

It is worth noting that, none of the publications listed above, analysed in detail the kinetics of cell cycle arrest or the timing of transactivation of p53-dependent downstream genes by observing multiple time points following exposure to IR. A study by Kachnic *et al.*, 1999, however, has shown that whilst SCID cells still have p53-dependent G1 checkpoint and that the levels of p53 rise and fall in the SCID mouse derived MEFs in the same manner as in control cells, the loss of DNA-PK activity attenuates the kinetics of p53 activating downstream genes, implying that DNA-PK plays a role in post-translational modification of p53 without affecting the increase in levels in response to IR. These findings are consistent with and complementary to the findings of Woo *et al* (1998) and suggest that the ability of p53 to activate downstream genes therefore appears to be, at least in part, dependent on DNA-PK

Alternatively, DNA-PK has been demonstrated to be capable of phosphorylating MDM2 on serine 17, in vitro, and therefore this might be an indirect mechanism by which it regulates p53 activity. Phosphorylation of MDM2 results in a loss of binding of MDM2 to p53 protein and this, in turn, prevents inhibition of p53 (Mayo *et al.*, 1997). These observations are not mutually exclusive and therefore the effect of a loss of DNA-PK activity could be a combined lack of phosphorylation of p53 (resulting in impaired transactivation) and lack of phosphorylation of MDM2 which then continues to attenuate p53 (rather than phosphorylation which results in less inhibition of p53).

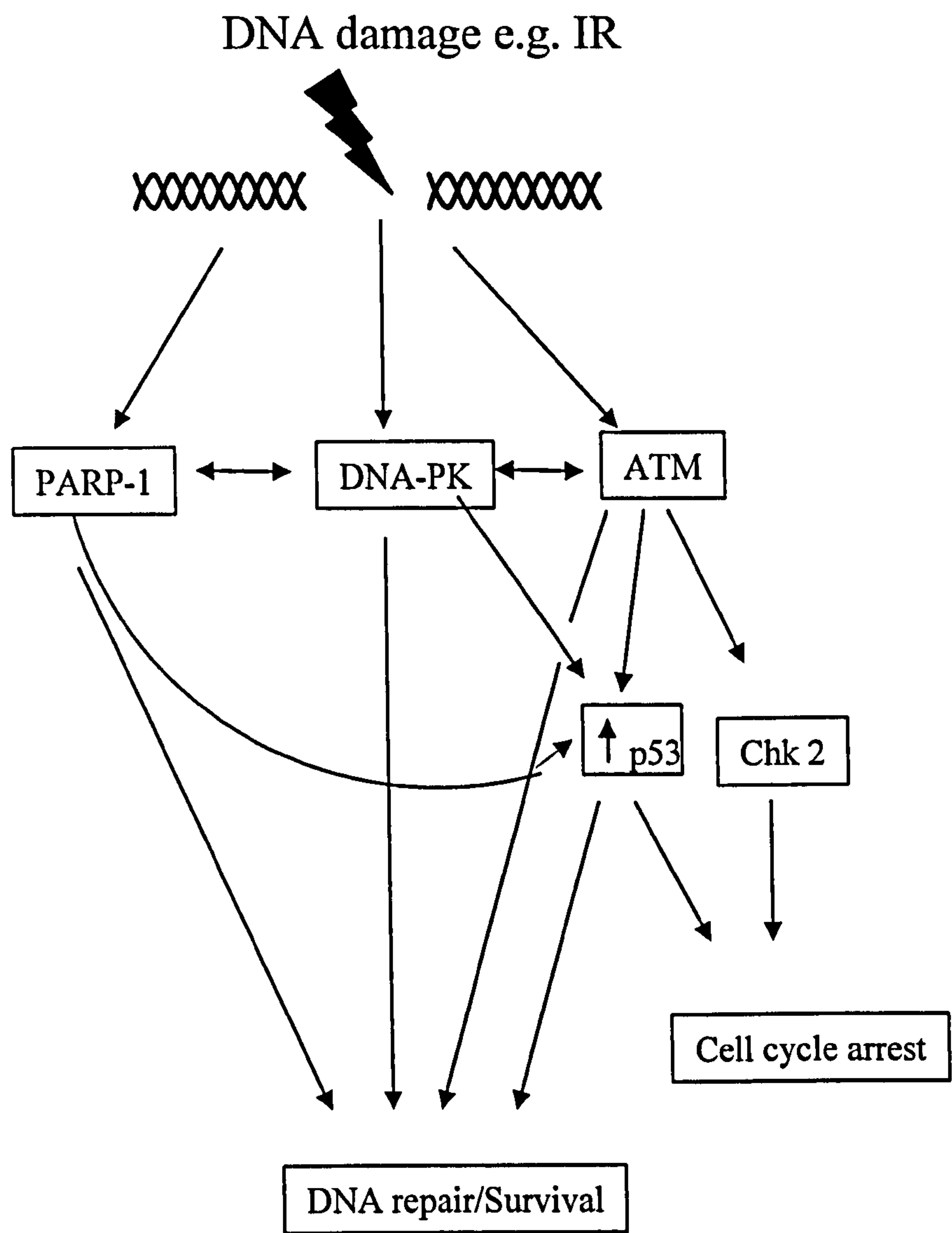


Figure 1.9 The role of PARP-1 and DNA-PK in the cellular responses to DNA damage

1.15 Aims of the studies described in this thesis

The primary aim of the work described in this thesis was to investigate the interactive effects of PARP-1 and DNA-PK in the cellular responses to ionising radiation-induced DNA damage. In order to achieve the aim, data presented in this thesis utilised cell lines proficient or deficient (by mutation or gene knockout) in PARP-1 or DNA-PK in combination with novel specific inhibitors of the two enzymes. The characterisation of the cell lines and inhibitors used in this thesis are described in Chapter 3. The effect on a number of biological endpoints known to be modulated by PARP-1/ DNA-PK function following DNA damage were investigated. These included clonogenic survival (Chapter 4) and DSB formation and repair (Chapter 5). In addition, the ability of the two enzymes to compete for strand breaks and utilise one another as a substrate was explored (Chapter 6).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General equipment

- β -Liquid scintillation counter Model 1409 DSA (Wallac, Milton Keynes, UK)
- Irradiator; Gammacell 1000 Elite (Nordian international Inc., Canada)
- FacScan (Beckton Dickinson, Oxford, UK)
- MR700 96-well microtitre plate reader (Dynatech, Sussex, UK)
- Coulter counter Model Z1 (Coulter Electronics, Beds., UK)
- Incubator (Heraeus Equipment Ltd., Beds., UK)
- Sonicator: MSE Soniprep (Fisher Scientific, Leics., UK)
- Spectrophotometer: Pye Unicam PV8610 (Pye Unicam, Cambridge, UK)
- Waterbath: Grant Model W28 (BDH, Dorset, UK)
- Centrifuges; Microcentrifuge Model S417R (BDH, Dorset, UK), MSE (Fisher Scientific, Leics., UK)
- X Cell Surelock electrophoresis tank and X cell II Blot module (Invitrogen, Paisley, UK)
- Power pack 200 (Bio-Rad, Herts., UK)
- Roller Mixer model SRT1 (Stuart Scientific, Surrey, UK)
- Automatic film developer (Fuji Photo Film Ltd., Japan)
- HPLC 2780 separation module (Waters alliance, Watford, UK)
- Multi-channel peristaltic pump Model 205S (Watson-Marlow, Cornwall, UK)
- Fraction collector 2211 Superfrac (Pharmacia LKB Technology, Sweden)
- Milipore filtration system (Milipore Corporation, Bedford, Mass., USA)

2.1.2 Chemicals/reagents/membranes

All chemicals and reagents were of analytical grade unless otherwise stated and were purchased from either Sigma-Aldrich Company Ltd. (Dorset, UK), or BDH (Dorset, UK), with the exception of the items listed below.

- p81 filter paper used in the DNA-PK assay was obtained from Whatman International Ltd., (Kent, UK)
- Nucleopore polycarbonate track-etch membranes utilised in the DNA strand break assay and microfibre filters used in the PARP assay were obtained from Whatman International Ltd., (Kent, UK)
- Isoton II and coulter pots were supplied by Coulter Electronics (Beds., UK)
- Optiphase scintillant was supplied by Wallac Scintillation Products (Milton Keynes, UK)
- Digitonin was purchased from Roche diagnostics (Sussex, UK)
- Trypan blue was used to obtain viable cell counts and was supplied by Northumbria Biologicals Ltd. (UK)
- BCA protein assay kit, albumin standard and enhanced chemiluminescence substrate (ECL) were purchased from Pierce-Perbio Science (Cheshire, UK)
- NUPAGE gels and markers for western blot analysis were obtained from Invitrogen Ltd. (Paisley, UK)
- Nitrocellulose membrane was purchased from Bio-Rad (Herts., UK)

2.1.2.1 Inhibitors

The PARP-1 inhibitor, AG14361, was synthesised as part of the Newcastle Anticancer Drug Development Group and Agouron Pharmaceutical collaboration. The DNA-PK inhibitor, NU7026, was synthesised by the Department of Chemistry, University of Newcastle Upon Tyne, UK. AG14361 and NU7026 were dissolved in 100 % v/v DMSO to give 10 mM and 5 mM stock solutions, respectively, and stored at -20 °C. All drugs were further diluted in dimethyl sulphoxide (DMSO), as necessary, such that when diluted in tissue culture medium, a final concentration of 1 % (v/v) DMSO was achieved. Drug free controls were exposed to 1 % (v/v) DMSO.

2.1.3 Radiochemicals

[2-¹⁴C] Thymidine (specific activity = 57 mCi/mmol or 50 μ Ci/ml), [methyl-³H] Thymidine (specific activity = 41 Ci/mmol or 1mCi/ml), [³²P] NAD⁺ (specific activity = 1000 Ci/mmol or 10mCi/ml) and [32P] ATP (specific activity = 3000 Ci/mmol or 10 mCi/ml) were purchased from Amersham International (Bucks., UK).

2.1.4 Enzymes

- Proteinase K was utilised in the DNA strand break assay and was obtained from Boehringer Mannheim Biochemica (Mannheim, Germany)
- Purified DNA-PK (500 μ g/ml), isolated from HeLa cells, was kindly supplied by G.C.M. Smith, KuDOS Pharmaceuticals (Cambridge, UK)
- Purified human PARP-1 (1 mg/ml) was kindly supplied by K.Maegley and Z. Hostomsky, Agouron Pharmaceuticals (California, USA)

2.1.5 Tissue culture supplies

All tissue culture plasticware were obtained from NUNC (Denmark). Tissue culture medium and constituents were purchased from Gibco-BRL Life Technologies Ltd. (Paisley, UK), with the exception of the foetal calf serum which was supplied by Globepharm Ltd. (Surrey, UK). Additional reagents and materials were supplied as follows:

- Dulbecco's phosphate buffered salts (modified) without Ca²⁺ and Mg²⁺ (DULA) were obtained from ICN Flow (ICN Biomedicals Inc., Irvine, UK)
- Bijoux were supplied by Bibby Sterlin Ltd. (Staffs., UK)
- Cells scrapers were obtained from Corning Co-Star (Bucks, UK)
- Haemocytometer (Neubauer, UK)

2.1.6 Cell lines and routine culture

The following cell lines were utilised in the studies described;

- AA8 Chinese hamster ovary. Derivative of the CHO-K1 cell line and the parent line for the repair deficient mutants V3
- V3 Chinese hamster ovary. Radosensitive mutant defective in the catalytic subunit of DNA-PK (gene product designated XRCC7) (Blunt *et al.*, 1995)

- **V3YAC** Chinese hamster ovary. V3 cell line transfected with a yeast artificial chromosome encoding human DNA-PKcs cDNA. This cell line and the radiosensitive mutant, V3, were a gift from Dr Penny Jeggo of the University of Sussex (Blunt *et al.*, 1995; Priestly *et al.*, 1998).
- **CHO-K1** Derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary from an adult Chinese hamster (Puck, 1957) Parent line for the repair deficient mutants xrs-6.
- **Xrs-6** Chinese hamster ovary. Radiosensitive mutant defective in Ku80, a subunit of the Ku protein and component of DNA-PK complex (gene product designated XRCC5) (Jeggo and Kemp, 1983).
- **Xrs-6-HamKu80** Chinese hamster ovary. Xrs-6 cell line transfected with hamster Ku80 cDNA (Singleton *et al.*, 1997).
- **PARP-1 +/+** Spontaneously immortalised mouse embryonic fibroblasts derived from primary cells kindly provided by Professor Gilbert de Murcia, Ecole Supérieure de Biotechnologie de Strasbourg, France.
- **PARP-1 -/-** Mouse embryonic fibroblast. Knockout for PARP-1 through disruption in exon 4 which encodes the DNA binding domain of this enzyme (Figure 3.1).

Specific details regarding the nature of any mutations in these cell lines are given in Chapter 3 (section 3.1.1). All cell lines were cultured as monolayers in plastic tissue culture dishes and grown in Dulbeccos modified eagles medium (DMEM) supplemented with 10 % (v/v) foetal calf serum (heat inactivated at 56°C for 30 minutes), 100 U/ml penicillin, 100 µg/ml streptomycin. Glutamine was added at a final concentration of 2 mM. All lines were sub-cultured twice weekly to maintain the cells in exponential phase of growth and kept at 37°C, 5 % CO₂ in a humidified incubator. During routine sub-culturing, and in experiments, the medium was aspirated from the dish, the cells washed with DULA and then removed from the plate using an appropriate volume of 0.25 % (v/v) trypsin in DULA/EDTA (dihydrate form of disodium salt, 0.02 % w/v). Trypsinisation was terminated by the addition of fully supplemented medium. The xrs-6 and V3YAC cell lines were maintained under antibiotic selection by an additional supplement of Genticin

Sulphate which was added at a final concentration, in medium, of 300 µg/ml and 500 µg/ml, respectively.

Mycoplasma, are a common contaminant of cell cultures. Although they can produce many abnormal changes within the cell including altered protein and nucleic acid synthesis and chromosomal breakage, their detection is difficult since a change in growth or morphology is not normally apparent. Consequently, all cell lines were routinely tested for mycoplasma spp on a 6 weekly basis.

2.1.6.1 Frozen cell stocks

Frozen cell stocks were stored in liquid nitrogen. Cells were preserved in 'freezing down' medium; DMEM, supplemented as described above, with the exception of the addition of 10 % (v/v) DMSO. Exponentially growing cells were harvested by trypsinisation, counted and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in an appropriate volume (approximately 10^6 cells/ml) of freeze medium into a cryovial and freezing was carried out overnight in a -80°C freezer at a rate of approximately 1°C per minute before transferring to liquid nitrogen. The slow freezing rate in combination with the use of DMSO prevents the formation of ice crystals that would otherwise rupture the cells. Recovery from liquid nitrogen was achieved by thawing cells rapidly in a waterbath at 37°C. The cells were then resuspended in 9 ml fully supplemented DMEM medium and centrifuged at 1500 rpm for 5 mins in order to remove traces of DMSO. The medium was aspirated and the pellet resuspended in an appropriate volume of medium and transferred to a 92mm plate.

2.2 COUNTING CELLS

In the studies described in this thesis, cells were counted using a haemocytometer, or where a large number of samples were to be counted, a Coulter counter was employed.

Principle A haemocytometer is a slide containing two mirrored chambers of known depth with a grid etched onto its lower surface. When a cover slip is placed over the

chambers, the depth is uniform (0.1 mm). Each chamber is divided into 9 large 1 mm² squares and therefore the volume of each large square is (1mm x 1mm) x 0.1 mm = 0.1 mm³. Since 1 mm³ = 1 µl, the conversion factor for 1ml = x 10⁴. Using this information, the cell number per ml of the original suspension can be calculated.

Counting Each sample was diluted 1:1 with 0.4 % v/v trypan blue (this gives an indication of the viability of the cells as only non-viable cells are stained blue) and then loaded into a counting chamber by capillary action. At least 100 cells were counted from either the 4 corner squares or all 9 squares, depending on the amount of cells and all counts were carried out in duplicate. Only viable cells were counted.
Mean cell count / number of squares x dilution factor = cells/ml x 10⁴

Principle A Coulter counter detects cells due to a change in electrical resistance when they pass through a small aperture in a glass tube. This change in electrical resistance is detected as a voltage pulse which is proportional in height to the volume of electrolyte displaced and therefore the size of the cell. The number of pulses are counted by the machine thus giving an accurate determination of the number of cells in a given volume. Whilst this technique is useful for the measurement of larger number of cells or for a large number of samples, a major limitation is the lack of discrimination between viable cells, dead cells and cell clumps as well as inanimate particles such as dust.

Counting For each sample, 500 µl of cell suspension was fixed in 500 µl of Carnoys fixative and diluted by the addition of 9 ml Isoton II to give a total volume of 10ml. The counter was set to count 1ml of the electrolyte solution at the appropriate threshold settings (see below) for the cell line. Duplicate counts of each sample were taken and the average value used to calculate the cell number, taking into account the dilution factor of 20.

An upper threshold (T_U) of 24 µm and a lower threshold (T_L) of 8 µm were used for all cell lines, as these settings were found to give optimum correlation with haemocytometer counts.

2.3 SULPHURHODAMINE B (SRB) ASSAY

Assay principle Sulphurhodamine B (SRB) is a bright pink aminoxanthine dye with two sulphonic groups, which binds electrostatically to the basic amino acid residues of proteins under mildly acidic conditions. The intensity of staining is therefore proportional to the amount of protein and therefore cell number. As described by Skehan *et al.*, (1990) this assay can be used as a rapid and sensitive means by which cellular growth rate and growth inhibition can be measured in microtitre plates.

2.3.1 Determination of cell growth rate

The amount of cell suspension seeded during routine subculture and in experiments is dependent on the growth rate of the cells, the confluent cell density and the duration of the experiment. A series of cell suspensions at concentrations ranging from 2×10^5 to 1.25×10^4 cells/ml were prepared and 100 μ l of cell suspension was then seeded into each of the inner 60 wells (each cell suspension across the plate to give 10 replicates) of a 96 well microtitre plate. Medium was added to the outer wells in order to compensate for “edge effect”, a phenomena which can result in abnormal growth (Dr N.J.Curtin, personal communication). A total of 6 plates were set up for each cell line and one plate was fixed per cell line at 24 hr intervals by aspiration of the medium from each well followed by the addition of 25 μ l 50 % trichloroacetic acid (TCA) to each well. The plates were then stored at 4°C, ready for staining.

Staining was achieved by the addition of 100 μ l of 0.4 % (w/v) SRB in 1 % acetic acid (v/v) to all wells. Plates were then incubated at ambient temperature for 30 minutes before removing the dye by rinsing five times with 1% acetic acid. Rinsing was performed gently but quickly in order to avoid dislodging adherent cells and to prevent desorption of protein bound dye. The plates were then air dried prior to the addition of 100 μ l 10 mM Tris base (pH 10.5) to each well. The addition of Tris base to the cells results in the solubilisation of the stain. Plates were read at 570nm using a MR700 96 well microtitre plate reader.

Calculation of results An average of the 10 absorbance readings obtained for each of the cell suspensions was calculated and these were plotted against time on an x-y plot. Doubling times (DT) were calculated by linear regression of the curve of log absorbance against time using GraphPad Prism software (Graphpad Inc, San Diego, CA, USA) and the following formula;

$$\frac{\log 2}{\text{slope}} = \text{DT hours.}$$

2.3.2 Growth Inhibition Assay

In studies described in this thesis, the SRB assay was used to investigate whether or not either of the inhibitors had any inherent inhibitory effects on cell growth. All CHO cell lines were seeded at 1.5×10^3 /well whilst the PARP-1 MEFs were seeded at 1.0×10^3 /well (results of cell growth rate studies). As before, 100 μ l of cell suspension was seeded into the inner 60 wells of a 96 well microtitre plate whilst medium alone was added to the outer wells to compensate for edge effect. The cells were incubated for 24 h to allow them to adhere. Medium was aspirated from the wells and then replaced with 100 μ l medium containing inhibitor(s) (dissolved in DMSO such that the final concentration of DMSO in medium was 1 % v/v). 6 wells per drug concentration were seeded and untreated controls were exposed to 1 % (v/v) DMSO with 12 replicates seeded. When the inhibitor(s) were added to the cells, a plate of untreated cells was fixed by the addition of 100 μ l 50 % TCA in order to measure the cell density at time 'zero'. Cells were exposed to the inhibitor for at least three full cell divisions (48 hr / 72 hr dependent on cell line) (see individual figure legends) before being fixed and stained as described above.

Calculation of results An average of the absorbance readings obtained for each of the 12 drug free controls gave a value for the number of control cells. Likewise, the values obtained for the 6 replicates for each drug concentration were averaged to give values for the number of treated cells. Cell growth, expressed as a percentage of untreated controls, was then calculated and plotted against inhibitor concentration on an x-y plot. The IC_{50} , defined as the concentration of inhibitor to give rise to a 50 % inhibition of growth of that shown by untreated controls, was calculated using a point to point plot using GraphPad Prism software.

2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Assay Principle HPLC is a fast, sensitive and versatile technique for the separation of compounds on the basis of their size, polarity, solubility or adsorption characteristics (Jones *et al.*, 1996).

Assay In order to evaluate the stability of NU7026, a reverse HPLC method was developed. Separation was achieved on a Hypersil BDS C18 5 μ m column (4.6 x 250 mm) using a mobile phase of 0.02 M ammonium formate buffer (pH 5.0) and acetonitrile (60:40 v/v). Detection was at 270 nm using a PDA996 detector with a retention time of approximately 8 minutes observed for NU7026.

PBS standards (1, 10 and 50 μ M in 1 % DMSO v/v) were used to determine λ_{max} and optimal separation conditions. Samples were set up in triplicate at 1, 10 and 50 μ M in DMEM medium, aliquots taken at various time points and extracted using acetonitrile precipitation (1:2 v/v sample : acetonitrile).

Calculation of results Results were processed using Waters Millennium 32 Software. See individual Figure legends for details.

2.5 CLONOGENIC ASSAY

Assay Principle Cell growth can be inhibited by both cytotoxic and cytostatic agents, however, only viable cells are able to carry out the 5-6 rounds of cell division required to form a visible colony. Colony forming assays show the difference between cytotoxic and cytostatic agents not shown in growth inhibition studies.

Assay Cells were plated onto 60 mm plates at a density that gave a final cell number of $3-5 \times 10^5$ cells/ml on the day of experiment. Dosing schedules were as described in figure legends. Inhibitors were added at final concentration of 1 % DMSO (v/v) in medium 1 hr prior to irradiation and controls were exposed to 1 % (v/v) DMSO. For γ -irradiation studies, cells were subjected to a ^{137}Cs source for appropriate exposure times (dose rate of 3.64 Gray/minute).

Following post-incubation at 37°C (see individual figure legends), the cells were trypsinised and resuspended in 5 ml of drug free medium and syringed using a 23G needle to ensure a single cell suspension. A 500 µl aliquot of each cell suspension was fixed and counted using a coulter counter (see section 2.2.2). Cells were diluted in medium to give suspensions of either 1x10⁴ cells/ml or 1x10³ cells/ml. A volume of the diluted cell suspension corresponding to a number of cells calculated to produce a countable number of colonies (50-200) were then seeded in triplicate onto 92 mm plates in 10 ml of fresh medium. The plates were then incubated for 7-14 days depending on the cell line, to allow for colony formation.

Following the formation of suitably large colonies (fifty cells or more), the medium was aspirated from the plates and the colonies fixed by the addition of 5 ml carnoys fixative. After 5 min the fixative was removed and the colonies were allowed to dry in air. Staining was achieved by the addition of 0.4 % (w/v) crystal violet in water for one minute. Excess stain was gently washed off under running water and the plates allowed to dry.

Calculation of results Absolute cell survival (plating efficiency) was calculated as the percentage of cells seeded which formed colonies i.e. (the number of colonies/the number of cells seeded) x 100. The relative survival of the treated cells was expressed as a percentage of untreated controls.

The LD₅₀ and LD₉₀ (defined as the dose of γ-irradiation to give 50 % and 90 % reduction in survival) were calculated using a point to point curve plot using GraphPad Prism software. The potentiation factor at 10% survival (PF₉₀) was calculated as follows:

$$PF_{90} = \frac{LD_{90} \text{ IR alone}}{LD_{90} \text{ IR} \pm \text{AG14361} \pm \text{NU7026}}$$

2.6 POTENTIALLY LETHAL DAMAGE RECOVERY ASSAY

Assay Principle Plateau phase cell cultures have a large proportion of non-proliferating G1 phase cells characteristic of in vivo tumours and therefore they are considered appropriate in vitro systems compared to exponentially growing cells for studying PLDR (Hahn and Little, 1972). The in vitro model used in the studies described in this thesis measured the increase in survival of irradiated growth arrested G1 phase cells following delayed plating for colony formation.

Assay In order to induce G1 arrest prior to irradiation, cells were seeded onto 60mm plates at an appropriate density ($2-5 \times 10^6$ /plate) and continuously cultured in the same media for 5 days until confluence was achieved. Cell cycle distribution to confirm G1 arrest in plateau phase cells was assessed by flow cytometric analysis (> 75 % cells in G1 with less than 20 % and 5 % in G2M and S, respectively) as described in section 2.7. Inhibitor(s) were added 1 h prior to irradiation in conditioned medium (medium obtained from confluent cells) and the cells were exposed to equitoxic doses of IR (see individual figure legends for dosing schedules). Cells were exposed to the inhibitor(s) during irradiation and up to the time of subculture. Following exposure to IR, cells were trypsinised and re-seeded for colony formation in the absence of inhibitor(s), either immediately following exposure to IR, or after a 24 h post-incubation at 37°C. The capacity for PLDR was evaluated by calculating the recovery ratio as follows:-

Recovery ratio

$$= \frac{\% \text{ survival 24 h post-IR}}{\% \text{ survival 0 h post-IR}}$$

% Inhibition of recovery

$$= 100 - [a/b \times 100]$$

$$a = (\text{survival} + \text{inhibitor(s) 24 h post-IR}) - (\text{survival 0 h post-IR})$$

$$b = \text{survival 24 h post-IR} - \text{survival 0 h post-IR}$$

2.7 FLOW CYTOMETRIC ANALYSIS OF THE CELL CYCLE EFFECTS OF AG14361 AND NU7026

Assay Principle Cell cycle distribution can be determined using flow cytometric analysis of cells stained with the fluorescent DNA binding dye, propidium iodide (PI) (Ormnerod., 1994). When a single cell suspension is passed through the flow cytometer, an argon laser excites the fluorescence on the cells labelled with PI. The fluorescent light emitted by the labelled cells is focused onto a photomultiplier tube and the electrical signal thus generated is proportional to the number of fluorescent molecules on each cell. As size and DNA content of the cells is characterised by light scatter and fluorescence emission, respectively, resolution of a scatter plot (forward scatter vs side scatter which gives the number of cells vs DNA content) allows the fraction of cells in G1, S and G2 to be estimated (Figure 2.1). An important feature of this technique is that it allows measurements to be made separately on each individual cell within the suspension in turn and not as average values for a whole cell population.

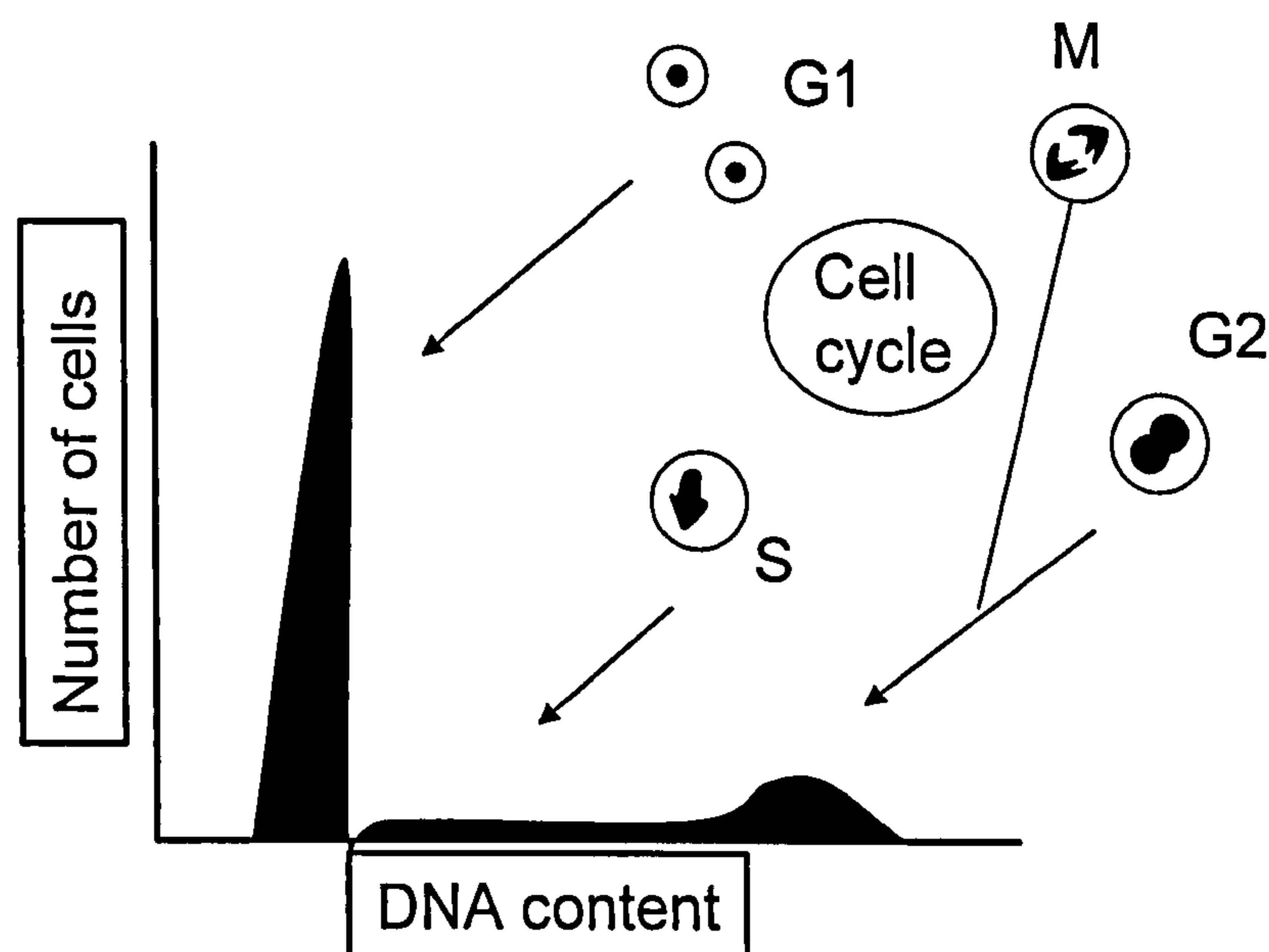


Figure 2.1 Schematic representation of a scatter plot illustrating the relationship between DNA content, and cell cycle phase

Sample preparation Cells were seeded on to 60mm plates at an appropriate density to allow at least 10,000 cells to be analysed per sample on the day of the experiment. Inhibitor(s) were added at a final DMSO concentration of 1% v/v (see individual figure legends for dosing schedules). The medium (containing floating cells) from each plate was transferred to a labelled bijoux. The cells from each plate were harvested by the addition of an appropriate volume of 0.25 % v/v trypsin combined with the medium collected earlier and then centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated off and the pellet resuspended in 1ml ice cold PBS before transferring to a labelled Eppendorf. The sample was then centrifuged for a further 5 minutes at 3000 rpm and the pellet fixed in 70 % (v/v) Ethanol in PBS and left on ice for at least 2 hrs. The pellet is stable for 1 month at 4°C. On the day of analysis, the cells were pelleted by centrifugation as above and resuspended in 800 µl PBS, 100 µl Ribonuclease A (Rnase 1 mg/ml) and 100 µl PI (400 µg/ml), and incubated at 37°C for 30 minutes. The cells were then placed on ice until analysis.

Flow Cytometry Flow cytometric analysis was performed using a Becton Dickinson FacScan equipped with an argon ion laser (excitation at 488 nm) using emission filter 600 nm (Pi). At least 10,000 individual cells from each cell suspension were analysed for their level of fluorescence and the data obtained were plotted on a FL2 (PI) plot using Cell Quest Version 1.2.2 to give cell cycle profiles. An example profile is shown in Figure 3.12.

2.8 DNA STRAND BREAK ASSAY

Assay Principle Neutral and alkaline elution are techniques which provide quantitative analysis of DNA double and single strand breakage, respectively (Bradley and Kohn, 1979, Kohn *et al.*, 1981). Fragments of DNA are separated on the basis of size using polycarbonate filters which impede the passage of larger DNA fragments, allowing smaller fragments to pass through more quickly. The rate of elution is therefore proportional to the relative size of the DNA strand. Alkaline elution is carried out at pH 12 which denatures the DNA, whilst neutral elution is carried out at a pH of 9.6 such that the DNA remains duplexed, allowing the quantification of double strand breaks. Adaptations of the technique also allow the

measurement of alkali labile sites, DNA-protein crosslinks and DNA interstrand crosslinks. The sensitivity of the assay has been calculated to be 1 DNA lesion/ 10^7 nucleotides. To increase the precision of the assay, an internal standard was used. This accounts for any differences in elution rate between samples which are a result of variation in pump channel efficiency thus eliminating intersample differences.

Preparation of the filters Polycarbonate filters with a 2 cm diameter and a pore size of 0.2 μ M were moistened in ice-cold phosphate buffered saline (PBS) pH 7.2 and placed shiny side up onto 16 x 25 mm polyethylene filtration funnels. These filters have a low capacity for protein or DNA adsorption. To prevent leakage of the system and create a tight seal, a rubber gasket was then centralised over the filter. This was followed by the filtration funnel top which was screwed on tightly and covered in foil since visible light can produce DNA strand breaks which would have the potential to interfere with the interpretation of results. A small volume (1- 2.5 ml) of PBS was passed through each of the filters to ensure that they were dripping correctly and then a 50 ml syringe barrel was connected to each funnel. Airlocks were released by placing a depressed Gilson tip into the hole of the funnel. 20 ml PBS was (then) added to each barrel and the filters allowed to drip freely by means of capillary action and gravitational pull until 10 ml remained. The eluent tube attached to each of the filter holders was clamped and the apparatus stored at 4°C.

Sample preparation Sample cells were plated onto 60mm plates at a density that gave a final cell number of 5×10^5 cells/ ml on the day of experiment. Internal standard cells were seeded into 92 mm plates to allow for an equivalent number of cells compared to the sample cells to be added to each filter on the day of experiment. Sample and internal standard cells were labelled for 1-2 generations. Sample cells were labelled with [2- 14 C] thymidine at a final concentration of 0.016 μ Ci/ml (this is preferred over [3 H] thymidine as it does not impede cell growth or affect DNA elution rate and is less damaging to DNA). Internal standard cells were labelled with [methyl- 3 H] thymidine at a final concentration of 0.1 μ Ci/ml. Following 24 h, the medium was aspirated and the cells were washed 1x with DULA, in order to remove any unincorporated label. The cells were then

resuspended in fresh medium and incubated at 37°C for at least 2 h to allow for the label to be chased into high molecular weight DNA.

For γ -irradiation studies, sample cells were trypsinised, resuspended in culture medium containing inhibitor(s) and transferred into bijoux bottles prior to irradiation (see individual figure legends for dosing schedules). Cells were kept on ice before, during and after irradiation to prevent cellular repair. Where cells were allowed to recover following irradiation, they were post-incubated at 37°C in a waterbath for the desired amount of time prior to elution, as described in the results. The internal standard cells were resuspended in PBS and irradiated at 100 Gy for neutral elution, 3 Gy for alkaline elution studies shortly before elution and held on ice until eluted. All handling of cells was carried out in the dark to prevent further damage to the DNA.

Elution protocol Approximately 5×10^5 sample cells were loaded onto each filter and an equal number of internal standard cells, as determined by haemocytometer count, were added to each filter. The tubing was unclipped and the cells allowed to drain onto filter. The cells were then washed with 2 ml lysis buffer or elution buffer (see appendix) for alkaline elution or neutral elution, respectively. The clips of the filtration funnels were fastened and the cells were treated with 1.5 ml lysis or elution buffer (alkaline or neutral elution, respectively, see appendix) containing 0.5 mg/ml proteinase K for 1 h in order to remove any protein associated with the DNA. The clips were unfastened and the cells washed with 2 mM EDTA, pH 10 (alkaline elution only). The eluent tubes from the filtration funnels were attached in numerical order to the corresponding tubing on a multichannel peristaltic pump that is in turn attached to a fraction collector. The syringe barrels were then reattached and 25 ml elution buffer was added to each barrel.

The pump was set at a slow and constant speed of (2 ml/h). The fraction collector was programmed to collect 8 fractions over 12 hrs at 90 minutes per fraction into scintillation vials containing 15mls scintillation fluid. Following 12 hrs the pump was continued for a further 20 minutes to collect any sample remaining in the tubes into fraction 9. The tubing was then disconnected from the filtration funnels and 0.4 M NaOH pumped through for another 20 minutes to flush the

system of any remaining counts, the filtration funnels were then dismantled and the filters placed on the bottom of fresh scintillation vials. 1 M HCl (0.4 ml) was added to each filter, the vial capped and the filter baked for 1 hour at 60°C to allow depurination of the DNA. Apurinic sites are converted to strand breaks by this procedure, causing the DNA to fragment and be released from the filters. To neutralise the acid, 2.5 ml 0.4 mM NaOH was then added to each vial and the vials shaken and left at room temperature for 1 hour. 15 ml of scintillant was added to each vial. All vials were capped, shaken and then placed in racks onto a scintillation counter. The levels of ^3H (fraction of internal standard DNA) and ^{14}C (fraction of sample DNA) present in each of the vials were determined using a 1 minute dual label count.

Calculation of results The total amount (T) of either ^3H or ^{14}C initially applied to the filter was calculated for each sample by the addition of the counts from all 10 fractions (where the tenth 'fraction' contains the filter). The number of counts retained on the filter after each fraction had eluted (R) was then determined using cumulative addition and the fraction of total (R/T) was then calculated. The data was plotted as the fraction of [^{14}C] thymidine remaining on the filter against the fraction of [^3H] thymidine using a double log scale and a reverse x axis, to give the elution profile (An example profile is given in Figure 2.2).

Regression analysis of each profile was performed to calculate the relative retention (R.R) of each sample i.e. the fraction of sample DNA retained on the filter when 50 % of the internal standard had eluted, using a point to point curve plot using GraphPad Prism software as shown in Figure 2.2. In order to put a numerical value on the difference of the elution profile of irradiated/drug treated samples compared to untreated controls, which would also facilitate inter-experimental comparisons, relative elution (the ratio of elution of DNA after treatment compared to an untreated control) was then calculated using the following equation :-

$$\text{Relative elution (RE)} = [\log \text{ control RR}] - [\log \text{ sample RR}]$$

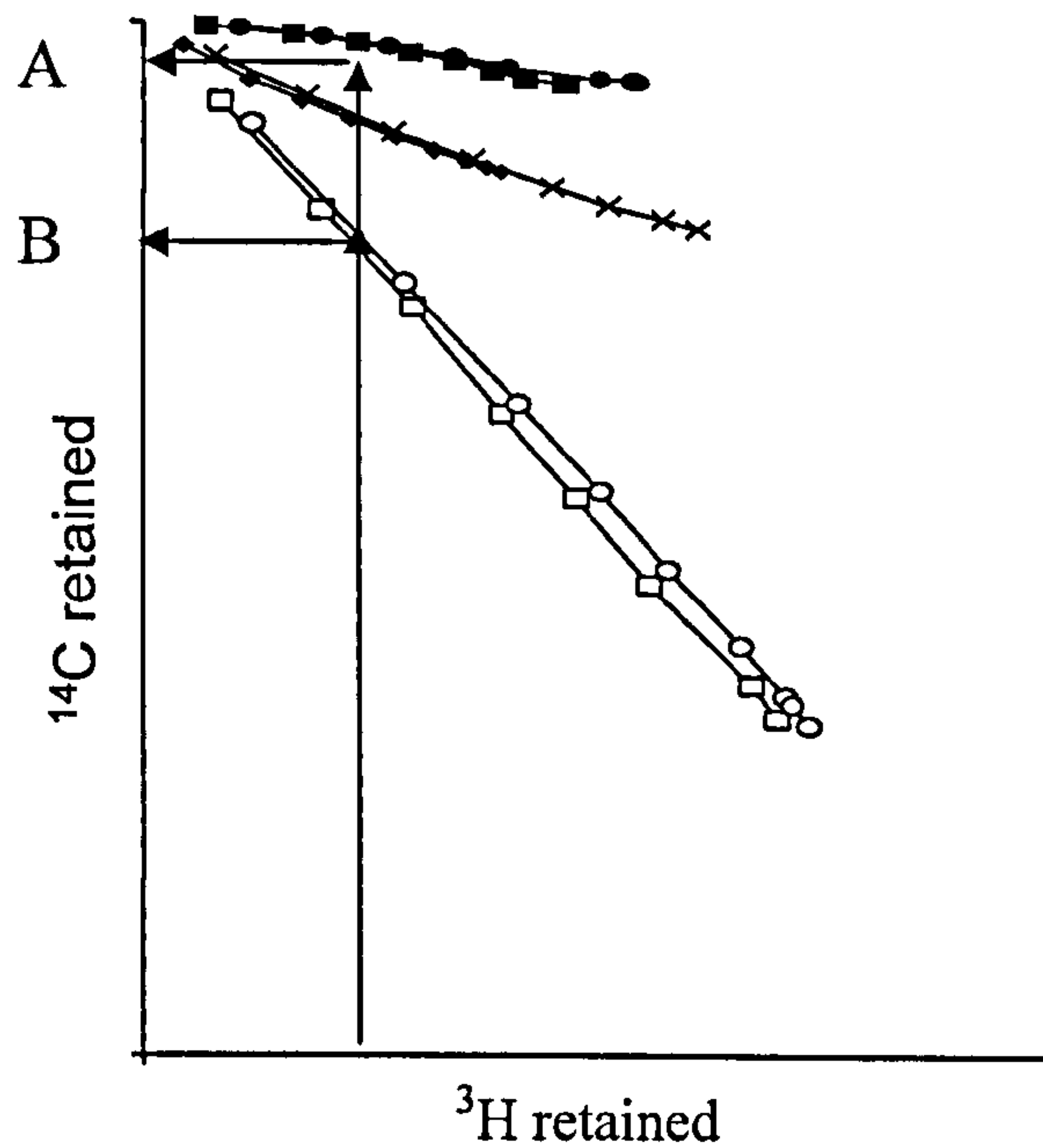


Figure 2.2 Calculation of Relative Retention

Representative elution profile and the readings taken in order to calculate the 'relative retention' (RR) for control untreated and treated samples, from which relative elution (RE) values can be calculated as ; $[\log A] - [\log B]$.

% Repair

$$= 100 - [a/b \times 100]$$

$$a = \text{RE} \times \text{h post-IR}$$

$$b = \text{RE} \ 0 \text{ h post-IR}$$

% Inhibition of repair

$$= 100 - [c/d \times 100]$$

$$c = (\% \text{ repair} + \text{inhibitor(s)} \times \text{h post-IR}) - (\% \text{ repair } 0 \text{ h post-IR})$$

$$d = \% \text{ repair} \times \text{h post-IR} - \% \text{ repair } 0 \text{ h post-IR}$$

2.9 WESTERN BLOTTING

Assay Principle Western blotting is the separation and immunodetection of specific proteins. The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity and its molecular mass. Proteins are first separated by mass under the denaturing conditions of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then specifically detected by immunoassay.

Sodium dodecyl sulphate (SDS) is an anionic detergent that binds to the non-polar regions of amino acids. Treatment of proteins with SDS dissociates them into linear polypeptide structures and the negative charges provided by the bound detergent predominate over the intrinsic polypeptide charges so that they have a constant mass:charge ratio. Migration of these anionic proteins within polyacrylamide gels in an electric field is thus dependent on the overall mass (size) of the proteins.

Western blotting is a method by which proteins, previously separated by SDS-PAGE are immobilised ('blotted') on a membrane support by electrophoresis transfer and subsequently detected using specific antibodies that will recognise and bind to the target protein on the membrane. The panel of antibodies used in the studies described in this thesis are shown in Table 2.1. Following application of these primary antibodies, enzyme conjugated secondaries were used. These secondaries are specific to the species of the primary antibodies. When conjugated with horseradish peroxidase (HRP), these secondary antibodies allow detection of target proteins using an ECLTM chemilluminiscent system and visualisation of the immune complex using X-ray film.

Sample preparation Cell lysates were prepared by the addition of 100 µl of SDS lysis buffer (see appendix) to a 2 ml plate. The cells were then collected into an Eppendorf using a cell scraper. (Cells were maintained on ice throughout sample preparation). Each cell lysate was sonicated for 10s at an amplitude of 10 microns and either stored on ice until loading onto the gel or frozen at -20°C. Following protein estimation (section 2.10), samples were diluted in lysis buffer and 4x sample buffer (see appendix) was added to give a final concentration of 2.5 µg/µl. Sample

buffer contains DTT which acts as a reducing agent, cleaving any disulphide bonds within/between the polypeptide chains. Samples were then boiled for 10 mins at 95°C in order to allow full denaturation of the proteins present in the sample.

Protein separation by SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Directly after heating, 20 µl (50 µg) samples were loaded onto either 4-20 % tris-glycine or 3-8 % tris-acetate precast polyacrylamide gels capable of separating proteins ranging from 4-250 kDa and 40-400 kDa, respectively, (choice dependent on size of protein to be separated). A pre-stained molecular weight maker (5 µl) was loaded. This permits the molecular weights of the translated proteins to be determined by comparing their mobility with the mobility of the marker peptides under the same conditions.

Gels were run in SDS electrode (running) buffer (see appendix) at a constant voltage of 200 V for approximately 45 minutes or until the dye front reached the bottom of the gel. Since the proteins within the sample are all negatively charged, they migrate from the positive anode (where they are loaded) to the negative cathode according to polypeptide size.

Immunoblotting Transfer of separated proteins from the gel to Hybond™ C nitrocellulose membrane was achieved by the application of an electric current which passed through the gel in the direction of the membrane, carrying with it the protein. The membrane was soaked in transfer buffer (see appendix) for 30 minutes prior to blotting. The transfer apparatus was set up as described in the manufacturers instructions and the proteins were transferred overnight in 1x transfer buffer using a constant voltage of 30 V or at 100 V for 1 h on ice.

Following electrophoretic transfer, the membrane was incubated (with constant agitation) for one hour in TBS-Tween (see appendix) containing 5 % non-fat milk. This was necessary to 'block' all of the unoccupied non-specific binding sites of the membrane prior to immunodection as it prevents background binding of antibodies to these sites. Primary antibodies were diluted (see table 2.1) in TBS-Tween 5 % milk and applied to the membrane. During this time, an antibody-antigen complex will form. Incubation in primary antibody was performed for one

hour. Following four 5 min washes in TBS-Tween, a peroxidase conjugated goat anti-mouse/rabbit secondary antibody (DAKO, UK), diluted 1:1000 in milk TBS-Tween (5 % milk), was added to the membrane and incubated for one hour. After this final incubation, four 15 minute washes in TBS-Tween were performed before detection of proteins by enhanced chemiluminescence (ECL).

PROTEIN	ANTIBODY	SPECIES	COMPANY	DILUTION
PARP-1	H250	Rabbit	Santa Cruz, CA	1:1000
Ku70	AHP 316	Rabbit	Serotec, Oxford, UK	1:1000
Ku80	AHP317	Rabbit	Serotec, Oxford, UK	1:1000
DNA-PKcs	Ab4 cocktail	Mouse	Strattech Scientific, Cambs, UK	1:1000
Topo IIα	18511	Rabbit	*	1:500
Actin	Clone AC-40	Mouse	Sigma, Dorset, UK	1:1000

Table 2.1 Table of primary antibodies

* Kindly provided by Dr Elaine Wilmore, Biochemictry and Genetics, University of Newcastle upon Tyne (Cowell *et al.*, 1998)

ECL development Chemiluminescence is the emission of light from a substance, which by chemical reaction, is caused to be in an excited state. The ECL™ detection system involves the oxidation of HRP (by peracid) followed by peroxidation and excitation of luminol, a cyclic diacylhydrazide. In alkaline conditions this excited luminol decays to ground state with the emission of light. The emission is detected by exposure to X-ray film.

Following the final wash in TBS-Tween, ECL detection was performed according to the instructions of the manufacturer. Briefly, the membrane was incubated in a solution comprising one part detection solution (1) and one part detection solution (2). After 1 minute the detection solution was poured off, the membrane wrapped in cling film and then exposed to X-ray film for the desired time (usually between 10 seconds and 5 minutes). The film was developed using an automatic developer.

2.10 PROTEIN ASSAY

Assay Principle The method utilised was the bicinchoninic acid (BCA) protein assay. This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in alkaline medium (the biuret reaction) with the colorimetric detection of the cuprous cation (Cu^{1+}) using a reagent containing bicinchoninic acid forming a purple colour which is proportional to the protein concentration.

Assay Albumin standard containing bovine serum albumin (BSA) at 2 mg/ml was used to prepare six standard solutions of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml. Samples were typically diluted 1:10 with dH_2O . Standards, samples and dH_2O blanks were loaded in 10 μl volumes in quadruplicate onto 96 well plates. BCA reagent A (25 mls) and BCA reagent B (5 mls) were combined and 190 μl of the mixture was added to each well and then mixed on a plate shaker for 30 seconds. The plate was covered in cling film and incubated at 37 °C for 30 minutes. Absorbance at or near 562 nm was read on a plate reader and unknown protein concentrations were calculated from the standard curve, taking into account the 10-fold dilution factor.

2.11 PARP PERMEABILISED CELL ASSAY

Assay principle PARP activity was measured in whole cells by using a modification of the published method by Halldorsson *et al.*, (1978) The principle of this technique relies on the incorporation of the ADP-ribose moiety of [^{32}P]- NAD^+ into acid-insoluble ADP-ribose polymer. The presence of a DNA oligonucleotide (see appendix) activates PARP-1 which uses both itself and intracellular proteins as

poly(ADP-ribose) acceptors. The amount of radiolabel incorporated into the polymer is directly proportional to PARP activity. As neither the NAD^+ nor oligonucleotide can enter intact cells, the plasma membrane was rendered 'permeable' by either hypotonic shock or treatment with digitonin (see appendix).

NAD^+ : A stock solution of approximately 6 mM NAD^+ was freshly prepared in sterile water on the day of each assay. The optical density (OD) at 260 nm of a 1/100 dilution of this stock NAD^+ solution was measured using spectrophotometer and a 1 cm silica cuvette. The Molar extinction coefficient (ϵ) for NAD/NADH at 260 nm = 18000 and therefore $18 \cdot \text{OD} = 1 \text{ mM } \text{NAD}^+$ (Beer Lambert Law). The absolute molarity of the stock solution was calculated and a working solution of 600 μM NAD^+ was prepared to which approximately 10 μCi [^{32}P] NAD^+ was added. The volume of radiolabel added was adjusted according to its current specific activity, a reflection of half life and rate of degradation.

Preparation of the permeabilised cells All cells were harvested by trypsinisation and the cells counted using a haemocytometer (see section 2.2). The cells were then washed once in ice cold DULA and the pellet resuspended into ice cold digitonin at a density of 3×10^7 cells/ml. Following 5 minute incubation on ice, 9 volumes of ice-cold isotonic buffer (see appendix) was added and the extent of permeabilisation was determined by a trypan blue exclusion haemocytometer count. If kept on ice, the cells remain useable for up to 1 hour.

Assay The components of the reaction mixture (5 μl oligonucleotide (see appendix), 50 μl NAD^+ and 8 μl 50 x inhibitor/100 % DMSO) were added to 15 ml polycarbonate conical bottomed tubes and positioned in a shaking waterbath at 26°C . The volume was made up to 100 μl with sterile water and the final concentrations of each of the reaction components are shown in Table 2.2. Each sample was performed in quadruplicate. The previously prepared cell suspension(s), were warmed at 26°C for 7 minutes. The reaction was initiated by the addition of 300 μl of the required cell suspension at 5 second intervals to each of the reaction tubes using an Eppendorf repeat dispenser. Each tube was then thoroughly vortexed and incubated for 5 minutes before the reaction was terminated by the addition of 2 ml ice cold TCA (10

% w/v TCA, 10 % w/v Na pyrophosphate). Assay blanks (T_0) which correct for non-specific binding of free $[^{32}\text{P}]\text{-NAD}^+$ to the filter were prepared by adding 2 ml TCA before the addition of cell suspension, thereby, preventing the reaction being initiated. Following the addition of TCA, all tubes were plunged into ice and left for at least an hour to allow precipitation of acid-insoluble material.

Table 2.2 Permeabilised PARP Assay Reaction Mixture

Reagent	Stock Concentration	Final Concentration
NAD^+	600 μM	75 μM
Oligonucleotide	200 $\mu\text{g/ml}$	2.5 $\mu\text{g/ml}$
Drug/DMSO	50 x drug/ 100 % DMSO v/v	1 x drug/ 1 % DMSO v/v

In order to separate the TCA –precipitated macromolecules which include poly(ADP-ribosylated) proteins from the soluble components such as free $[^{32}\text{P}]\text{-NAD}^+$, the reaction mixtures were filtered using a Millipore filtration system GF/C (25 mm) microfibre filters were moistened with 10 % TCA and placed rough side upwards onto the filter holder of the Millipore filtration apparatus. The funnel assembly was then secured tightly on top. The contents of the tubes were added to individual funnels and each was rinsed 5 times with TCA (1 % w/v TCA, 1 % w/v Na pyrophosphate) under gentle suction pressure. The filters were removed, air dried and placed into scintillation vials containing 10 ml scintillation fluid. Four standards (S) containing 5 μl of the 600 μM NAD^+ + $[^{32}\text{P}]\text{-NAD}^+$ solution were prepared. All vials were counted on a β particle scintillation counter for 2 minutes.

Calculation of results The number of DPM which represent 1 pmol NAD⁺ (C) was calculated from the mean DPM of the four standards (S_M), which for a 600 μM NAD⁺ stock (5 μl of a 600 μM = 3000 pmol) is;

$$S_M/3000 = \text{DPM}/\text{pmol NAD}^+ = C$$

The pmol NAD⁺ present in each of the samples (C_S) was then calculated by first subtracting the mean of the T₀ samples (T_{0M}) from each of the sample counts thus removing non-specific background counts. The value obtained (P₀) for each sample was then divided by the number of DPM equivalent to 1 pmol NAD⁺ (C) to give pmol NAD⁺ in each sample (C_S). As 300 μl of cell suspension was used per sample, this was converted to pmol NAD⁺ in 1 ml of cell suspension. Finally, using the cell count taken prior to the experiment, results were expressed as pmol NAD⁺ incorporated/10⁶ cells.

2.12 PURIFIED PARP-1 ASSAY

Assay Principle The activity of purified PARP-1 was assayed by modification of the permeabilised cell assay. The enzyme was activated by the same oligonucleotide(s) and activity was again measured by the incorporation of [³²P]-NAD⁺ into acid insoluble material. The reaction components were also the same, except for the addition of histones that serve as the acceptor proteins for the ADP-ribose polymers.

Purified PARP-1 working solution: Immediately before the assay, purified PARP-1 protein (see section 2.1.4) was diluted 1/500 in PARP-1 buffer (see appendix) to give a 2 μg/ml working solution.

NAD⁺ working solution: A fresh solution of 1mM NAD⁺ (see section 2.11) was made up on the day of assay and approximately 10 μCi [³²P]-NAD⁺, s.a.=1000 Ci/mmol, was added. As in the permeabilised cell assay, the volume of radiolabel added was adjusted according to its current specific activity, a reflection of half life and rate of degradation. 1 ml NAD⁺, 5 μl histone solution and 25 μl oligonucleotide

(see Table 2.3 for stock concentrations) were then added to 1.5 ml NAD⁺ buffer (see appendix).

Assay 50 µl aliquots of the NAD⁺ working solution were dispensed into 15 ml polycarbonate conical bottomed tubes. 1 µl 100 x inhibitor/100 % DMSO was then added to each tube and the tubes warmed in a shaking waterbath at 26°C. Each sample was carried out in quadruplicate. The reaction was initiated by the addition of 50 µl purified PARP-1 working solution to each tube at 5 second intervals using an Eppendorf repeat dispenser. The final concentration of each of the reaction components are shown in Table 2.3. Each tube was then thoroughly vortexed and incubated for 5 minutes before the reaction was terminated by the addition of 25 µl ice cold TCA (99 % w/v TCA, 1 % w/v Na pyrophosphate). Assay blanks (T₀) were prepared by the addition of 25 µl TCA prior to the addition of purified PARP-1 protein, thereby preventing the reaction from being initiated and accounting for non-specific binding of free [³²P]-NAD⁺ to the filter.

Table 2.3 Purified PARP-1 Assay Reaction Mixture

Reagent	Stock Concentration	Final Concentration
NAD ⁺	1 mM	200 µM
Oligonucleotide	200 µg/ml	1 µg/ml
Drug/DMSO	100 x/100 % DMSO v/v	1x/1 % DMSO v/v
Histones	1 mg/ml	1 µg/ml
PARP-1 protein	1 mg/ml	1 µg/ml

The tubes were left on ice for 30 minutes to allow precipitation of acid insoluble material. The samples were then filtered using a Millipore filtration system as in the permeabilised cell assay. Each sample was washed 5 times with 5 % w/v TCA/ 1% w/v Na pyrphosphate. Four standards (S) containing 5 µl of the 1 mM NAD⁺ + [³²P]-NAD⁺ solution were prepared. All vials were counted on a β particle scintillation counter for 2 minutes.

Calculation of results The mean of the To samples (T_{0M}) values were subtracted from sample counts. The values thus obtained were converted to pmol NAD⁺ in each sample by reference to mean standard counts (S_M) (5 µl 1 mM NAD⁺ = 5000 pmol), as described for the permeabilised cell assay. Results were expressed as pmol NAD⁺ incorporated/µg protein.

2.13 IMMUNO DOT-BLOT TO DETECT ADP-RIBOSE POLYMER FORMATION

Assay Principle This is an assay which is based on a technique first described by Afar *et al.*, 1998, and has been optimised in the Northern Institute for Cancer Research It relies on the immunodetection of ADP-ribose polymer bound to a membrane using a specific antibody which shows no cross reactivity with DNA, RNA and monomers of ADP-ribose or NAD⁺ (Kawamitsu *et al.*, 1984; Menard and Poirier, 1987). Although the methodology is very similar to the PARP permeabilised cell assay, this assay has the advantage of requiring much smaller cell numbers and it does not involve the use of radioactivity.

	³² P-NAD ⁺ assay	Dot blot
Cell permeabilisation	Digitonin	Digitonin
PARP activity	Maximally stimulated by oligonucleotide	Maximally stimulated by oligonucleotide
Substrate	75 µM NAD ⁺ with ³² P-NAD ⁺	75 µM NAD ⁺
Reaction time	6 minutes	6 minutes
Product measured	Amount of ³² P-NAD ⁺ incorporated in reaction time	Amount of polymer >10 subunits detected with monoclonal antibody
Detection method	β emission scintillography	Chemiluminescence
Quantification	3000 pmol hot NAD standard	PAR purified polymer
Cells required	1 million/reaction	3-10,000/reaction

Table 2.4 Comparison of PARP permeabilised cell assays

Preparation of permeabilised cells All cells were harvested by trypsinisation and the cells counted using a haemocytometer (see section 2.2). The cells were washed once in ice cold PBS and the pellet resuspended into ice cold permeabilisation buffer (see appendix) supplemented with 0.015 % digitonin at a density of 3×10^5 cells/ml. Following 1 minute incubation on ice, 4 ml ice cold permeabilisation buffer/ml cells was added and the cells pelleted by centrifuging at 1500 rpm for 10 minutes at 0°C. The pellet was then resuspended in ice cold permeabilisation buffer at 1.25×10^7 cells/ml and the extent of permeabilisation determined by a trypan blue exclusion haemocytometer count. If kept on ice, the cells remain useable for up to 1 hour.

Assay The cells were warmed to 30°C in a shaking waterbath. 34 µl aliquots of reaction buffer, 25 µl aliquots of oligonucleotide and 1 µl 100 x inhibitor/100 % DMSO were dispensed into 15 ml polycarbonate conical bottomed tubes. The final concentration of each of the reaction components are shown in Table 2.5. The reaction was initiated by the addition of 5×10^5 cells (40 µl) to each tube and terminated after 6 minutes by the addition of an excess of AG14361 (termination buffer, see appendix). The tubes were vortexed and transferred to ice.

Table 2.5 Immuno dot-blot Assay Reaction Mixture

Reagent	Stock Concentration	Final Concentration
NAD	1 mM	300 µM
Oligonucleotide	200 µg/ml	50 µg/ml
Drug	100 x/100 % DMSO v/v	1x/1% DMSO v/v
Termination buffer (AG14361)	1.25 µM	1 µM

Immunodetection of ADP-ribose polymer All samples were diluted in PBS and 1000, 3000 or 5000 cells (total volume 50 µl) / sample were loaded in duplicate onto Hybond-N membrane by addition to separate wells of a 96-well manifold. 100 µl TCA (10 % w/v TCA, 2 % w/v Na pyrophosphate) was then added to each well followed by 200 µl 70 % v/v ethanol. The wells were aspirated and the membrane was rinsed in PBS three times and then incubated (with constant agitation) for one hour in PBS-tween containing 5 % (see appendix) non fat milk in order to block all

of the unoccupied non-specific binding sites of the membrane. The membrane was then incubated overnight at 4°C (with constant agitation) in primary antibody (see appendix) at a final concentration of 2.5 µg/ml in PBS-milk tween. Following two 5 minute washes in PBS-tween, an anti-mouse secondary antibody (DAKO) diluted 1/1000 in PBS-milk tween was added to the membrane and incubated for one hour at room temperature. After this final incubation, four 15 minute washes in PBS-Tween were performed before enhanced chemiluminescence (ECL) detection on photographic film as described in section 2.9.

2.14 PURIFIED DNA-PK ASSAY

Assay Principle Upon activation by DNA strand breaks, DNA-PK phosphorylates several DNA binding substrates *in vitro* including the tumour suppressor protein, p53, the SV40 large T antigen and several transcription factors using ATP as a substrate (Anderson and Lees-Miller, 1992). In this assay, the presence of a double stranded oligonucleotide, activates DNA-PK which phosphorylates both itself and a wt p53 peptide substrate flanking serine 15 on native p53 (EPPLSQEAFADLLKK) (Finnie *et al.*, 1995). The amount of [³²P] incorporated into the peptide substrate and therefore bound to the filter is proportional to DNA-PK activity and is determined by liquid scintillation counting.

Assay The components of the reaction mixture (1 µl of ; purified DNA-PK, oligonucleotide, peptide and inhibitor/DMSO) were added on ice into an eppendorf and the volume made up to 9 µl with DNA-PK buffer (see appendix). The reaction was initiated by the addition of 1 µl 500 µM ATP containing 0.5 µCi [³²P] ATP (s.a 3000 Ci/mMol). Each tube was thoroughly vortexed and incubated for 10 minutes at 30°C. The reaction was terminated by the addition of 10 µl 30 % (v/v) acetic acid and enzyme activity quantitated by spotting half the reaction onto phosphocellulose paper, washing and subjecting to liquid scintillation counting. Assay controls were prepared by carrying out the reaction in the absence of oligonucleotide. Four standards (S) containing 5 µl of the 500 µM ATP + [³²P]-ATP solution were also prepared by spotting onto phosphocellulose paper and eliminating the washing step.

Table 2.6 Purified DNA-PK Assay Reaction Mixture

Reagent	Stock Concentration	Final Concentration
ATP	500 µM	50 µM
Oligonucleotide	100 µg/ml	10 µg/ml
Drug	10 x/10 % DMSO v/v	1x/1% DMSO v/v
Peptide	2 mM	200 µM
DNA-PK	500 µg/ml	50 µg/ml

Measurement of DNA-PK activity in nuclear extracts For comparison of enzyme activities in the cell lines, DNA-PK activity was assayed in nuclear extracts. Nuclear extracts were obtained using a cell lytic nuclear extraction kit and following manufacturers instructions (Sigma-Aldrich Company Ltd. Dorset, UK). DNA was removed from the extracts using high salt elution (fast flow DEAE sepharose, Amersham Pharmacia, Bucks, UK). Protein concentrations of extracts were quantified using a standard protein assay protocol as described in section 2.10. Samples were diluted to give stock concentrations of 50 mg/ml. 50 µg aliquots were then assayed for DNA-PK activity.

Calculation of Results Results were expressed as pmol ATP incorporated /µg protein. The mean of the no oligonucleotide assay control values were subtracted from sample counts. The values thus obtained were converted to pmol ATP in each sample by reference to mean standard counts (S_M) (5 µl 500µM ATP = 2500 pmol) as described for the permeabilised cell assay.

2.15 COMBINED PURIFIED PARP-1 AND DNA-PK ASSAYS

Based on the known molecular weights of the enzymes (116 kDa for PARP-1 and 623 kDa for the DNA-PK holoenzyme), enzymes were added at 1;1, 1;2, and 1;3 molar ratios (see Figure legends for details).

When PARP-1 activity was assayed in the presence of DNA-PK, the enzymes were mixed prior to addition to the reaction mix and carried out as for the purified PARP-1 assay described in section 2.12. Where specified, ATP (50 μ M final concentration) was added to the reaction mix. Conversely, when DNA-PK was assayed in the presence of PARP-1, the assay was carried out as described in section 2.14, with the exception that the buffer used was the same as the final buffer used in the purified PARP-1 assay (see appendix for buffer constituents). DNA-PK activity (when ATP and p53 peptide were present) was not compromised by the alterations in the constituents of the assay buffers. The enzymes were mixed prior to addition to the reaction mix. Where specified, NAD⁺ (200 μ M final concentration) was added to the reaction mix.

2.16 PURIFIED ENZYME ASSAYS FOR THE COMPARISON OF WORTMANNIN AND NU7026 AGAINST THE PIKK FAMILY OF ENZYMES

The following assays were performed by staff at KuDOS pharmaceuticals, Cambridge, UK. Mammalian DNA-PK (500 ng/ μ l) was isolated from HeLa cell nuclear extract following chromatography utilising Q-sepharose, S-sepharose and Heparin agarose. DNA-PK (250 ng) activity was measured at 30°C, in a final volume of 40 μ l, in buffer (see appendix) and 1 mg of the substrate GST-p53N66 (the amino terminal 66 amino acid residues of human wild type p53 fused to glutathione S-transferase) in polypropylene 96 well plates. Varying concentrations of inhibitor (in DMSO at a final concentration of 1 % v/v) were added to the reaction mix. After 10 minutes of incubation, ATP was added to give a final concentration of 50 μ M along with a 30 mer double stranded DNA oligonucleotide (final concentration of 0.5 ng/ml) to initiate the reaction. After 1 h with shaking, 150 μ l of phosphate buffered

saline (PBS) were added to the reaction and 5 µl then transferred to a 96 well opaque white plate containing 45 µl of PBS per well, where the GSTp53N66 substrate was allowed to bind to the wells for 1 h. To detect the phosphorylation event on the serine 15 residue of p53 elicited by DNA-PK, a p53 phosphoserine-15 antibody (Cell Signaling Technology, Beverly, MA) was used in a basic ELISA procedure. An anti-rabbit HRP conjugated secondary antibody (Pierce-Perbio Science, Cheshire, UK) was then employed in the ELISA before the addition of chemiluminescence reagent (Renaissance, New England Nuclear) to detect the signal as measured by chemiluminescent counting via a TopCount NXT (Perkin Elmer-Packard).

The protocols used to detect ATM kinase and ATR kinase activities were performed essentially according to the methodologies described previously (Banin *et al.*, 1998; Tibbetts *et al.*, 1999). ATM and ATR were immunoprecipitated using rabbit polyclonal antisera raised to the COOH-terminal 400 amino acids of ATM, and antisera raised to amino acids 400-480 of ATR respectively. The PI 3-Kinase assay was performed essentially as described previously (Wymann *et al.*, 1996), using baculoviral derived recombinant p110α and p85α (a kind gift from Prof. Mike Waterfield, Ludwig Institute, London).

The concentration(s) of inhibitor(s) to inhibit kinase activity by 50 % (IC₅₀ values) for the compounds in all the enzymes assays were derived from sigmoidal plots using the graphic package Prism, in which the enzyme activity in the varying concentration of compounds was plotted against the concentration of compound.

2.17 PERMEABILISED DNA-PK ASSAY

Assay Principle A DNA-PK permeabilised cell assay was developed by modification of the purified DNA-PK assay described in section 2.14 and the PARP permeabilised cell assay, described in section 2.11. The enzyme was activated by the same 30 bp double stranded blunt-ended oligonucleotide (see appendix) and activity was measured by the binding of ³²P labelled phosphate groups onto phospho-specific cellulose filter paper as described for the purified DNA-PK assay.

Preparation of the permeabilised cells All cells were harvested by trypsinisation and the cells counted using a haemocytometer (see section 2.2). The cells were then washed once in ice cold DULA and the pellet resuspended into ice cold digitonin at a density of 6×10^7 cells/ml. Following a 5 minute incubation on ice, 9 volumes of ice-cold isotonic buffer (see appendix) was added and the extent of permeabilisation was determined by a trypan blue exclusion haemocytometer count. If kept on ice, the cells remain useable for up to 1 hour.

Assay The components of the reaction mixture (25 μ l ATP spiked with 0.5 μ Ci [32 P]- ATP (s.a 3000 Ci/mMol), 10 μ l oligonucleotide, 4 μ l 50 x inhibitor/100 % DMSO and 4 μ l NAD^+) were added to 15 ml polycarbonate conical bottomed tubes and positioned in a shaking waterbath at 30°C. The volume was made up to 50 μ l with sterile water. The previously prepared cell suspension(s) were warmed at 30°C for 7 minutes. The reaction was initiated by the addition of 150 μ l of the required cell suspension at 5-second intervals to each of the reaction tubes using an eppendorf repeat dispenser. The final concentrations of each of the reaction components are shown in Table 2.7. Each tube was then thoroughly vortexed and incubated for 10 minutes at 30°C before the reaction was terminated by the addition of 100 μ l 60 % (v/v) acetic acid. Assay blanks (T_0) which correct for non-specific binding of free [32 P] to the filter were prepared by adding 100 μ l 60 % (v/v) acetic acid before the addition of cell suspension, thereby preventing the reaction being initiated. Following addition of acetic acid, all tubes were plunged into ice before spotting half the reaction onto phosphocellulose paper, washing and subjecting to liquid scintillation counting. Four standards (s) containing 5 μ l of the ATP + [32 P] ATP solution were also prepared by spotting onto phosphocellulose paper and eliminating the washing step.

Table 2.7 Permeabilised DNA-PK Assay Reaction Mixture

Reagent	Stock Concentration	Final Concentration
ATP	400 µM	50 µM
Oligonucleotide	200 µg/ml	10 µg/ml
Drug	50 x / 100 (v/v) % DMSO	1 x / 1 % (v/v) DMSO
NAD ⁺	3.75 mM	75 µM

Calculation of Results Results were expressed as pmol ATP incorporated/ 10⁶ cells. The number of DPM which represent 1 pmol ATP (C) were calculated from the mean DPM of the four standards (S_M), which for a 400 µM ATP stock (5 µl of 400 µM = 2000 pmol) is: -

$$S_M/2000 = \text{DPM/pmol ATP} = C$$

The pmol ATP present in each of the samples (C_s) was then calculated by first subtracting the mean of the T₀ samples from each of the sample counts thus removing non-specific background counts. The value obtained for each sample was then divided by the number of DPM equivalent to 1pmol ATP (C) to give pmol ATP in each sample. As 150 µl cell suspension was used per sample, this was converted to pmol ATP in 1 ml of cell suspension (Taking into account the fact that only half of the reaction was spotted onto the filter). Finally, using the cell count taken prior to the experiment, results were expressed as pmol ATP incorporated per 10⁶ cells.

2.18 CURVE FITTING AND STATISTICAL ANALYSIS

All graphs presented in this thesis were prepared using GraphPad Prism (GraphPad Software, Inc, California, USA), and either point to point, linear or sigmoidal dose response curves were fitted using linear or non-linear regression analysis.

To test for differences between groups of data and assess their significance, unpaired Student’s t-tests were used unless stated otherwise. A Student’s two-tailed unpaired t-test assumes a Guassian distribution and equal standard deviations. A p

value of <0.05 was deemed to mean that the groups of data were significantly different at a 5 % confidence interval.

CHAPTER 3

CHARACTERISATION OF CELL LINES AND THE NOVEL PARP-1 AND DNA-PK INHIBITORS, AG14361 AND NU7026

3.1 Introduction

As a major approach to elucidating the physiological roles of PARP-1 and DNA-PK in DNA repair, a number of techniques have been developed which result in aberrant or reduced cellular PARP-1 or DNA-PK activity. These are described in sections 1.8 and 1.11 and include mutant or knockout cell lines deficient in either of the two enzymes and small molecule inhibitors. Molecular strategies include the development of anti-sense constructs and dominant negative approaches. The studies presented in this thesis utilised cell lines proficient or deficient (by mutation or gene knockout) in PARP-1 or DNA-PK in combination with novel specific inhibitors of the two enzymes.

3.1.1 Choice of cell lines

Comparative studies of cell lines that have a common origin but different radiosensitivity provide valuable model systems where a greater understanding of the relationship between radiation damage and biological effects is sought. In order to facilitate data comparison, the Chinese hamster ovary (CHO) cell lines (see section 2.1.6) were selected for study in this thesis as they have been previously used in many studies investigating the cellular responses to IR. Moreover, well-established hamster cell lines are easily manipulated and have high plating efficiencies, properties that are advantageous when using techniques such as the clonogenic assay as an endpoint.

Radiation sensitive mutants have been isolated and categorised into 11 complementation groups designated XRCC1-11. The Chinese hamster xrs-6 and V3 cell lines used in the studies described in this thesis are deficient in the genes XRCC5 and XRCC7, which encode the Ku80 and DNA-PKcs components of DNA-PK,

respectively. The xrs-6 cell line was the first radiation sensitive rodent mutant to be described (Jeggo and Kemp, 1983) and analysis of these mutants has provided a link between DNA-PK and DSB repair (Taccioli *et al.*, 1994a). These cells have been shown to harbour a splice site mutation that results in a 13 bp insertion and frame shift thus giving rise to a 'null' phenotype (Singleton *et al.*, 1997). Although these cells are only partially complemented by transfection with human Ku80 cDNA, full complementation can be achieved with hamster Ku80 cDNA (Singleton *et al.*, 1997) and therefore the cell line used in studies described in this thesis is transfected with hamster cDNA. The V3 cells are derived from the hamster CHO AA8 cell line. They have an inactivating mutation (premature termination at residue 4024 important for kinase function) in the C-terminal region of one allele important for DNA-PKcs function (Blunt *et al.*, 1995). The DNA-PKcs deficiency was complemented by the stable transfection of a yeast artificial chromosome (YAC) encoding the human XRCC7 gene.

As a means to evaluate the biological contribution of PARP-1 during embryonic development, postnatal life and following genotoxic stress, various groups have generated PARP-1 knockout (KO) mice by interruption of Exon 1, Exon 2 or Exon 4 (section 1.8.5). The PARP-1 knockout mouse embryonic fibroblasts (MEFs) used in the studies described in this thesis are disrupted in exon 4 of the PARP-1 gene by homologous recombination (Figure 3.1). Confirmation that these cells are disrupted in exon 4 has been carried out within the department (P. Jowsey, unpublished results). Briefly, the wild type PARP-1 gene contains two EcoRI sites, which upon digestion give rise to a 9.6 kb fragment. The disruption of the PARP-1 gene by the insertion of a neomycin resistance cassette (phosphoglycerate kinase promoter followed by the neo gene) into exon 4 of the PARP-1 locus introduces an extra EcoRI site which upon digestion produces a 3.3 kb fragment. Southern blotting using a radiolabelled probe (kindly provided by Gilbert de Murcia, Ecole Supérieure de Biotechnologie de Strasbourg) produced bands at the corresponding fragment sizes thus confirming the genotype of the PARP-1 ^{-/-} cells.

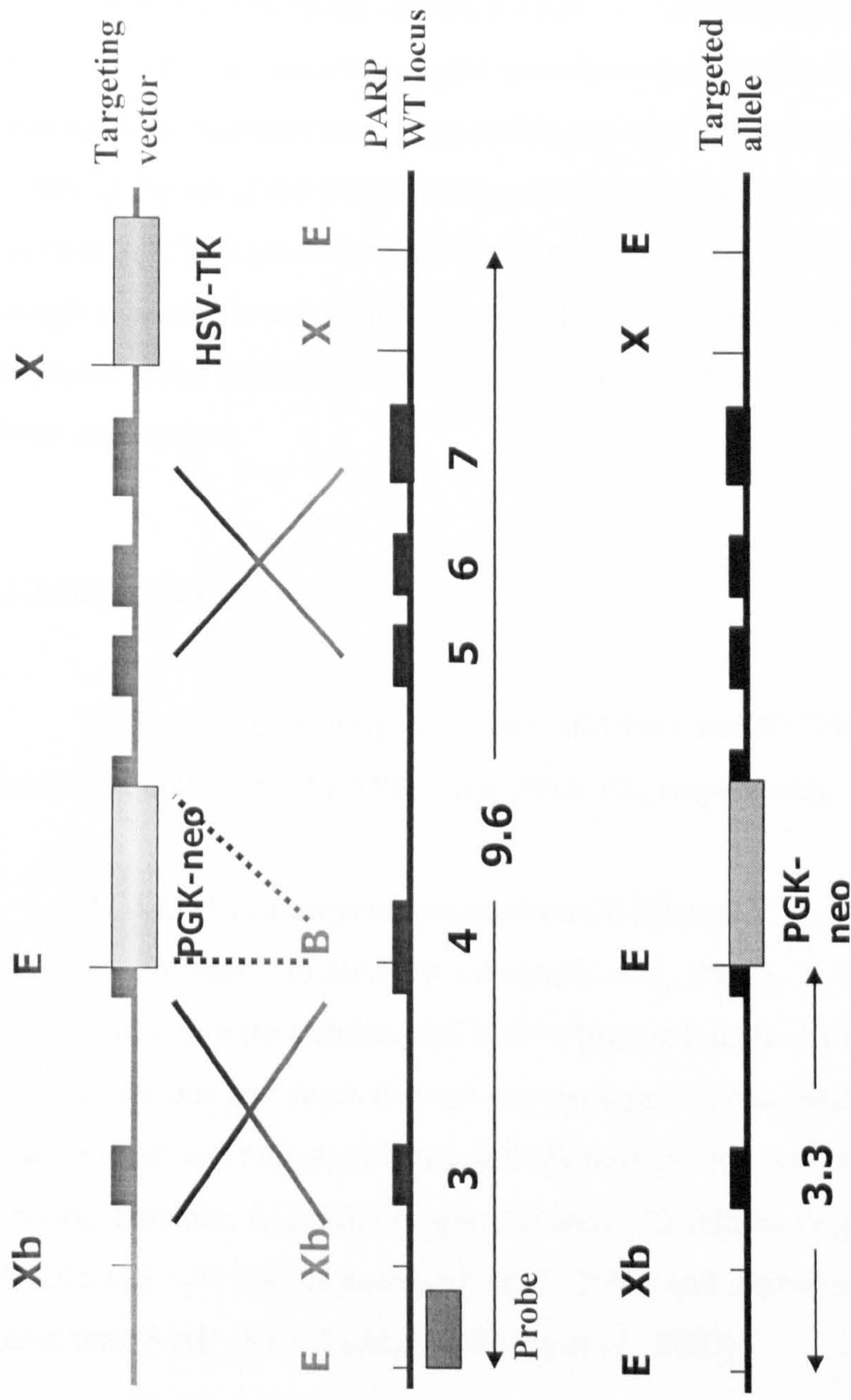


Figure 3.1 Disruption of Exon 4 in PARP-1 $-/-$ MEFs by Homologous Recombination

Inactivation of the PARP-1 gene by homologous recombination. Demonstration that the insertion of a neomycin resistance cassette into exon 4 of the PARP-1 gene gives rise to an extra EcoRI site. Scheme of targeting construct (Top), the PARP-1 gene and hybridization of the probe (middle) and the targeted allele (bottom). Xb, Xba restriction enzyme site; E, EcoRI restriction enzyme site; B, BamH restriction enzyme site; X, Xho restriction enzyme site; pGK-neo, neomycin resistance gene driven by the pGK promoter; HSV-TK, thymidine kinase gene driven by the herpes simplex virus promoter. Adapted from

Menissier de Murcia *et al*, 1997..

In view of the fact that the primary MEFs were slow growing (doubling time approximately 50 h) and were unable to form colonies, spontaneously immortalised derivatives were derived. Analysis of the p53 status of these cell lines during the course of this research established that the p53 basal levels were unusually high in the PARP-1 +/+ cells compared to the PARP-1 -/- cells. The p53 gene was sequenced and it was found that the PARP-1 -/- had a wt p53 sequence but the PARP-1 +/+ had an inactivating p53 mutation which rendered p53 unable to act as a transcriptional transactivator ; Asp to Glu substitution at codon 278 within the conserved region of the DNA binding domain (P. Jowsey, unpublished results). This mutation may be a product of the development of the cells from embryonic cells through senescence and immortalisation. The significance of a difference in the functional status of p53 with respect to the results obtained in this thesis is explored where appropriate.

3.1.2 Inhibitors

At the time of writing this thesis AG14361 and NU7026 were the current benchmark inhibitors of PARP-1 and DNA-PK, respectively.

AG14361 is a tricyclic benzamidazole (Figure 3.2) and is a competitive inhibitor with respect to NAD^+ (Canonkoch *et al.*, 2002). It is highly soluble and exhibits good in vitro stability and in vivo (mouse) pharmacokinetics. Additionally, at concentrations and doses that are non toxic per se, this inhibitor demonstrates potent chemo-and radiosensitising activity both in vitro and in vivo (Calabrese *et al.*, in press). Notably, AG14361 is approximately 10-fold more potent ($K_i < 5\text{nM}$) than NU1025 ($K_i = 50\text{ nM}$) (Canonkoch *et al.*, 2002) and approximately 1000-fold more potent than 3-AB ($K_i = 3\text{ }\mu\text{M}$) (Skalitzky *et al.*, 2003).

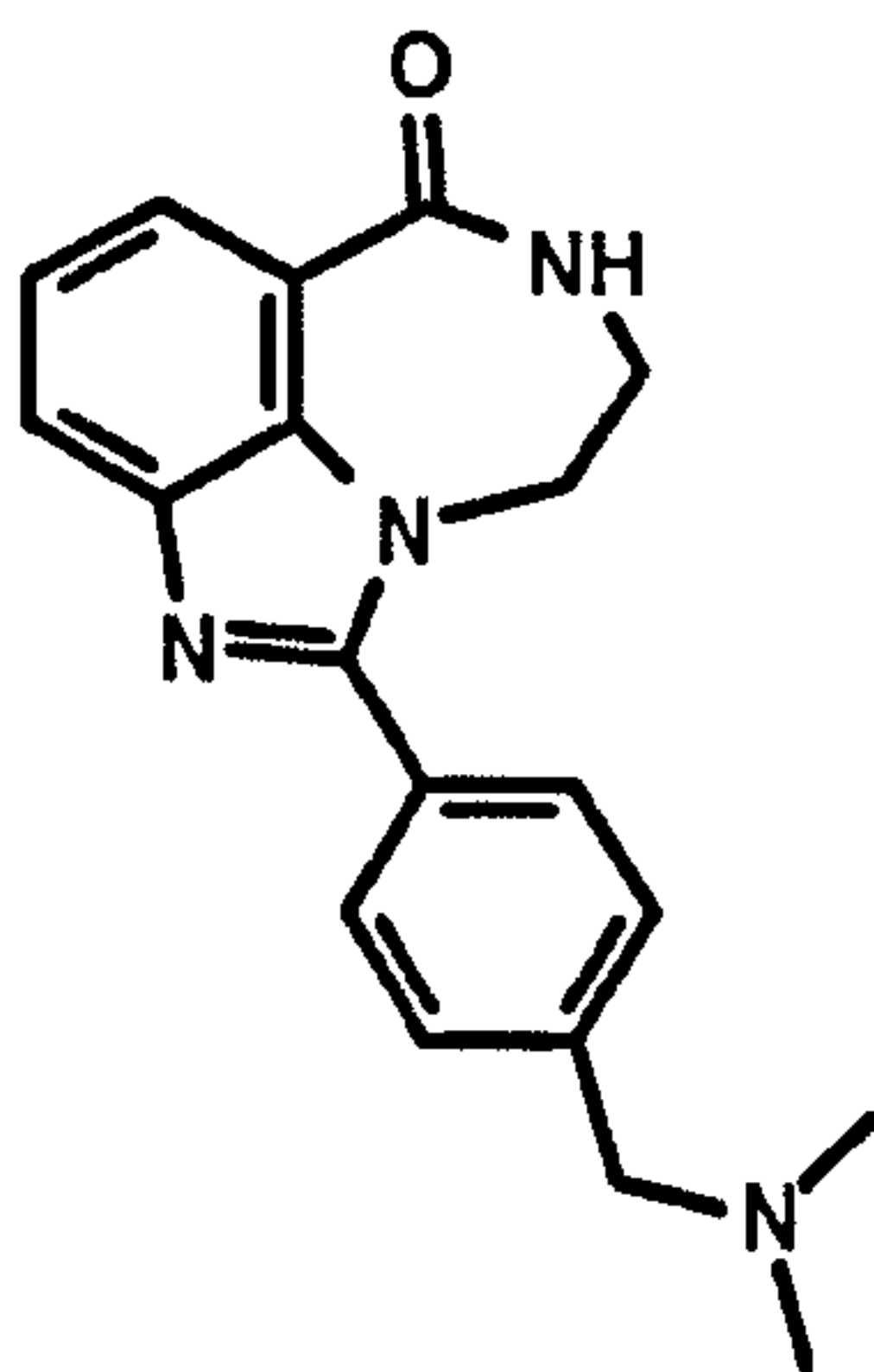


Figure 3.2 Structure of AG14361

NU7026 is an analogue of the PI3-kinase inhibitor LY294002 (2-(morpholin-4-yl)-8-phenyl-chromen-4-one) and is a competitive reversible inhibitor with respect to ATP.

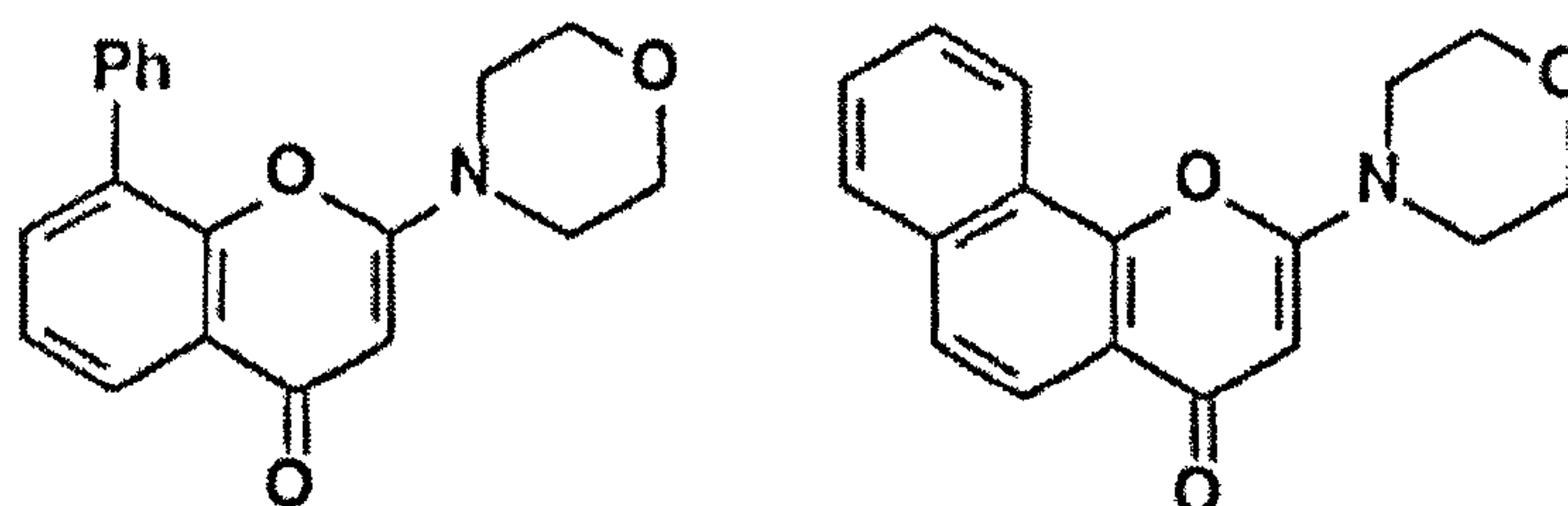


Figure 3.3 Comparison of the structures of LY294002 and NU7026

The relative inhibitory potencies and specificities of NU7026 and Wortmannin against the PIKK family of enzymes (PI3-K, DNA-PK, ATM and ATR) were compared (Table 3.1). Wortmannin and NU7026 are approximately equipotent against DNA-PK. However, whilst wortmannin is primarily a PI 3-K inhibitor, being at least 90-fold more active against PI3-K than DNA-PK or ATM, NU7026 is more selective for DNA-PK with a 60-fold greater potency against this enzyme than PI 3-K and inactive against both ATM and ATR. Thus in contrast to wortmannin, NU7026 demonstrates excellent specificity for DNA-PK.

Enzyme	NU7026 IC ₅₀ (μM)	Wortmannin IC ₅₀ (μM)
DNA-PK	0.23 ± 0.01	0.26 ± 0.02
PI 3-K	13.0 ± 3.00	0.003 ± 0.00
ATM	> 100	0.30 ± 0.05
ATR	> 100	4.4 ± 0.18

Table 3.1 Comparison of inhibitor potencies and specificities against the PIK-K family enzymes.

Reproduced with permission from G.C.M Smith, KuDOS Pharmaceuticals, Cambridge, UK. Each IC₅₀ value represents the mean of 3 independent experiments ± SEM (section 2.16).

3.2 Aims

The aim of this initial results Chapter was to fully characterise the cell lines and enzyme inhibitors used in this thesis, in order to identify optimal cell line and inhibitor combinations/concentrations for subsequent investigations. The following parameters were evaluated:-

Cell line characterisation

- Growth rates
- Western blotting of PARP-1, Ku80, Ku70 and DNA-PKcs protein levels
- PARP and DNA-PK enzyme activity
- Radiosensitivity

Inhibitors

- Effect of inhibitors on PARP and DNA-PK enzyme activity
- Stability of NU7026 under assay conditions
- The effect of inhibitors per se on growth and cell survival
- The effect of the inhibitors per se on cell cycle distribution

3.3 Results

3.3.1 Cell growth rate of cell lines

The density of cell suspension seeded during routine subculture and in experiments is dependent on the growth rate of the cells, the density at which the cells reach confluence and the duration of the experiment. The mean cell growth rate in DMEM medium was determined for each of the cell lines using the SRB assay as described in section 2.3.1. This enabled the exposure period to the inhibitors to be adjusted to ensure that each cell line was exposed, in the exponential phase of growth, for a period equivalent to 3 cell doublings in growth inhibition studies (section.2.3.2). An average growth rate of 12 ± 1 h was observed for all CHO cell lines. The PARP-1 +/+ and PARP-1 -/- MEFs both had a mean growth rate of 23 ± 1 h. (Table 3.2).

Cell line	Doubling Time (Hours)
AA8	11
V3	13
V3YAC	12
CHOK1	11
Xrs-6	12
Xrs-6 Ku80	13
PARP-1 -/-	22
PARP-1 +/-	24

Table 3.2 Control cell doubling times

Cell growth rates were measured by SRB assay and doubling times were calculated by linear regression of the curve of log absorbance against time as described in section 2.3.1.

3.3.2 Protein expression

The protein levels of Ku80, Ku70, DNA-PKcs and PARP-1 were determined in all cell lines by Western blotting. Briefly, cell lysates were prepared from exponentially growing cells and loaded onto denaturing polyacrylamide gels as described in section 2.9 DNA-PKcs was resolved on 3-8 % Tris-acetate gels and all other proteins were separated on 4-20 % Tris Glycine gels. Following electrophoresis, proteins were electrotransferred onto nitrocellulose and probed for target proteins using specific antibodies (Table 2.1). Purified PARP-1 and DNA-PKcs were also run as controls on the gel, where appropriate.

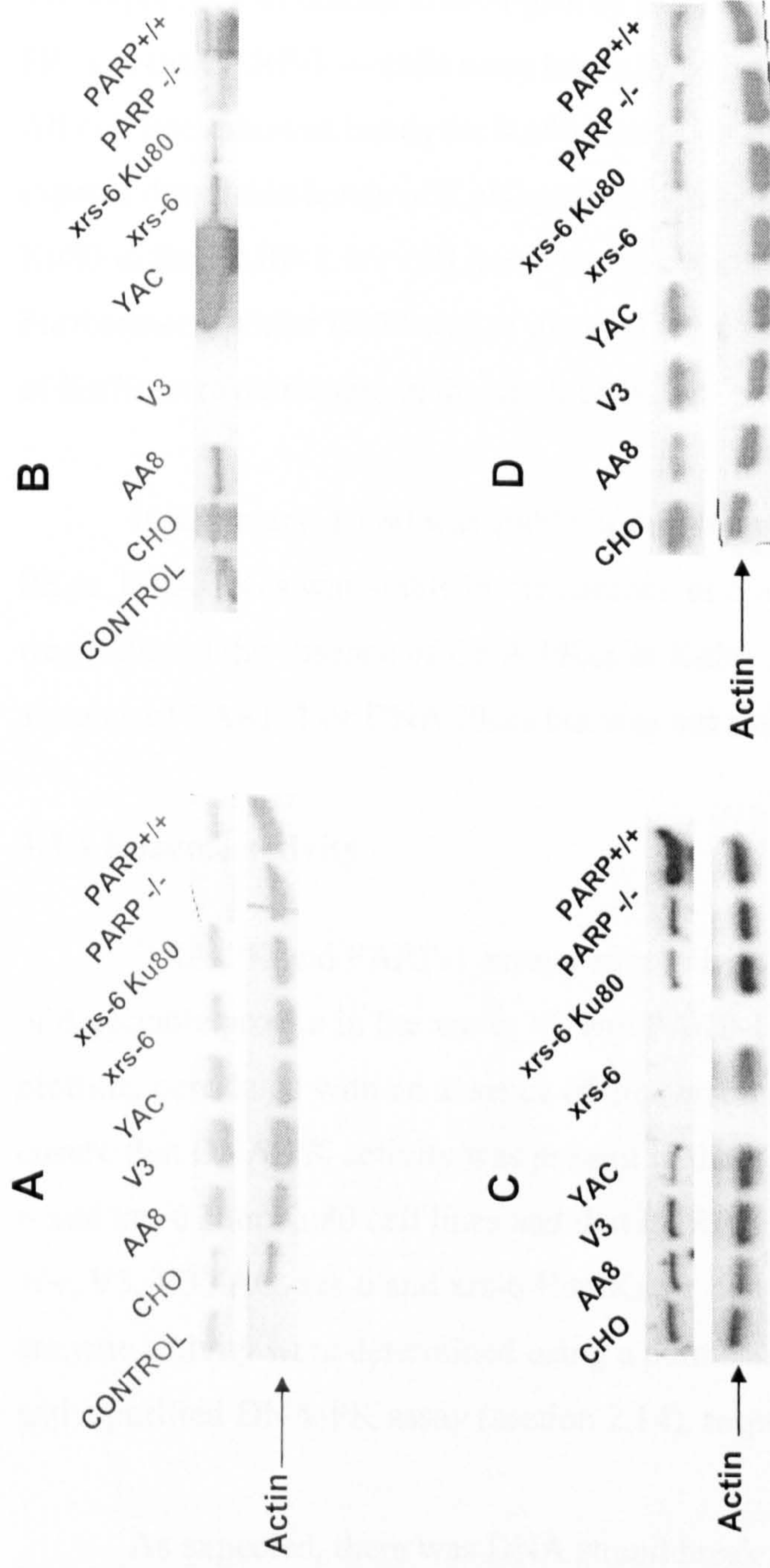


Figure 3.4 Comparison of protein levels in the cell lines studied

Western blot analysis of :- A, PARP-1; B, DNA-PKcs; C, Ku80; D, Ku70. Whole cell protein lysates were collected for each cell line and 50 μ g sample was separated by electrophoresis on denaturing polyacrylamide gels as described in section 2.9. Purified PARP-1 and DNA-PKcs were also run as controls on the gel (A and B). Blots were probed using specific antibodies against PARP-1, DNA-PKcs, Ku80 and Ku70. As a loading control, an anti-actin antibody was used.

As expected, PARP-1 $-/-$ MEFs contained no detectable PARP-1 protein and no DNA-PKcs protein was detected in extracts from V3 cells. However, both proteins are present in all other cell lines as demonstrated by bands of 116 kDa and 470 kDa, respectively (Figure 3.4). The levels of DNA-PKcs in the V3YAC cells were greater than that observed in the parental AA8 cell line and this may reflect overexpression of human DNA-PKcs by the YAC. Conversely, the levels of DNA-PKcs in the PARP-1 $-/-$ cells were lower than that observed in the PARP-1 $+/+$ cells. All cell lines showed bands for Ku80 except for the xrs-6 cells which failed to express detectable levels of Ku80 protein. Intriguingly, there were higher levels of Ku80 in the PARP-1 $+/+$ cell line when compared to the PARP-1 $-/-$ cell line. Furthermore, whilst Ku70 was of parental level in all cell lines, only very low levels of Ku70 were detectable in the xrs-6 cells.

In summary, Ku80 was stable in the absence of both PARP-1 and DNA-PKcs, DNA-PKcs was stable in the absence of both Ku80 or PARP-1 and PARP-1 was stable in the absence of DNA-PKcs or Ku80. Finally, Ku70 was stable in the absence of PARP-1 or DNA-PKcs but was not stable in absence of Ku80.

3.3.3 Enzyme activity

DNA-PK and PARP-1 assays were carried out to determine whether the lack of detectable protein in the xrs-6, V3 and PARP-1 $-/-$ cells, as assessed by Western blotting, correlated with an absence of the corresponding enzyme activities and to ensure that DNA-PK activity was present in the V3YAC, PARP-1 $+/+$ and PARP-1 $-/-$ and xrs-6 HamKu80 cell lines and that PARP-1 activity was present in the PARP-1 $+/+$, V3, V3YAC, xrs-6 and xrs-6 HamKu80 cell lines. PARP and DNA-PK enzyme activity were determined using a permeabilised PARP assay (section 2.11) and a purified DNA-PK assay (section 2.14), respectively.

As expected, there was DNA strand break activatable PARP activity in all cell lines except for the PARP-1 $-/-$ MEFs (Figure 3.5). PARP activity ranged from 100 pmol NAD incorporated per 10^6 cells for the xrs-6 cells to 150 pmol NAD per 10^6 cells for the V3 cells. PARP activity was slightly but significantly different in the V3YAC and V3 cells (129 ± 7 pmol compared to 149 ± 2 pmol $p = 0.06$). The xrs-6

cells had approximately 25 % lower PARP activity than the xrs-6 HamKu80 cells (102 ± 10 pmol compared to 135 ± 8.6 pmol $p = 0.02$). It should be noted, however, that the PARP-1^{-/-} cells retained a small but significant residual PARP activity (4 ± 0.3 pmol compared to 132 ± 3.8 pmol in the PARP-1^{+/+} cells) that was not stimulated by the oligonucleotide (figure for – oligo = 5.6 ± 2.8) and is therefore strand break independent. This minority activity is probably attributable to PARP-2 which together with PARP-1 has also been implicated in BER (Schreiber *et al.*, 2002). Indeed, unlike PARP-1, PARP-2 activity is not stimulated by blunt DNA ds ends (de Murcia, personal communication) but is stimulated by a variety of other BER intermediates, including nicks, flaps and cruciforms. The oligonucleotide used in this thesis would therefore not be expected to activate PARP-2. Activity in the absence of oligonucleotide possibly represents PARP-2 stimulation by strand breaks in cellular DNA arising endogenously or as a result of cell manipulation.

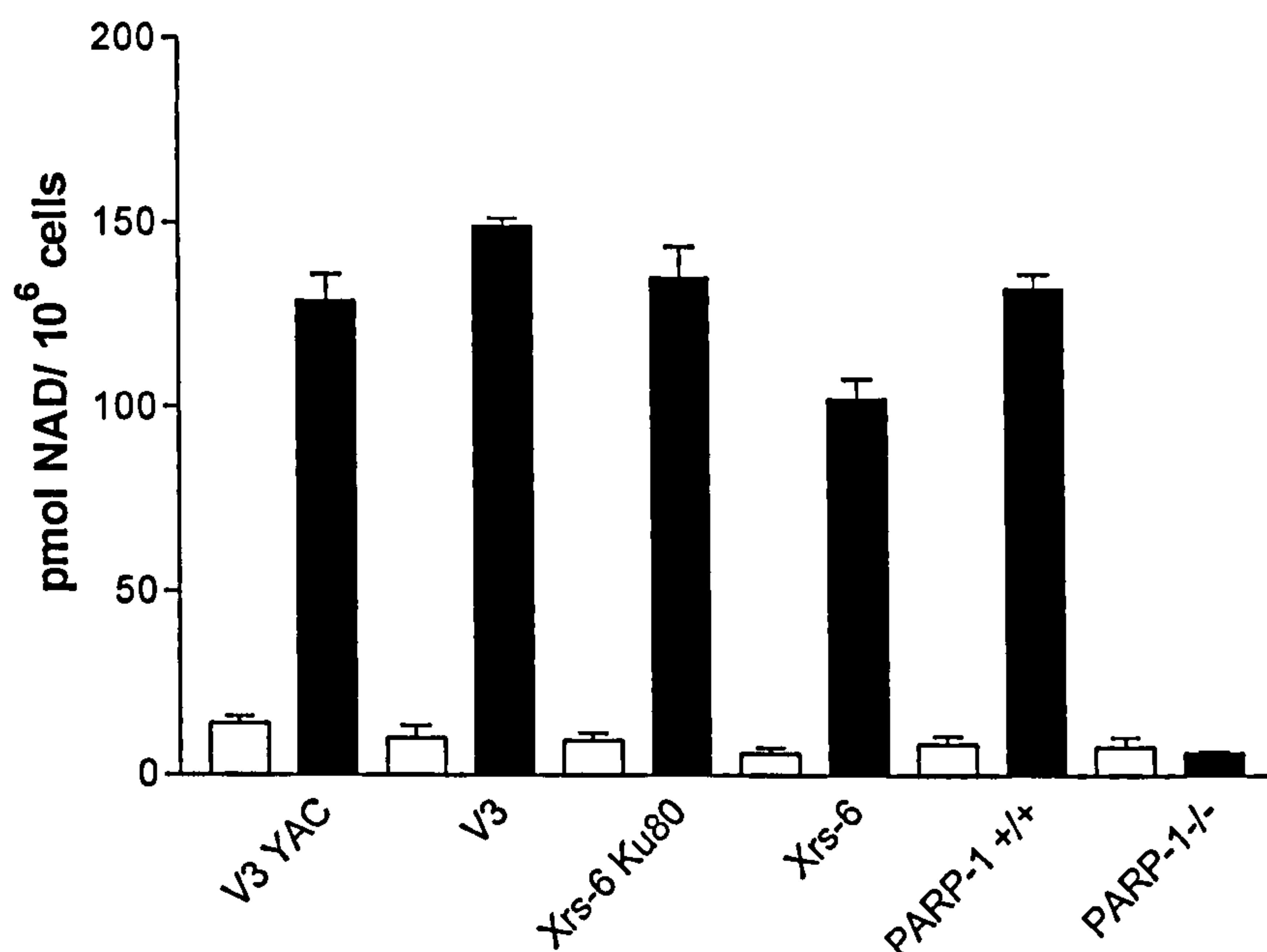


Figure 3.5 Comparison of PARP activity in all cell lines

Comparison of PARP activity in all cell lines in the absence (white bar) or presence of oligonucleotide (black bars) determined using the permeabilised PARP assay described in section 2.11. Data are the mean \pm SEM of 3 independent experiments.

DNA-PK activity (Figure 3.6) was measured using nuclear extracts as described in section 2.14. No activity was measured in the absence of oligonucleotide (data not shown) which served as a control. DNA-PK activity reflected that of protein expression and ranged from 500 - 750 pmol ATP incorporated per μg nuclear protein. There was no significant detectable DNA-PK activity compared to a -oligo control in the xrs-6 ($p = 0.43$) or V3 ($p = 0.72$) cell lines, deficient in Ku80 and DNA-PKcs, respectively, thus indicating that both the DNA binding activity of the Ku70/80 heterodimer and DNA-PKcs protein are essential for the kinase activity of the DNA-PK complex. Furthermore, whilst DNA-PK activity was detectable in all other cell lines, it was higher in the V3YAC cell line (750 pmol ATP/ μg protein) compared to the other DNA-PK proficient cell lines (~500 pmol ATP/ μg protein). As suggested for protein expression, this could reflect overexpression of human DNA-PKcs by the YAC. Despite lower levels of DNA-PKcs protein in the PARP-1^{-/-} cells, there was no significant difference in the DNA-PK activity between the PARP-1^{+/+} and PARP-1^{-/-} cell lines ($p = 0.63$). In summary, these results confirm that transfection of DNA-PKcs or Ku80 cDNA into the V3 and xrs-6 cell lines, respectively, restores kinase activity

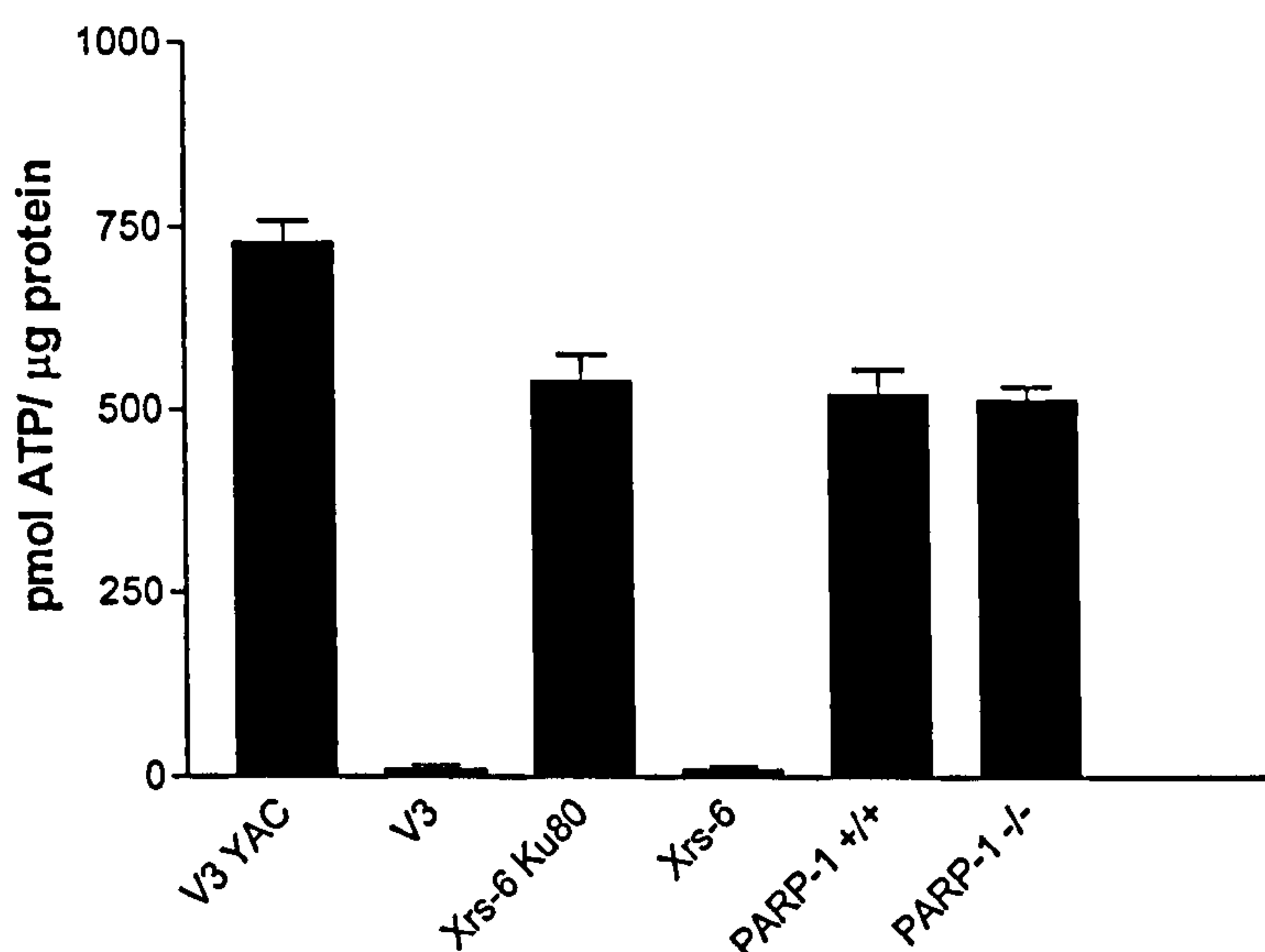


Figure 3.6 Comparison of DNA-PK activity in all cell lines

Comparison of DNA-PK activity in all cell lines. DNA was extracted from nuclear extracts using hi-trap columns (section 2.14) and DNA-PK activity was quantified using the purified DNA-PK assay. Data are the mean \pm SEM of 3 samples from a single experiment

3.3.4 Radiosensitivity of cell lines

The impact of the loss of Ku80, DNA-PKcs or PARP-1 on the radiosensitivity of the cell lines was measured using a clonogenic survival assay as described in section 2.5. In exponentially growing cells, IR induced a dose dependent reduction in cell survival in all of the cell lines (Figure 3.7). The IR doses required to reduce survival by 50 % (LD₅₀) and 90 % (LD₉₀) are summarised in Table 3.3. The LD₉₀ values ranged between 1 – 6.5 Gy. The xrs-6 cells, PARP-1 ^{-/-} MEFs and V3 cells were significantly more sensitive to IR than the enzyme-proficient transfected cell lines (4.4, 3.3 and 3.7-fold, respectively, $p < 0.05$). The Ku80 deficient cells were the most sensitive to IR with an LD₉₀ of 1.0 ± 0.01 compared to 6.5 ± 0.3 in the parental CHOK1 cell line. Transfection of Ku80 into the xrs-6 cells significantly increased the radioresistance of this cell line ($p = 0.045$) and they were only 1.5-fold more sensitive than parental CHOK1 cells. Similarly, DNA-PKcs transfected V3 cells were significantly more radioresistant than the V3 cells alone ($p = 0.034$) and only 1.3-fold more sensitive to IR than parental AA8 cells. These data are consistent with the hypothesis that PARP-1 and DNA-PK are required for the recovery from radiation induced DNA damage.

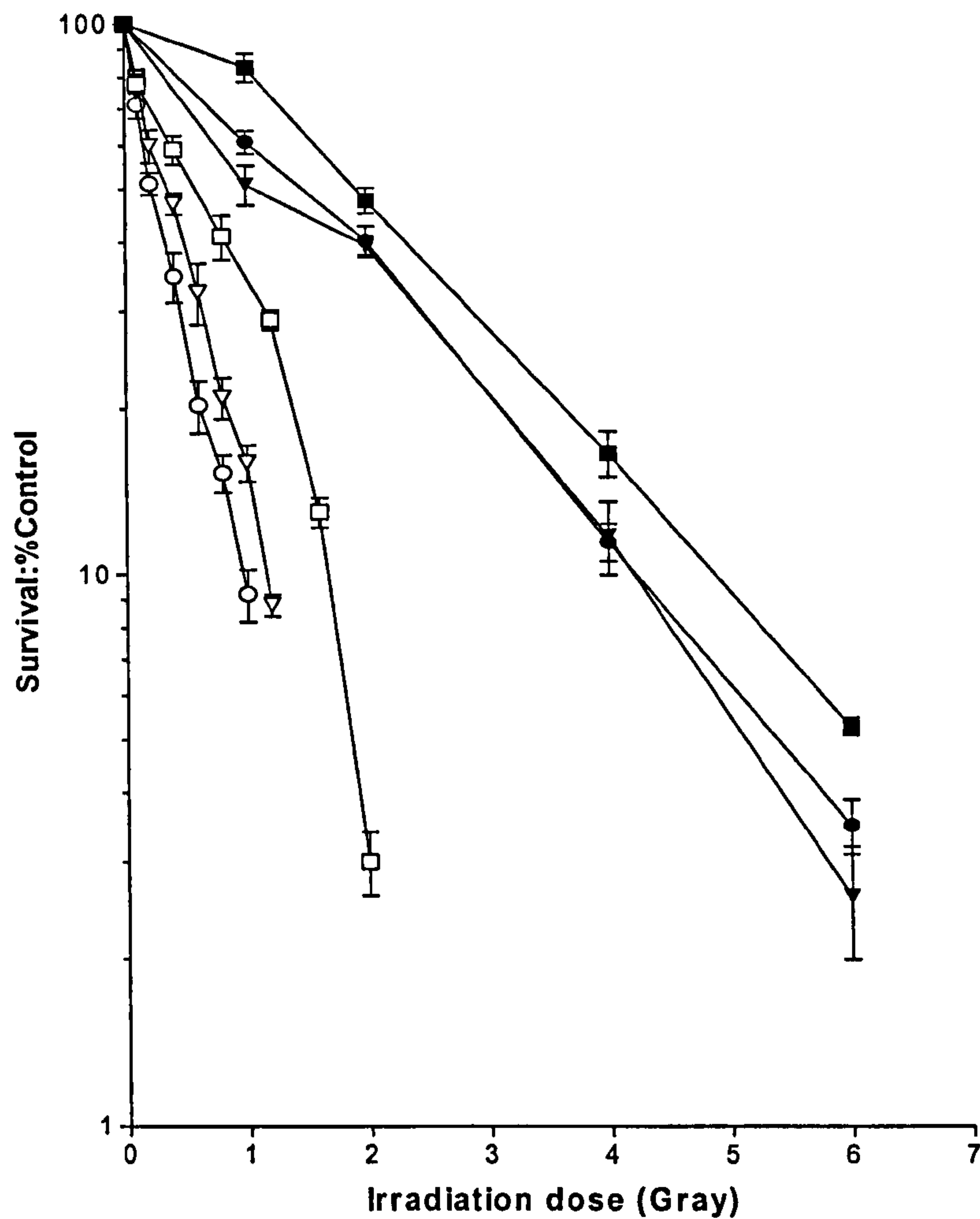


Figure 3.7 Cytotoxicity of IR in cell lines with different PARP-1 or DNA-PK status

The effect of increasing doses of IR on the clonogenic survival of exponentially growing:- ■ PARP-1 +/+ cells; □ PARP-1 -/- cells; ▼ V3YAC cells; ▽ V3 cells; ● xrs-6 Ku80 cells; ○ xrs-6 cells. Cells were irradiated and reseeded for colony formation immediately. Data are the mean \pm SEM of at least 3 independent experiments.

Cell line	LD ₅₀ (Gy)	LD ₉₀ (Gy)	Fold sensitivity
AA8	2.9 ± 0.10	5.6 ± 0.07	1.3
V3YAC	1.3 ± 0.00	4.4 ± 0.04	
V3	0.4 ± 0.01	1.2 ± 0.01	
CHOK1	2.7 ± 0.20	6.5 ± 0.3	1.5
Xrs-6 Ku80	1.5 ± 0.00	4.4 ± 0.2	
Xrs-6	0.27 ± 0.01	1.0 ± 0.01	
PARP-1+/+	2.4 ± 0.22	5.5 ± 0.2	3.3
PARP-1 -/-	0.9 ± 0.01	1.7 ± 0.00	

Table 3.3 The effect of increasing doses of IR on clonogenic cell survival

Comparison of the LD₅₀ and LD₉₀ values resulting from the exposure of exponentially growing cells to increasing doses of IR. The values represent the mean ± SEM of at least 3 independent experiments. Fold sensitivity was calculated as a ratio of:- LD₉₀ of parental cell line/ LD₉₀ of transfected cell line or LD₉₀ of transfected cell line/ LD₉₀ of enzyme deficient cell line

3.3.5 Effect of AG14361 and NU7026 on PARP-1 and DNA-PK enzyme activity

AG14361 and NU7026 were used at 0.4 µM and 10 µM in all experiments described in this thesis. For AG14361, this represents a standard dose for which Agouron Pharmaceuticals screened all potential inhibitors and was selected because it caused ≥ 80 % depletion of cellular PARP activity, and was sufficient to cause maximum potentiation of temozolomide (a clinically used monofunctional DNA methylating agent) whilst being less than 1/10 of the concentration of AG14361 which caused detectable growth inhibition (Calabrese *et al.*, in press). For NU7026, the choice of dose was based upon clonogenic assays which determined the minimum dose required to produce maximal potentiation in whole cells following 4 Gy IR. These experiments are described in section 4.3.1.1. In order to ensure that

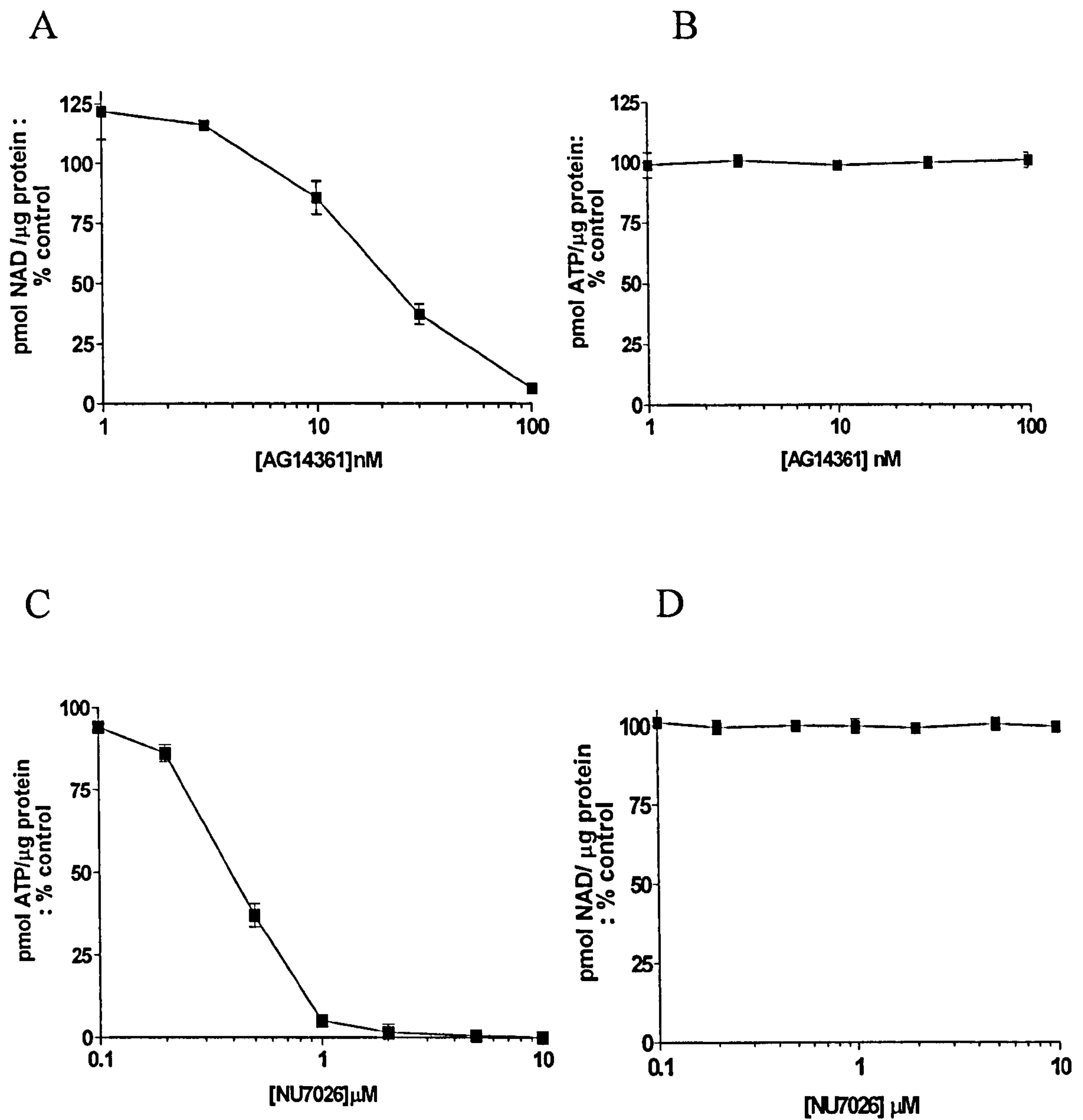


Figure 3.8 The effect of AG14361 and NU7026 on purified PARP-1 and DNA-PK activity

The effect of AG14361 on purified PARP-1 (A) and DNA-PK (B) activity and the effect of NU7026 on purified DNA-PK (C) and PARP-1 activity (D). Data are the mean \pm SEM of at least 3 independent experiments.

PARP-1 and DNA-PK activity were sufficiently depleted by the concentrations of inhibitors used in the experiments described in this thesis, PARP-1 and DNA-PK activity were measured in the presence and absence of the inhibitor(s).

Briefly, the concentration(s) of AG14361 and NU7026 required to inhibit PARP-1 and DNA-PK activity, respectively, by 50 % (IC_{50}) were determined by using purified enzyme assays. In addition, the effect of AG14361 on DNA-PK activity and the effect of NU7026 on PARP-1 activity were tested. Figures 3.8 C and D show that NU7026 inhibited purified DNA-PK activity with an IC_{50} of 0.44 ± 0.01 μ M whilst NU7026 had no inhibitory effect on purified PARP-1 activity up to 10 μ M. Similarly, Figure 3.8 A shows that AG14361 inhibited purified PARP-1 activity with an IC_{50} of 20.3 ± 2 nM. AG14361 had no inhibitory effect on purified DNA-PK activity up to 100 nM (Figure 3.8B).

Permeabilised PARP assays were carried out to assess the effects of AG14361 in whole cells as described in section 2.11. The IC_{50} values were; 68.4 ± 4 nM, 56.3 ± 4 nM, 59.6 ± 3 nM and 15 ± 2 nM for the V3YAC, V3, PARP-1 +/+ and PARP-1 -/- cells, respectively (Data not shown). Permeabilised cells are not in the physiological state, however, and therefore it could be argued that that this assay does not demonstrate the entry and activity of the inhibitor in intact cells. Studies were therefore carried out in V3YAC cells treated with AG14361 before washing with PBS to remove residual inhibitor and subsequently permeabilising the cells. The assay was then performed in the absence of added inhibitor. As can be seen in Figure 3.9, there was no significant difference in the potency of AG14361 against PARP whether the inhibitor was present prior to or following permeabilisation (Compare IC_{50} pre-permeabilisation of 71.6 ± 2.3 nM to the IC_{50} post permeabilisation of 68.4 ± 3.5 nM) $p = 0.1$.

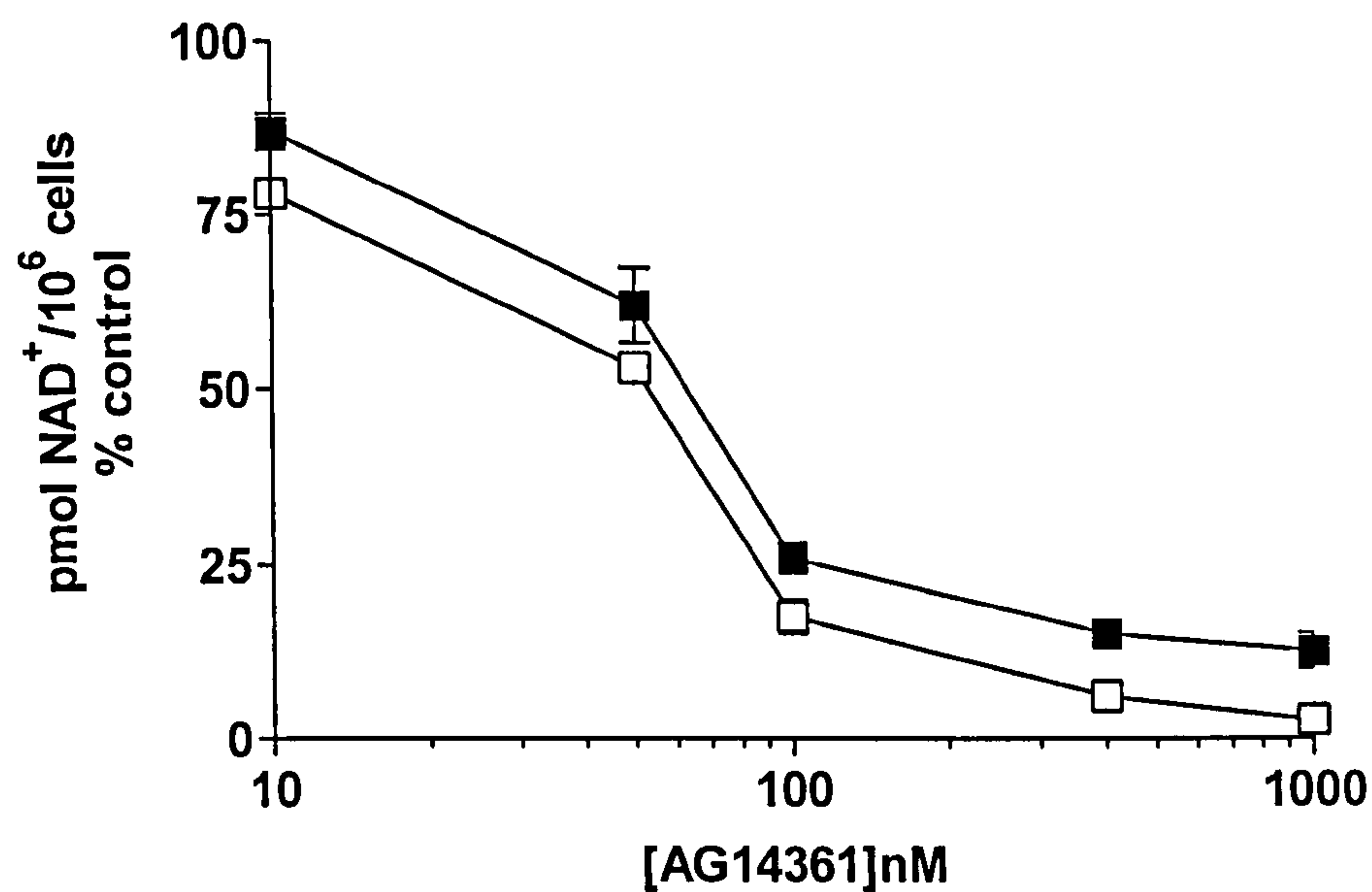


Figure 3.9 Effect of AG14361 on PARP activity in permeabilised V3YAC cells

Dose response to AG14361 in permeabilised V3YAC cells treated with inhibitor; ■ before permeabilisation ; □, after permeabilisation. Permeabilised cell assay was carried out as described in section 2.11. Data are the mean \pm SEM of at least 3 independent experiments.

3.3.6 Stability of NU7026 under assay conditions

Studies assessing the stability of AG14361 under various assay conditions, including light and medium, have been carried out by Agouron Pharmaceuticals and within the department. Importantly, the compound was shown to be stable under these conditions (personal communication; Calabrese *et al.*, in press). However, at the initiation of these studies, details concerning the stability of NU7026 were unknown. The stability of NU7026 under the assay conditions of medium, light and freeze thawing was therefore evaluated by developing a reverse phase HPLC method (see section 2.4). For medium and light stability studies, triplicate samples were set up in DMEM medium or PBS, respectively, at 1, 10 and 50 μ M and aliquots were taken every 2 h up to 24 h. Freeze thaw samples were set up in 100 % DMSO (n=1) at 1, 10 and 50 μ M and stability tested up to 3 freeze thaws. At time zero, the area under the peak is directly proportional to the concentration of NU7026 (Figure 3.10). For example, in the freeze thaw study, the area under the peak for 50 μ M is 5 times larger than the corresponding area for 10 μ M (1715048 compared to 376565.7) and this is true for all parameters tested.

There were no significant differences in the area under the peaks whether the samples had been exposed to light or maintained in the dark for 24 h (For example, Figure 3.10B; 223300 for 10 μ M compared to 239598 for 10 μ M, $p = 0.09$) and this was true for all concentrations of NU7026. Similarly, there was no significant difference in peak area, at all doses of NU7026, between samples that were maintained in medium for 24 h and those that were analysed immediately (For example, Figure 3.10A; 279721 for 10 μ M compared to 289314 for 10 μ M, $p = 0.08$). Finally, there was no significant change in peak area for all doses of NU7026 following 3 freeze thaws $p = > 0.05$ (Figure 3.10C).

In summary, these data indicate that no degradation of compound occurred under any of the assay conditions tested and therefore demonstrates that NU7026 is stable, by HPLC, in DMEM medium at room temperature for 24 h, is unaffected by exposure to light for 24 h and is not influenced by freeze thawing up to 3 times.

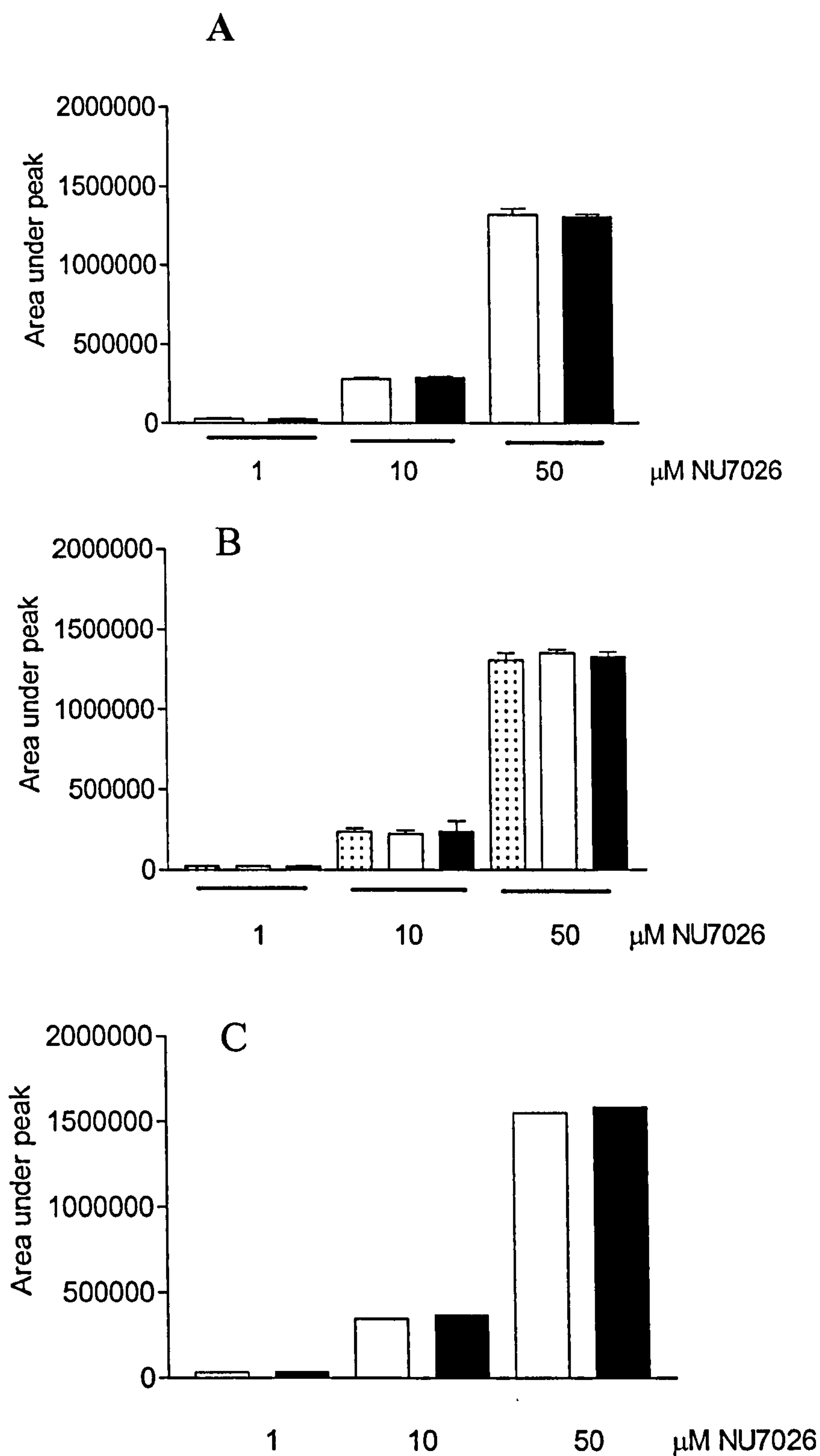


Figure 3.10 Stability of NU7026 under assay conditions of medium, light and freeze-thaw
A, Medium stability, 0 h (white bars), 24 h (black bars); B, Light stability, 0 h (chequered bars), 24 h light (white bars), 24 h dark (black bars); C, Freeze thaw, 0 (white bars), 3 (black bars)

3.3.7 The effect of the inhibitors on growth and survival

Although it has been demonstrated that mice deficient in PARP-1 or DNA-PK are viable, the possibility that the use of a chemical inhibitor of either of the enzymes could result in non-specific effects on the growth or viability of the cells remained. The effects of the inhibitors on growth was assessed by SRB assay (see section 2.3.2) in all cell lines. Briefly, exponentially growing cells were continuously exposed to increasing concentrations of AG14361 (0.1, 1, 10 and 100 μM) or NU7026 (1, 2, 5, 10 and 50 μM) for ≥ 3 cell doublings (CHO cells lines, 48 h, PARP MEFs, 72 h). Using the percentage of control cell growth, the concentrations of AG14361 and NU7026 required to inhibit cell growth by 50 % (GI_{50}) was calculated for all cell lines and the results are summarised in Table 3.4. Owing to its limited solubility, the highest achievable concentration of NU7026 at 1 % (v/v) DMSO in aqueous medium was 50 μM . Both inhibitors inhibited the growth of all cell lines in a concentration-dependent manner. In response to NU7026, there was no evident growth inhibition up to 10 μM whilst a modest but significant growth inhibition of approximately 20 % was seen at 20 μM in all cell lines. Similarly, exposure to AG14361 did not cause any growth inhibition up to 1 μM whilst a modest growth inhibition (approximately 10 %) was seen at 10 μM in all cell lines. Representative graphs for the growth inhibitory effects of NU7026 and AG14361 are shown in Figure 3.11 a and b, respectively. Figure 3.11a shows that there was no significant difference in the sensitivity of matched cells proficient or deficient in DNA-PK to NU7026 ($p = 0.8$). Similarly, Figure 3.11b shows that there was no significant difference in the sensitivity of matched cells proficient or deficient in PARP-1 to AG14361 ($p = 0.65$).

In summary, there were no noteworthy differences in GI_{50} values for AG14361 (ranging from 30 – 34 μM). Similarly, no marked differences in GI_{50} values were seen for NU7026 with values ranging from 30 to >50 μM , 50 μM being the limit of solubility for NU7026 (Table 3.4).

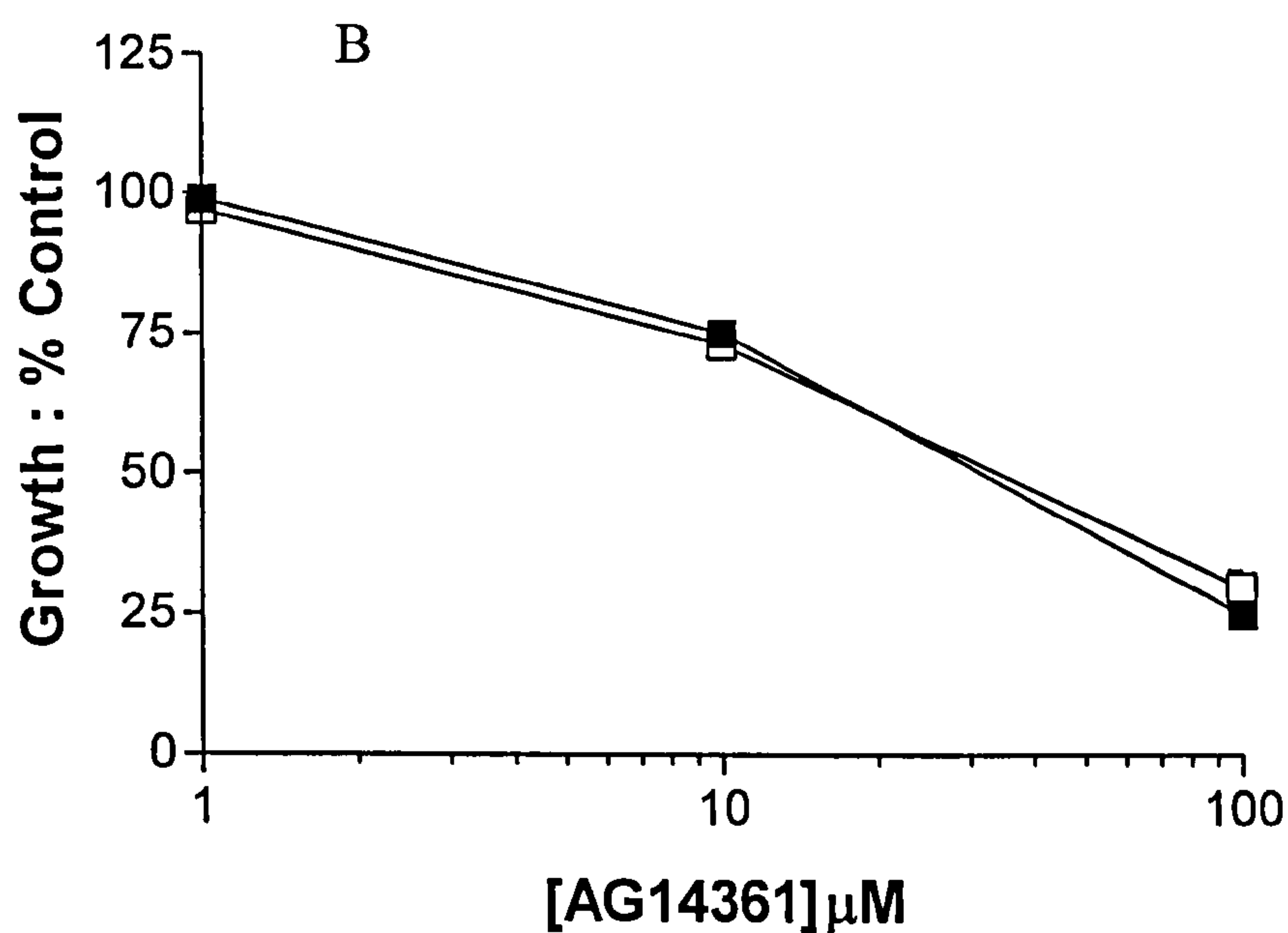
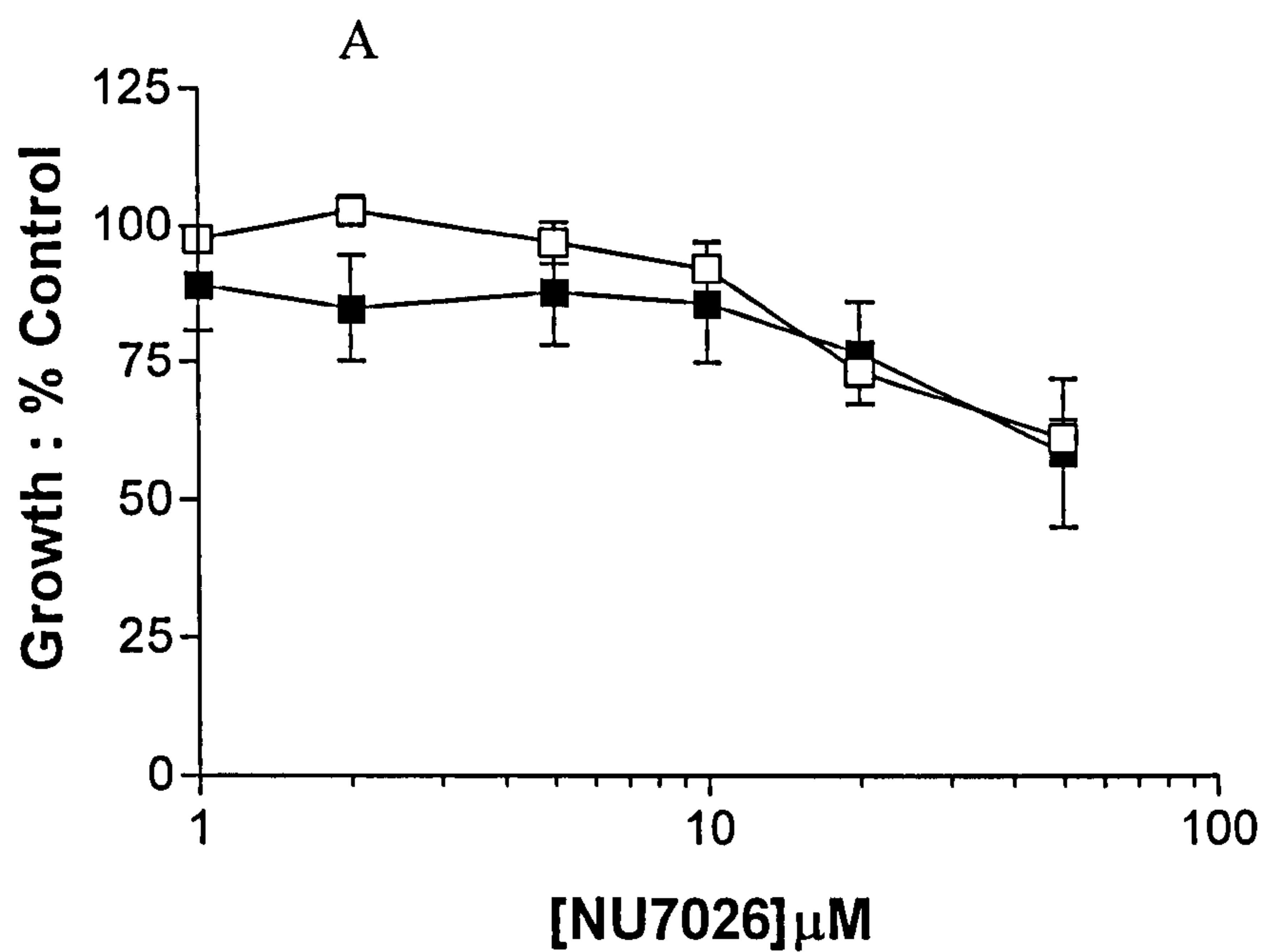


Figure 3.11 Growth inhibition by NU7026 and AG14361

A, Growth of V3YAC cells (■) and V3 cells (□) following a 48 h continuous exposure to increasing concentrations of NU7026. B, Growth of PARP-1 +/+ cells (■) and PARP-1 -/- cells (□) following a 72 h continuous exposure to increasing concentrations of AG14361. Data are normalised to DMSO alone controls and are the mean \pm SEM from at least 3 independent experiments.

Cell line	AG14361 GI ₅₀ (μM)	NU7026 GI ₅₀ (μM)
AA8	30.5 ± 3.9	>50
V3YAC	32.0 ± 4.9	>50
V3	31.6 ± 2.4	>50
CHOK1	33.5 ± 1.8	35.1 ± 5.8
Xrs-6 Ku80	31.5 ± 2.9	30.0 ± 3.7
Xrs-6	32.5 ± 2.2	31.2 ± 5.4
PARP-1 +/+	33.4 ± 1.8	>50
PARP-1 -/-	34.1 ± 1.5	>50

Table 3.4 The effect of AG14361 and NU7026 alone on cell growth

Comparison of the GI₅₀ values of AG14361 and NU7026 following continuous exposure for at least 3 cell doublings to increasing concentrations of either AG14361 or NU7026. The level of growth inhibition was assessed as compared to 1 % DMSO alone controls by SRB assay. Data are the mean ± SEM from at least 3 independent experiments.

The plating efficiencies, which reflect the ability of the cells to form colonies, in the presence of AG14361 or NU7026, alone, at the concentrations used in all experiments (0.4 and 10 μM, respectively) were measured in order to evaluate their relative cytotoxicity. The exposure time for clonogenic assays was 16 hrs (approximately one cell cycle) as opposed to the 48/72 h incubation time used for the evaluation of growth inhibition. Since both inhibitors were dissolved in DMSO prior to adding to medium, the plating efficiencies in the presence of 1 % (v/v) DMSO were also measured. On comparing the plating efficiencies (Table 3.5), there is no significant difference between colony formation in the presence or absence of AG14361 or NU7026 compared to 1 % (v/v) DMSO controls (p > 0.05).

Furthermore, the plating efficiencies in the presence of 1 % (v/v) DMSO were not significantly different from the DMSO-free controls ($p = > 0.05$).

All drug exposures shown in the results Chapters were therefore conducted at a final concentration of 1 % (v/v) DMSO and growth inhibition or cytotoxicity data were normalised to 1 % (v/v) DMSO alone or drug alone controls.

Cell line	Control PE	PE + 1 % (v/v) DMSO	PE + 0.4 µM AG14361	PE + 10 µM NU7026
V3YAC	73.7 ± 1.5	73.0 ± 1.1	72.4 ± 2.1	74.3 ± 0.9
V3	74.6 ± 1.2	74.1 ± 1.5	73.2 ± 2.2	73.1 ± 0.1
Xrs-6 Ku80	87.0 ± 1.5	86.9 ± 1.4	87.2 ± 1.5	86.3 ± 4.8
Xrs-6	82.9 ± 1.4	83.1 ± 1.4	81.0 ± 3.6	84.1 ± 1.8
PARP-1 +/+	68.0 ± 1.5	67.0 ± 2.4	66.0 ± 3.2	68.0 ± 1.2
PARP-1 -/-	64.0 ± 1.2	63.0 ± 2.4	63.0 ± 1.1	64.0 ± 0.8

Table 3.5 Plating efficiencies (PE) of cell lines in the presence or absence of 1 % (v/v) DMSO, 0.4 µM AG14361 or 10 µM NU7026

Cells were continuously exposed for 16 h to either 1 % (v/v) DMSO, 0.4 µM AG14361 or 10 µM NU7026 and cell survival determined by colony formation (section 2.5). Data are the mean ± SEM of at least 5 independently dosed samples from at least 3 independent experiments.

3.3.8 The effect of the inhibitors on cell cycle phase distribution

PI3-kinase is required for many cellular processes that include growth factor signalling (Cospedal *et al* 1999; Puglianello *et al* 2000) and inhibition of PI3- kinase has been shown to result in G1/S block of the cell cycle (Chang *et al.*, 2003).

Accordingly, inhibitors of PI3-kinase induce cell cycle arrest. Although NU7026 is more selective for DNA-PK than PI3-kinase, *in vitro*, (Table 3.1), at higher concentrations it is able to inhibit PI3- Kinase. The 10 μ M dose used in whole cell studies could therefore potentially affect PI3- Kinase activity which would be manifested as a cell cycle blockade. Similarly, it is possible that AG14361 could exhibit non-specific effects that could result in cell cycle effects.

Flow cytometric analysis (FACS) was used to investigate whether NU7026 or AG14361 (at the concentrations used in all experiments) were associated with an arrest of cell division in a specific phase of the cell cycle. Initially, exponentially growing cells were seeded onto 92 mm dishes and allowed to adhere for 24 h before addition of inhibitor(s). The cell cycle distribution was then followed for 96 h, with samples taken for FACS analysis at 24 h intervals (section 2.7). The mean cell cycle phase distribution was calculated for all samples and a representative cell cycle profile is shown in Figure 3.12. The mean cell cycle phase distributions of the inhibitor treated cells were compared to that of untreated control cells. Figure 3.13 shows that, in the absence of DNA damage, there was no significant difference in cell cycle distribution between any of the cell lines. Moreover, 0.4 μ M AG14361 or 10 μ M NU7026, alone or in combination, produced no significant changes in the cell cycle phase distribution in any of the cell lines tested ($p = >0.05$).

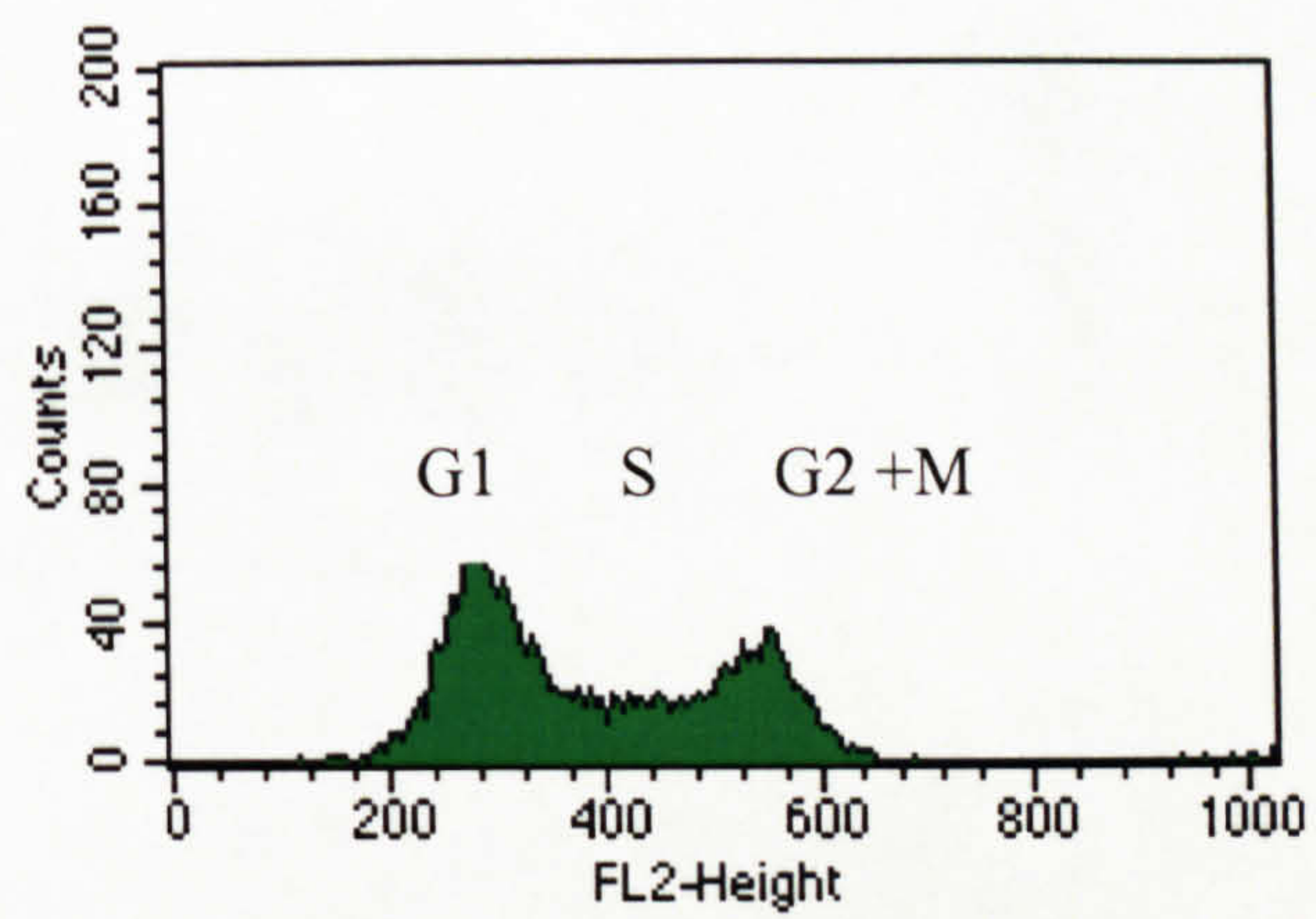


Figure 3.12 Representative cell cycle profile of exponentially growing cells

Cells were seeded and flow cytometric analysis carried out as described in section 2.7.

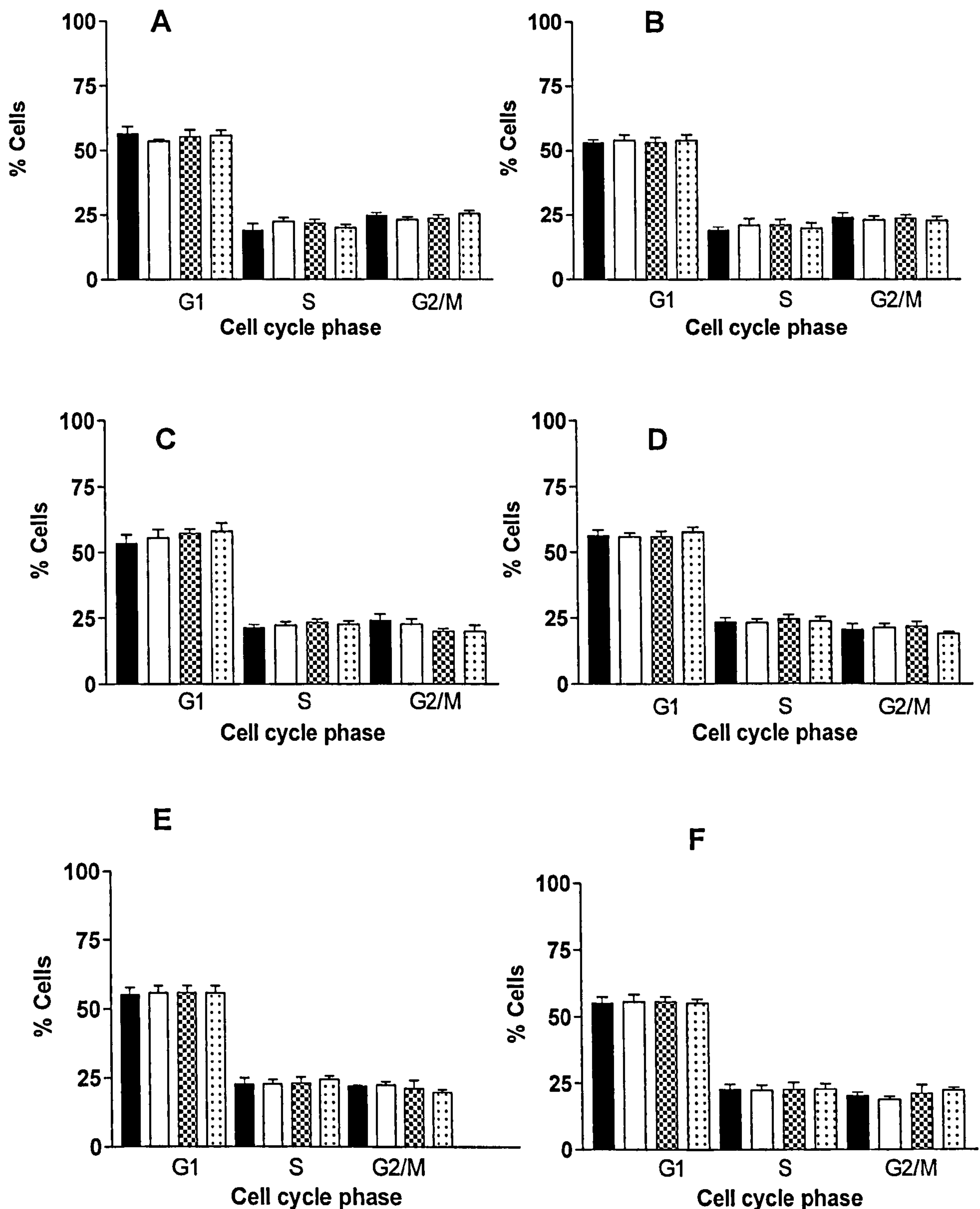


Figure 3.13. The effects of AG14361 and NU7026 on cell cycle phase distribution
A, V3YAC cells; B, V3 cells; C, PARP-1^{+/+} cells; D, PARP-1^{-/-} cells; E, xrs-6 Ku80 cells; F, xrs-6 cells. Bars represent:- Black bars, Cells alone ; White bars, + 0.4 μ M AG14361 ; Checkered bars, + 10 μ M NU7026; Dotted bars, +AG14361+NU7026. Each bar represents the mean \pm SEM of samples taken at 24, 48 and 96 h, and analysed by flow cytometry, as described in section 2.7.

3.4 Discussion

The major aim of this Chapter was to confirm the protein and enzyme activity status of all the cell lines. A lack of Ku80, DNA-PKcs and PARP-1 protein expression in the xrs-6, V3 and PARP-1^{-/-} cells, respectively, was confirmed and a corresponding lack of DNA-PK or PARP-1 activity demonstrated. PARP-1 and DNA-PK activity were present in all other cell lines. The results demonstrate clearly that all the cell lines are of the correct phenotype.

The Ku complex was stable in the absence of either PARP-1 or DNA-PKcs. This is in line with previous observations which have shown that both Ku protein and DNA end binding activity are found in DNA-PKcs defective mutants (Danska *et al.*, 1996; Rathmell and Chu, 1994). In the absence of Ku80, lower levels of Ku70 protein were observed. It has been suggested that the two Ku components stabilise one another, and therefore in agreement with data from others, these data suggest that inactivation of Ku80 leads to dramatic destabilisation of both itself and Ku70 (Errami *et al.*, 1996; Blunt *et al.*, 1996; Danska *et al.*, 1996). In support of this suggestion, it has been demonstrated that co-translation of both subunits is required for heterodimerisation of active Ku and therefore individual Ku subunits may be degraded when not dimerised. Although there was no detectable Ku80 protein in the xrs-6 cells, evidence in the literature suggests that these cells harbour an intact but nonexpressed Ku80 allele which can be reactivated by treatment with azacytidine (Singleton *et al.*, 1997). This indicates that the gene has been silenced by hypermethylation at promoter regions. Moreover, these cells may revert spontaneously in cell culture and therefore in line with this notion, all cells were used at passage 15 or lower in all experiments and periodic Western blot analyses were carried out to re-confirm the phenotype of the cells.

The V3YAC cells expressed levels of DNA-PKcs protein which were greater than that observed in parental hamster cells. This could reflect overexpression of DNA-PKcs by the YAC. Alternatively, rodent cells have as much as 5-fold less DNA-PKcs than human cells and therefore the higher levels of DNA-PKcs in the V3YAC cells could be a reflection of this. In contrast, however, the levels of Ku protein in hamster and human cells are comparable (Chan *et al.*, 1996; Yumoto *et al.*, 1998). The levels of kinase activity approximately reflected that of protein

expression whereby the V3YAC cells had higher levels of DNA-PK activity than the other DNA-PK proficient cell lines.

The PARP-1 +/+ cells expressed considerably higher Ku80 and DNA-PKcs protein levels than the PARP-1 -/- cells. A number of publications have demonstrated interactions between DNA-PK and PARP-1 or Ku and PARP-1 (Galande and Kowhi-Shigomatsu, 1999; Ruscetti *et al.*, 1998) and therefore the presence of one protein may enhance the stability of the other protein in a similar manner to Ku70 and Ku80, described above. This observation was not investigated further, however, it would be interesting to investigate the effect AG14361 on Ku80 or DNA-PKcs protein levels following exposure to IR in PARP-1 +/+ cells. Despite similar levels of PARP-1 protein, the level of PARP-1 activity was approximately 25 % lower in the xrs-6 cell line compared to the xrs-6 Ku80 cell line. This implies a functional association of these two proteins in response to DNA damage. In contrast, despite having lower levels of DNA-PKcs protein, PARP-1 -/- cells had the same amount of DNA-PK activity compared to the PARP-1 +/+ cells. Similarly, the V3 cells have the same amount of PARP-1 activity as the V3YAC cells thus indicating that the activity of one enzyme does not require the presence of the other for stimulation. It should also be noted that there was no loading control for the DNA-PKcs Western blot and therefore the different protein levels could simply reflect differences in loading accuracy.

The enzyme assays also demonstrated that the PARP-1 -/- cells possess approximately 3.9 ± 0.5 % PARP activity compared to their wild type counterparts which is not oligonucleotide inducible and which cannot be attributed to PARP-1. This activity may be attributed to the activity of alternative PARP proteins that have recently been described. Indeed, there are now thought to be at least 18 members of the PARP family and these include PARP-2, V-PARP and tankyrase although PARP-2 is the only homologue which has been so far reported to be activated by DNA damage (section 1.9).

The presence of an alternative PARP activity in PARP-1 $-/-$ MEFs (developed by Wang *et al.*, 1995, and disrupted in exon 2) has been previously described by Shieh *et al.*, 1998, who showed that following treatment with MNNG, these cells were able to synthesise polymer that was indistinguishable in structure from that produced in the PARP-1 $+/+$ cells. In addition, a reduction in intracellular NAD^+ concentration occurred with the formation of polymer thus supporting the suggestion that the polymer formation in these cells was due to PARP protein. As with PARP-1, this residual activity was induced by DNA damage, however, this is in contrast to the studies described in this Chapter which have shown that the residual PARP activity in the PARP-1 $+/+$ cells was not stimulated by oligonucleotide (Figure 3.5). The difference in these results could be due to the fact that the authors utilised a different technique for stimulating PARP activity, preferring to damage endogenous DNA using MNNG rather than introducing an exogenous oligonucleotide as was used in the studies described here. Furthermore, the authors did not permeabilise the cells as a means to measure PARP activity but measured the accumulation of PAR instead.

Studies carried out by Ame *et al.*, 1999, in the de Murcia cells, disrupted in exon 4, have attributed this activity to PARP-2 which is both DNA-damage and strand break inducible despite the absence of a zinc finger. As described in section 3.3.3, PARP-2 would not be activated by the blunt ds oligonucleotide used in the studies described in this thesis although endogenous strand breaks or those arising as a result of the permeabilisation process could activate it. Therefore PARP-2 could be the alternative PARP activity in these cells.

A more recent study by Sallmann *et al.*, 2000, using the Wang cells, have described an alternative PARP activity that is independent of strand breaks but can be induced by DNA damage. The cloned cDNA of the PARP protein was identical to the catalytic domain of PARP-1 but the DNA binding domain was absent. The authors proposed that the protein was an alternative product of the PARP gene produced by transcription of the PARP gene from an alternative transcription start site downstream of the disrupted area of exon 2 in these particular PARP-1 $-/-$ MEFs. The group named this PARP, sPARP-1. Significantly, the disrupted area of the PARP-1 gene is also upstream of this putative alternative transcription site in the exon 4 PARP-1 $-/-$ MEFs used in the studies described in this thesis and therefore

sPARP-1 could possibly be produced in these cells. Moreover, the characteristics of sPARP-1 are more consistent with the data presented in this Chapter and could therefore be responsible for the residual activity detected in the PARP-1 $-/-$ cells.

The residual activity in the PARP-1 $-/-$ knockout cells is inhibited by 0.4 μ M AG14361 (Figure 6.4). In terms of interpretation of results, therefore, the effects of AG14361 cannot be attributed solely to the inhibition of PARP-1. However, correlations with results from the PARP-1 $-/-$ knockout cells will enable conclusions to be drawn about the function of PARP-1 bearing in mind this constraint.

Studies into the roles and functions of PARP-1 and DNA-PK in DNA repair have demonstrated that a relationship exists between enzyme status and IR sensitivity as described in sections 1.8.4 and 1.12.2. The effect of increasing doses of IR on cell survival was evaluated in all cell lines using a clonogenic assay as described in section 2.5. In agreement with published data, cell lines deficient in DNA-PKcs, Ku80 or PARP-1 were approximately 4-fold more sensitive to IR than their proficient counterparts (Table 3.3) demonstrating that mutation in either PARP-1 or DNA-PK confers sensitivity to IR. Transfection with a YAC encoding human DNA-PKcs restored the radioresistance of V3 mutant cells. Similarly, in line with previous studies which have shown that hamster Ku80 cDNA can significantly restore the radioresistance in the xrs-6 mutants (Singleton *et al.*, 1997), the xrs-6 Ham Ku80 cells were only 1.5-fold more sensitive than the parental CHOK1 cell line.

PARP-1 and DNA-PK inhibitors have helped to elucidate the function and physiological effects of the two enzymes (sections 1.7.1 and 1.11). A number of hypotheses regarding the relative functions of PARP-1 and DNA-PK in DNA repair now exist and these are discussed in detail in sections 1.64 and 1.10.4. More recently, there has been interest in the clinical potential of inhibitors of PARP-1 and DNA-PK as potential radio- or chemo-sensitisers (Griffin *et al.*, 1995; Boulton *et al.*, 1999). The potential of AG14361 and NU7026 to act as radiosensitisers has been investigated in detail in Chapter 4. Since completion of these studies, more potent inhibitors of PARP-1 and DNA-PK have been developed. However, potency is not necessarily the single most important factor in the selection of a radiosensitiser for clinical trials. Other criteria of importance are:

- Lack of inherent cytotoxic or cytostatic properties in vitro
- Ability to selectively potentiate cytotoxic drug action in vivo
- Solubility, stability and ease of formulation
- Extent of metabolism and activity of metabolites
- Pharmacokinetic considerations such as half life in the plasma and biodistribution

A major criterion for a compound to act as a radiosensitiser requires that inherent cellular cytotoxicity and growth inhibitory effects are low. Before attempting to modulate the cytotoxicity of IR by the use of the PARP-1 inhibitor, AG14361, or the DNA-PK inhibitor, NU7026, (alone and in combination), the direct cellular effects of these inhibitors were investigated by means of growth inhibition studies carried out in all cell lines using the SRB assay (section 2.3.2).and cytotoxicity studies using colony forming assays (section 2.5.).

Each of the inhibitors reduced cell growth in a concentration dependent manner. Critically, both AG14361 and NU7026 were found to be non-growth inhibitory or cytotoxic per se at the concentrations (0.4 μ M and 10 μ M, respectively) and duration of experiments carried out in this thesis. Significantly, 0.4 μ M AG14361 is as much as 75-fold lower than the mean cell GI₅₀ and at this dose, the mean cell growth as a percentage of control is 100 %. Similarly, 10 μ M NU7026 is 3.5-fold lower than the mean cell GI₅₀ and at this dose, the mean cell growth rate as

% of control is >95 %. Higher concentrations of the inhibitors, however, did significantly reduce cell growth in all the cell lines tested.

Since DNA is continually undergoing repair of endogenous damage, it could be speculated that the cellular effects of either AG14361 or NU7026, alone, are a result of the inhibition of repair of intrinsic levels of DNA damage. However, AG14361 was equally growth inhibitory in both PARP-1 +/+ and PARP-1 -/- and similarly, NU7026 was equally growth inhibitory in DNA-PK proficient and deficient cell lines and therefore the cytostasis associated with the inhibitors cannot be attributed to inhibition of target enzymes.

Previous data in the literature also found no evidence to imply that inhibition of endogenous PARP-1 activity is responsible for the cytostatic effects of PARP-1 inhibitors at higher concentrations. Hunting *et al.*, 1985, carried out growth inhibition analysis on a number of PARP-1 inhibitors and showed that there was no correlation between the extent of growth delay and the potencies of the compounds as inhibitors of PARP-1. It cannot be ruled out, however, that the growth inhibitory effects of AG14361 could be attributable to the inhibition of other PARP family members that might be critical for growth or survival.

It is also possible that AG14361 is acting on other crucial NAD⁺ utilising enzymes in the cell such as the mono(ADP-ribosyl) transferases and ADP-ribosyl cyclase. As these enzymes also bind NAD⁺ and mediate the catalytic cleavage to ADP-ribose and nicotinamide, it is likely that some features of the NAD⁺ binding site (and hence inhibitor binding site) will be held in common. Rankin *et al.*, 1989, showed that PARP-1 inhibitors at micromolar concentrations were specific for PARP-1 but that at millimolar concentrations, mono(ADP-ribosyl) transferases were also inhibited. A later study by Banasik *et al.*, 1992, comparing the ability of a wide range of compounds to inhibit PARP-1 and mono(ADP-ribosyl) transferase, also showed that at micromolar concentrations PARP-1 was inhibited whilst at millimolar concentrations, mono(ADP-ribosyl) transferase was also inhibited. Mono(ADP-ribosyl) transferase can ADP-ribosylate the GTP-binding regulatory G_i protein (Tanuma *et al.*, 1988). G proteins play a major role in cell signalling and similarly, cyclic ADP-ribose, formed by the action of ADP-ribosyl cyclase, also plays an

important role in cell signalling (Lee *et al.*, 1999). Thus, inhibition of mono(ADP-ribosyl) transferase or ADP-ribosyl cyclase could disrupt cellular signal transduction pathways, potentially resulting in growth inhibition and cell death.

Similarly, the observation that NU7026 caused growth delay at high concentrations in all cell lines, including the DNA-PKcs deficient V3 cells and Ku80 deficient xrs-6 cells, implies that although NU7026 has direct growth inhibitory activity, it is not a DNA-PK related cytostasis. It is more likely that NU7026 has some non-specific effects through inhibition of other pathways that are required for cell growth. For example, PI3- kinase is required for signalling through several growth factor receptor pathways and inhibition of this enzyme results in a G1/S cell cycle block (Chang *et al.*, 2003). As discussed in section 1.13, DNA-PK shares homology with PI3- kinase and NU7026 is known to inhibit this enzyme at higher concentrations (Table 3.1). The growth inhibitory effects of both AG14361 and NU7026 did not result in cytotoxicity, however, as even a 16 h exposure to each of the drugs did not cause a significant reduction in clonogenic survival (Table 3.5).

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In purified enzyme assays, 10 μ M NU7026 completely inhibited DNA-PK activity whilst having no effect on PARP-1 activity and, similarly, 0.4 μ M AG14361 completely inhibited PARP-1 activity whilst having no effect on DNA-PK activity. No assay for the detection of polymer in intact cells was available for the measurement of PARP-1 activity. As an alternative, however, a permeabilised cell assay was used to measure the inhibitory effect of AG14361 on PARP-1 activity as this is considered to reflect the intact cell situation better than cell-free assays with recombinant or purified protein. AG14361 was more potent in a purified PARP-1 assay compared to the permeabilised cell assay (lower IC₅₀ value), however, 0.4 μ M fully inhibited PARP activity in the permeabilised cell assay.

Permeabilised cells are not in the physiological state, however, and it is therefore possible to argue that this assay does not demonstrate the entry and activity of inhibitor(s) in intact cells. Studies were therefore carried out where cells were treated with inhibitor before washing with PBS to remove residual inhibitor and subsequently performing the assay in the absence of added inhibitor. Any PARP-1 inhibition can only result from inhibitor that has transported into cells. The potency

of AG14361 was similar in the two assays with IC_{50} of 71.6 ± 2.3 and 68.4 ± 3.5 , respectively. These data suggest that AG14361 easily traverses the cell membrane (as predicted on the basis of size and charge). In addition, this is probably a reflection of the inhibitor being bound very tightly to PARP-1 prior to the process of permeabilisation. Similar experiments were not carried out with NU7026 since no adequate whole cell or permeabilised DNA-PK assay was available, however, 10 μ M is at least 20-fold the purified IC_{50} and should therefore sufficiently deplete cells of DNA-PK activity. This has been confirmed by studies in the following Chapter which have used maximal potentiation of a fixed dose of IR as an endpoint. In these experiments 10 μ M NU7026 was sufficient to maximally potentiate IR cytotoxicity.

In summary, the studies described in this Chapter confirm that the concentrations of inhibitor(s) used in the studies described in this thesis are sufficient to inhibit the cellular enzymes completely. Furthermore, they are both non-growth inhibitory or cytotoxic at these doses. Moreover, the inhibitors were not associated with the arrest of cell division in a specific phase of the cell cycle. This confirms that NU7026 does not target PI3-K at the dose used since a G1/S block would have been observed. Furthermore, these data suggest that in the absence of DNA damage, neither PARP-1 or DNA-PK are required for cell cycle regulation.

3.5 Summary

The studies described in this Chapter have shown that;-

- Cell lines deficient in either PARP-1 or DNA-PK are approximately 4-fold more sensitive to IR than parental cell lines
- The Ku complex is stable in the absence of functional PARP-1 or DNA-PKcs protein, however Ku70 is unstable in the absence of Ku80 protein.
- There is no detectable DNA-PK activity in the absence of Ku80 or DNA-PKcs
- Concentrations of NU7026 and AG14361 that cause profound inhibition of DNA-PK and PARP-1, respectively, are not cytotoxic or growth inhibitory and do not affect cell cycle phase distribution per se.

- AG14361 does not inhibit DNA-PK activity and NU7026 does not inhibit PARP-1 activity.

CHAPTER 4

RADIO- AND CHEMO-SENSITISATION BY INHIBITORS OF PARP-1 AND DNA-PK IN CELL LINES PROFICIENT OR DEFICIENT IN PARP-1 OR DNA-PK

4.1 Introduction

DNA is the molecular target for many cancer therapies. Indeed, anticancer agents that target DNA are some of the most effective agents in clinical use. DNA is an important subcellular target of ionising radiation (IR). IR induces a wide array of different DNA lesions which result in the production of single and double stranded DNA breaks either directly or indirectly through the generation of reactive free radicals or as a consequence of DNA repair pathways (reviewed in section 1.3.4.1). The integrity of cellular DNA is of paramount importance for survival and cells have evolved efficient mechanisms for both DNA damage recognition and repair in order to ultimately maintain genomic integrity (see section 1.5). In response to IR, cells initiate a complex response that includes the arrest of cell cycle progression and the activation of DNA repair pathways.

Although a detailed review of cell cycle function and control is beyond the scope of this thesis, the function of the DNA damage checkpoints is of importance when considering carcinogenesis, cancer therapy and DNA repair. If DNA damage checkpoints fail, the cell will experience the consequences of replicating a damaged template, whether that is mutation fixation resulting from replication of modified bases, loss/rearrangement of genetic material due to replication of a broken template or failure to engage apoptosis in the presence of high levels of DNA damage. DNA damage checkpoints are at four stages of the cell cycle; one at the G1/S transition, one during progression through S phase, one at the G2/M boundary and the mitotic spindle assembly checkpoint (Figure 4.1). Cell cycle arrest is therefore critically important to allow for cellular repair of IR induced damage before replication.

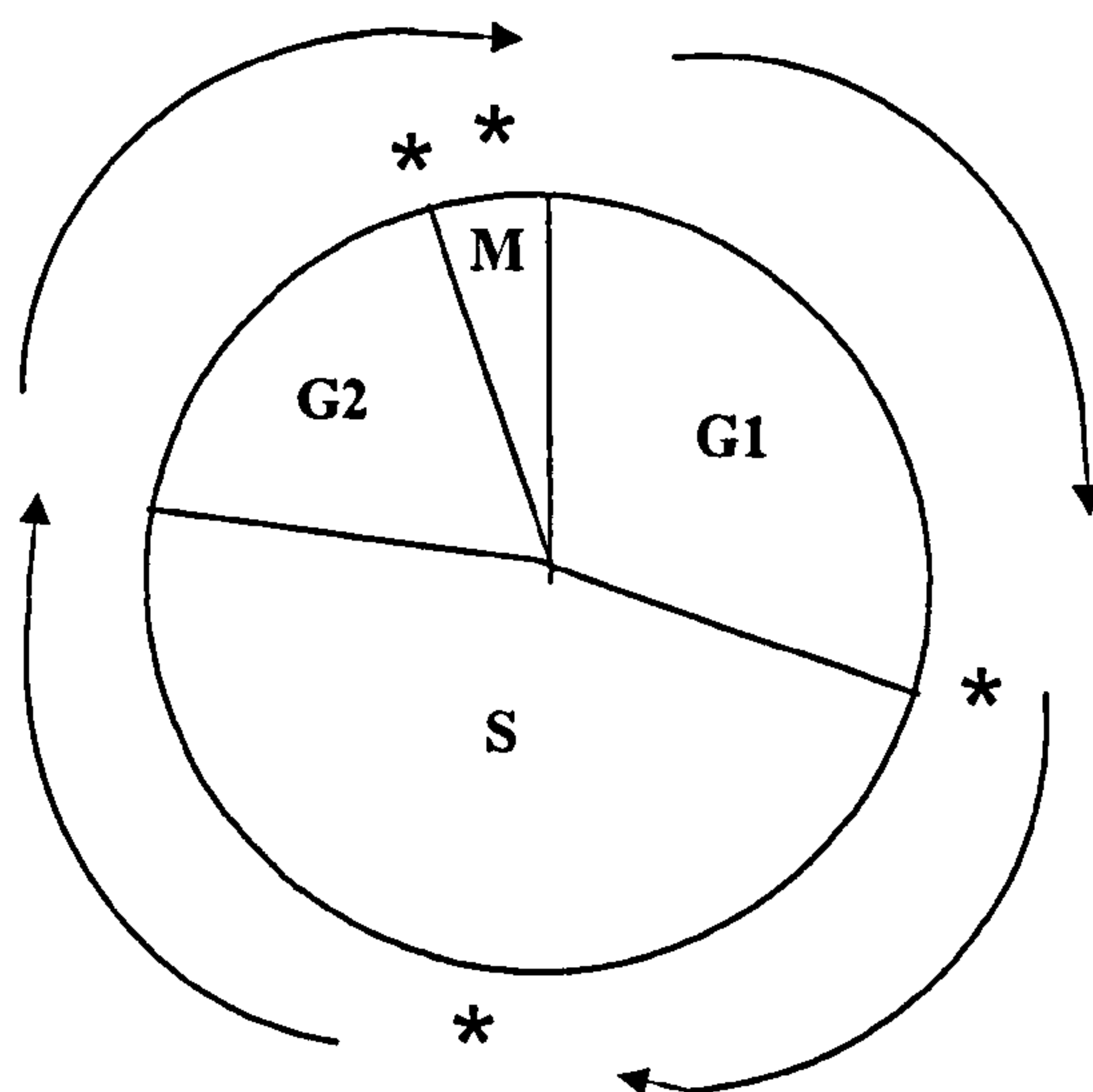


Figure 4.1 Schematic representation of the cell cycle

Diagram illustrating the different phases of the cell cycle and their relative duration. Cell cycle checkpoints are represented by an asterix.

Radiotherapy is the treatment of choice for many solid tumours and remains one of the most effective tools in the treatment of cancer. However, there are considerable differences in the outcome for treatment of tumours of differing type and tumour radioresistance is the main obstacle to clinical success. Consequently, intrinsic radiosensitivity of a tumour is an important determinant of its response to radiotherapy. Mutant cell lines that are sensitive to IR have contributed considerably to our understanding of intrinsic radiation sensitivity and have pinpointed a major role for DNA repair in this process (sections 1.8.4 and 1.12.2). Indeed, the capacity for repair has been shown to correlate with tumour responses to radiotherapy (Fox and Roberts, 1987, Barret and Hill, 1998). Resistance of cancer cells to radiotherapy is therefore thought to be largely a consequence of their ability to repair radiation-induced DNA damage. DNA repair mechanisms should therefore provide useful pharmacological targets as a means of sensitising tumour cells to IR.

PARP-1 and DNA-PK are two enzymes that are activated by DNA strand breaks and considered to be key players in DNA damage recognition and signalling (Watters *et al.*, 1999). As confirmed in Chapter 3 (Figure 3.7), mutant or knockout cell lines deficient in either of these enzymes are highly radiosensitive. Although their relative roles in DNA repair are not clearly defined, inhibition of these enzymes has serious effects on the survival of cells following exposure to various DNA

damaging agents. PARP-1 and DNA-PK therefore represent attractive targets for the development of potent and specific inhibitors as chemo and radio-sensitisers for cancer therapy.

4.1.1 Inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase as radiosensitisers and chemopotentiators.

The purpose of the following two sections is to review the literature tracing the use of inhibitors of PARP-1 and DNA-PK as radio-and chemo-sensitisers. The development of these inhibitors is described in sections 1.7 and 1.11.

4.1.1.1 Inhibitors of PARP-1

Nicotinamide was the first compound to be identified as a PARP-1 inhibitor (Fujimura *et al.*, 1967 ; Clark *et al.*, 1971). The use of a PARP-1 inhibitor as a means to potentiate DNA damage was originally suggested by Durkacz *et al.*, in 1980. In a classic study, it was demonstrated that 4 classes of PARP-1 inhibitor (nicotinamides , benzamides, methylantrines and the pyrimidine derivative, thymidine) and deprivation of NAD^+ can inhibit DNA strand break rejoining and potentiate cytotoxicity in L1210 cells following treatment with the monofunctional alkylating agent dimethyl sulphate (DMS). Indeed, 3-aminobenzamide (3AB) inhibited repair and enhanced cytotoxicity of DMS 4-fold whereas its non PARP-1 inhibitory analogue 3 aminobenzoic acid did not. Radiopotential by nicotinamide was first described in 1970 (Calcutt, 1970). Subsequently, Berger *et al.*, 1982, showed that L1210 leukaemia bearing mice co-treated with nicotinamide or 6-aminonicotinamide and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) had a higher survival than those treated with BCNU alone. The validity of these results, however, were questionable since nicotinamide is a substrate for other enzymes.

Benzamide, an analogue of nicotinamide, has been found to enhance the cell killing of the radiomimetic drug, bleomycin in HeLa cells and mice bearing Ehrlich ascites tumours (Sakamoto, 1983). Furthermore, in vivo potentiation of the alkylating agent dacarbazine (DTIC) and bleomycin in Ehrlich ascites tumours with 3-AB has

been demonstrated (Kato *et al.*, 1988). 3-methoxybenzamide was shown to potentiate the effects of bleomycin (Huet and Laval, 1985a) and the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Jacobson, 1984). Although crucial to early studies probing the function of PARP-1, benzamide and its derivatives have a number of drawbacks including a lack of potency and these have been described in more detail in section 1.7. 3AB potentiates IR but not UV, consistent with a role for PARP-1 in BER and not NER. However, potentiation has also been reported with this group of inhibitors for agents that are not repaired via the BER pathway. The cross linking agent, cisplatin, for example (Chen and Pan 1988).

Since 1980, many studies have shown that a variety of PARP-1 inhibitors can potentiate the cytotoxicity of a wide range of DNA damaging agents (Molinete *et al.*, 1993; Ding and Smulson, 1994; Curtin *et al.*, 2000). Consequently, the clinical potential of this class of agents has led to the development of diverse classes of potent PARP-1 inhibitors (Tentori *et al.*, 2002; Griffin *et al.*, 1995; Skalitzky *et al.*, 2003).

The 5' substituted dihydroquinolinones of which 3,4-dihydro-5-methoxyisoquinalin-1(2H)-one (PD128763) is the lead compound were developed by Suto *et al.*, 1991. This compound was demonstrated to potentiate cytotoxicity and inhibit the repair of DNA damage caused by IR (Arundel-Suto *et al.*, 1991) and monofunctional alkylating agents (Sebolt-Leopold and Scavone, 1992). Using structure based design, the Newcastle Drug Development Group have developed novel PARP-1 inhibitors for use as chemo- and radiosensitisers. The quinazolinones are 2-3 orders of magnitude more potent than the benzamides. Boulton *et al.*, 1995, compared NU1025 with 3-AB in L1210 cells and demonstrated that NU1025 was 50-fold more potent at potentiating the cytotoxicity of the alkylating agent, temozolomide. Importantly, this work correlated PARP-1 with the repair of single strand breaks (SSBs). Delaney *et al.*, 2000, showed that NU1025 potentiated the cytotoxicity of temozolomide and the topoisomerase I inhibitor, topotecan in a panel of 12 human tumour cell lines with equal potency to PD128763. A study by Bowman *et al.*, 1998, tested mechanistically diverse agents and showed that NU1025 potentiated the cytotoxicity of the methylating agent 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC), IR and bleomycin 3.5, 1.4 and 2-fold,

respectively. Another compound, NU1064 increased the activity of MTIC to a similar extent. A later study by Bowman *et al.*, 2001, demonstrated differential effects of NU1025 on topoisomerase I and II poisons. The cytotoxicity of the topo I poison, camptothecin, was increased 2.6-fold but the cytotoxicity of the topo II poison, etoposide was not increased when NU1025 was co-incubated. This reinforced the role of PARP-1 in the repair of SSBs.

Convincing evidence for in vivo radiopotentialiation with a PARP-1 inhibitor was obtained using PD128763 (Leopold and Sebolt-Leopold, 1990). Using a mouse tumour model, Leopold and Sebolt-Leopold demonstrated that PD128763 + 2.5 Gy IR caused a 10-15 day increase in tumour growth delay of SCC7 sarcomas compared to irradiation alone. However, acute hypotension and hypothermia were observed in the mice following treatment suggesting that this inhibitor had other cellular targets apart from PARP-1.

Despite having IC₅₀ values in the micromolar range, the benzamides need to be used at high concentrations in the millimolar range to potentiate the cytotoxicity of DNA damaging agents in vitro. Furthermore, there was considerable overlap between the concentrations required to potentiate drug cytotoxicity and the growth inhibitory effects of the compounds when used alone. Similarly, the quinazolinones have IC₅₀ values in the nanomolar range but need to be used at high micromolar concentrations in cell culture for effective potentiation. In contrast, the PARP-1 inhibitor used in the studies described in this thesis, AG14361, has been demonstrated to exhibit high activity in in vitro whole cell assays at sub-micromolar concentrations (Calabrese *et al.*, 2003, in Press).

4.1.1.2 Inhibitors of DNA-PK

Wortmannin (WM), a sterol like fungal metabolite, shown previously to be a potent inhibitor of PI3-kinase was one of the first compounds demonstrated to be capable of inhibiting DNA-PK in vitro. Indeed, WM has been shown to potentiate IR-induced cytotoxicity at concentrations that inhibited cellular DNA-PK activity (Boulton *et al.*, 1996a; Boulton *et al.*, 1999; Chernikova *et al.*, 1999; Rosenzweig *et al.*, 1997). Boulton *et al.*, 1999, showed that WM inhibited DSB repair and not SSB

repair thus adding weight to the argument that DNA-PK is involved in DSB and not SSB repair. Furthermore, Muller *et al.*, 1998, demonstrated that WM sensitised murine cells to bleomycin and Plumb and Kaye, 1999, have shown that WM is able to increase DSBs caused by the topo I poison, topotecan (topo I poison only induces SSBs, which are considered to convert to DSBs at the replication fork). Boulton *et al.*, 2000 showed that WM potentiated etoposide cytotoxicity in parental CHOK1 cells whilst no potentiation was seen in the Ku80-deficient xrs-6 cells which are deficient in DNA-PK activity. Thus, with regard to its effects on DNA DSB repair and cell killing, DNA-PK is most likely the target of WM. However, WM is primarily a PI3-K inhibitor and is known to inhibit other PI3-kinase family members including the ataxia telangiectasia gene product, ATM or ATR (Table 3.1). Therefore, although WM clearly inhibits DNA-PK, the ability of WM to potentiate IR induced cytotoxicity could also be mediated, at least in part, via an inhibition of ATM or ATR (Hartley, 1995).

The compound LY294002 (2-(4-morpholynyl-2-pyridone) and its derivatives are chemically unrelated to WM (section 1.11, Figure 1.8) and are reversible competitive inhibitors with respect to ATP (Vlahos *et al.*, 1994). LY294002 has been shown to sensitise cells to IR (Rosenzweig *et al.*, 1997) and to topotecan (Plumb and Kaye, 1999). In addition, these compounds have been shown to increase the DNA damage caused by other cytotoxic agents which generate DSBs such as bleomycin (Hosoi *et al.*, 1998) and the nitrogen mustards (Muller *et al.*, 2000). As stressed for WM, however, there is no clear evidence that the potentiation seen with LY294002 or any of its derivatives is due to an inhibition of DNA-PK and not due to the inhibition of other PIKK family members.

There is a requirement for more potent and specific PARP-1 and DNA-PK inhibitors to probe further the physiological functions of PARP-1 and DNA-PK and to address the therapeutic potential of PARP-1 and DNA-PK inhibition for the treatment of human cancer. AG14361 represents the first inhibitor of PARP-1 with the required pharmacological properties of potency, specificity and solubility and in vivo anti-tumour activity. NU7026 represents the first potent and specific inhibitor of DNA-PK.

4.1.2 Potentially lethal damage recovery (PLDR)

Non-proliferating cells exposed to IR demonstrate substantially higher survival than proliferating cells, or cells induced to start a proliferative cycle shortly after irradiation (Phillips and Tolmach, 1966; Little, 1969). Consistent with this, slow growing tumours are more radioresistant than rapidly growing cancers as they have a larger proportion of non-cycling cells, a consequence of local tumour 'microenvironments' such as cell and vascular density as well as availability of oxygen and nutrients. Indeed, quiescent (Q) cells constitute the majority of the tumour cell population of some of the most radioresistant tumours which include connective tissue and bone carcinomas (Guichard *et al.*, 1984). These 'microenvironments' are thought to cause enhancement of recovery from 'potentially lethal damage' (PLDR) (Hahn and Little, 1972).

Potentially lethal damage (PLD) has been operationally defined as damage that can be either modified to non-lethal damage or 'fixed' to lethal damage by altering conditions following exposure to IR (Phillips and Tolmach, 1966). Significantly, an increase in PLDR has been shown to be proportional to an increase in non-cycling or Q cells which are thought to be the resistant compartment within a tumour (Nakatsugawa and Dewey, 1984). Moreover, radiation survival analysis of synchronously dividing cells has demonstrated that cells irradiated during the G2 and M phases of the cell cycle are more radiosensitive compared to cells irradiated during G1 and S (Sinclair, 1969). In agreement with these findings, Hahn and Little, 1974, demonstrated that the process of PLDR occurred primarily in the G1 phase of the cell cycle. Recovery from PLD is therefore an important phenomenon that probably enhances the resistance of tumour cells to IR.

Plateau phase cell cultures have a large proportion of non-proliferating G1 phase cells, characteristic of *in vivo* tumours, and therefore they are considered appropriate *in vitro* systems compared to exponentially growing cells for studying PLDR (Little *et al.*, 1973). In this model, plateau phase cultures are exposed to IR and then allowed to recover for various periods of time before being counted and seeded, at low density, in order to induce proliferation and hence colony formation.

This gives a measure of the radiosensitivity of the cells. This model was first used with established human and rodent cell lines (Hahn and Little, 1974) and later applied to cell lines derived from specific human tumours (Weichselbaum *et al.*, 1977; Weichselbaum *et al.*, 1980; Weichselbaum *et al.*, 1982a; Weininger *et al.*, 1978). The in vitro model used in the studies described in this Chapter measured the increase in survival of irradiated growth-arrested G1 phase cells following delayed plating for colony formation (Figure 4.2).

The existence of PLDR following irradiation has been demonstrated in various experimental human tumour cells in vitro (Guichard *et al.*, 1984; Kinsella *et al.*, 1984; Arundel and Leith, 1986) and in vitro studies have demonstrated that significant PLDR occurs in human tumour cells exposed to doses of IR which are similar to the doses used in clinical radiotherapy. For example, Courtenay *et al.*, 1982, studied PLDR in 9 tumour cell lines derived from tumours of varying radiocurability. Three cell lines from tumours considered incurable showed a considerably higher recovery from PLD than 3 cell lines obtained from tumours considered curable. In line with this observation, Weichselbaum *et al.*, 1977, reported a human osteosarcoma line with a 24-fold PLDR following 7 Gy IR. Based on these findings, tumours which are radioresistant are more likely to contain cells proficient in PLDR. Guichard *et al.*, 1984, proposed that PLDR may contribute to radiotherapy failure under certain conditions.

Recovery comparable in magnitude to that found in vitro has been shown in in vivo experimental tumours. The existence of PLDR in vivo was first described in several different rodent tumour systems (Guichard *et al.*, 1982; Phillips and Tolmach, 1966). Guichard *et al.*, 1984, investigated PLDR in human tumour xenografts. Tumours were implanted subcutaneously into the flank of athymic mice and removed at various times from 0 to 24 h after IR. The tumours were then dissociated into single cell suspensions, counted and seeded at low densities for 'ex vivo' colony formation. The recovery ratio was compared for tumours of low and intermediate curability. In line with in vitro studies, the authors demonstrated that the capacity for PLDR was greater in those tumours of lower curability. Furthermore, this group, along with others, demonstrated that the larger the tumour, the greater the amount of recovery (Hahn and Little, 1972; Little *et al.*, 1973; Tubiana *et al.*, 1977).

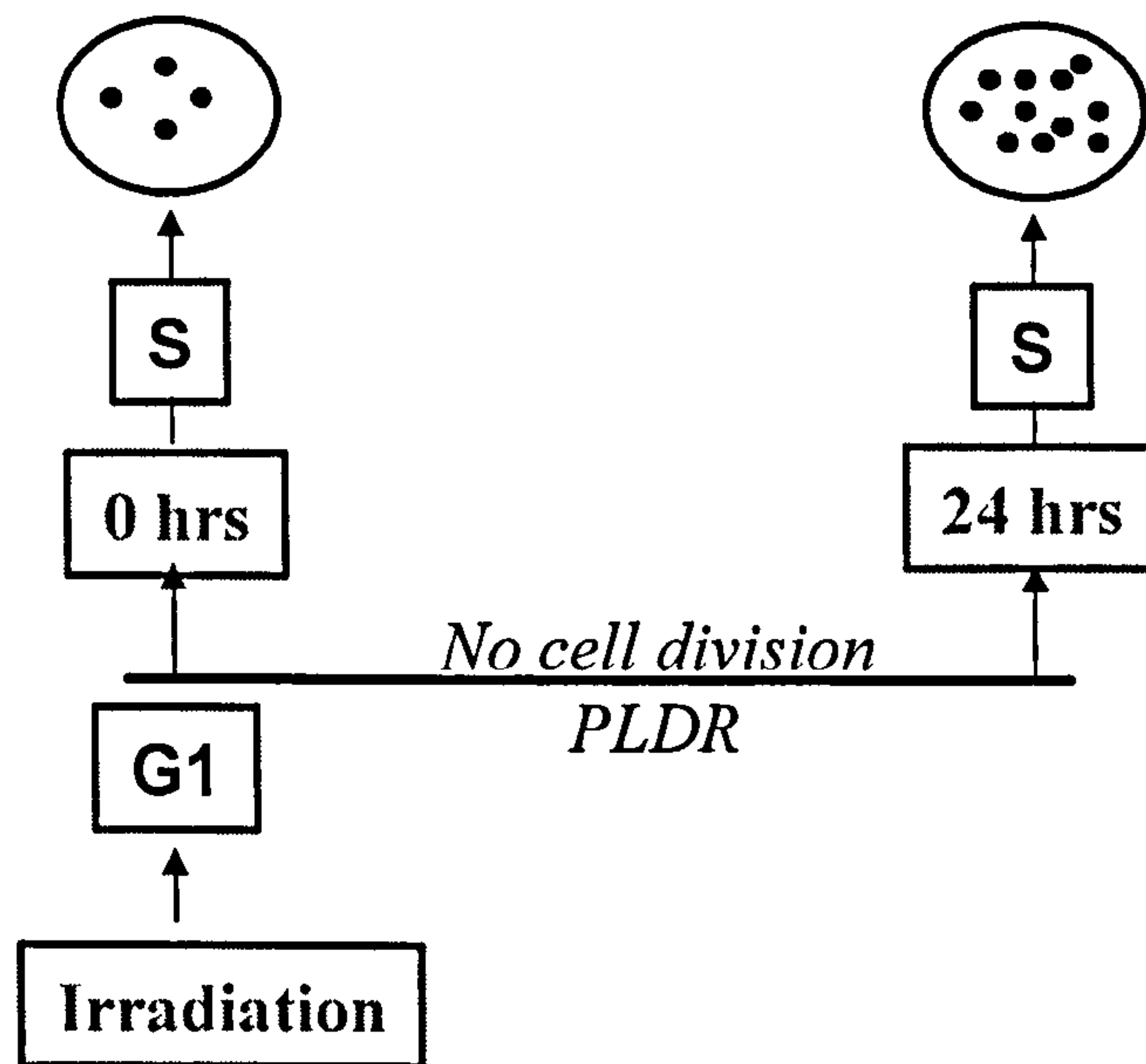


Figure 4.2 Schematic representation of the proposed mechanism for potentially lethal damage recovery (PLDR)

Non-proliferating cells exposed to IR are known to undergo PLDR, leading to substantial increases in survival. This diagram depicts the PLDR model used in the studies described in this thesis. Following arrest in G1, cells were irradiated and either re-seeded for colony formation immediately or following 24 h recovery. Cell survival was assessed by colony formation as described in section 2.5. The proposed mechanism for PLDR is that when cells are maintained in a non-cycling state following irradiation they are able to repair ‘potentially lethal damage’ that would otherwise be fixed to lethal damage following progression through S phase of the cell cycle.

Consistent with this, larger tumours generally have a higher proportion of G1 phase cells as a consequence of local tumour micro-environments, including oxygen and nutrient deprivation. Furthermore, in vivo PLDR in human tumour xenografts is similar to or greater than that observed in rodent tumours (Hahn and Little, 1972; Little *et al.*, 1973; Tubiana *et al.*, 1977; Shipley *et al.*, 1975; Urano *et al.*, 1976)).

The ability to enhance the radiosensitivity of radioresistant cell lines has considerable potential for clinical application. Current interest in clinical radiosensitisation has focused on hypoxic radiosensitisers and chemical radiosensitisers such as the halogenated pyrimidine analogs (Adams, 1992; Gregoire *et al.*, 1999; Horsman, 1995). Little effort, however, has been made to develop new therapeutic techniques that might reduce PLDR in human tumours. Several early reports, using either cell lines mutationally inactivated in DNA-PK, or PARP-1 inhibitors, showed a reduction in PLDR in non-proliferating cells (Illkias, 1990; Li *et al.*, 1998; Huet and Laval, 1985; Arundel-Suto *et al.*, 1991; Rudat *et al.*, 1998). The potential of novel potent small molecule inhibitors of PARP-1 and DNA-PK to prevent PLDR is therefore an important consideration in the assessment of their therapeutic application.

Early studies of PLDR have investigated the effects of PARP-1 inhibitors. These studies used the classical PARP-1 inhibitors, nicotinamide (Ben-Hur *et al.*, 1984a) or the aromatic amide, 3-AB following IR (Burgman and Konings, 1989; Utsumi and Elkind, 1994; Huet and Laval, 1985; Brown *et al.*, 1984; Ben Hur *et al.*, 1984b). All showed that inhibition of PARP-1 could reduce the PLDR capacity of cells. In one study, the effect of four chemical classes of PARP-1 inhibitors including nicotinamide and 3-AB on IR-induced PLDR in plateau phase CHO cell cultures were compared. Following 12 Gy, untreated cells exhibited a 5-6 fold increase in survival over a 6 h period compared to cultures that were re-plated immediately. 10 mM 3-AB or nicotinamide reduced PLDR by at least 50 % compared to controls, with 3-AB being a more potent inhibitor than nicotinamide (Brown *et al.*, 1984). A different study by Huet and Laval, 1985, however, showed that although 3-AB could significantly reduce PLDR in CHO cells, nicotinamide did not. No studies have been carried out with inhibitors of DNA-PK, to date. However, it has been demonstrated that cell lines deficient in DNA-PK have a reduced capacity

for PLDR thus suggesting a role for DNA-PK in PLDR. These studies are described in more detail in section 4.4.

4.2 Aims

Overview The area of mammalian DNA repair and its relationship to cancer and therapeutic approaches is rapidly growing. One of the main aims of the Newcastle Drug Development Group is to develop novel compounds that increase the efficacy of anticancer agents and such ‘resistance modifying agents’ should ideally be inherently non-toxic and selectively sensitise tumour cells to IR and chemotherapeutic agents.

The aim of this results Chapter was to evaluate the ability of the novel PARP-1 and DNA-PK inhibitors AG14361 and NU7026, respectively, to act as chemo- or radio-sensitisers and PLDR inhibitors:-

- Investigate the effects of the inhibitors on cell survival following IR in exponentially growing cell lines proficient or deficient in DNA-PK or PARP-1
- Investigate the effects of the inhibitors on PLDR following IR in growth arrested cell lines proficient or deficient in DNA-PK or PARP-1
- Investigate the effects of the inhibitors on cell cycle progression following subculture of irradiated G1 arrested cells
- Investigate the effect of NU7026 on cell survival following treatment with the clinically relevant chemotherapeutics etoposide and bleomycin in exponentially growing cell lines proficient or deficient in DNA-PK

4.3 Results

The initial in vitro screening of NU7026 and AG14361 utilised purified human enzymes or conditions which facilitated access of the compound to their respective targets, permeabilised cells, for example. This only provides data on the inhibitory potency of the compounds. Other considerations include interactions with cellular components and intracellular metabolism as well as permeation of the inhibitors through the cell membrane, all of which influence the inhibitory potency of a novel compound in intact cells. It is therefore important to assess the ability of a novel compound to act in an intact cell situation. Data presented in this Chapter investigated the whole cell effects of AG14361 and NU7026 in response to IR. Paired cell lines (described in section 3.1.1) proficient or deficient in PARP-1 or DNA-PK were used as models to confirm that PARP-1 and DNA-PK are the targets of AG14361 and NU7026, respectively.

4.3.1 Radiosensitisation by AG14361 and NU7026, in vitro

The roles of PARP-1 and DNA-PK in the cellular responses to IR has been extensively reported in the literature. Clonogenic survival (as described in section 2.5) was used as the biological endpoint for the assessment of the cytotoxicity of IR and the effect of AG14361 and NU7026 (alone or in combination) in exponentially growing cells.

4.3.1.1 Effect of increasing doses of NU7026 in the presence or absence of a fixed dose of IR

Cell survival following exposure to increasing concentrations of the DNA-PK inhibitor, NU7026, alone, and in combination with a fixed dose of IR was investigated in the DNA-PK deficient and proficient V3 and V3YAC cell pair and the PARP-1 proficient and knockout cell lines. An equitoxic dose of IR was used for each cell line that would cause only moderate cellular cytotoxicity alone (approximately 30 % cell kill) (see Figure 3.7) but that would allow potentiation of IR cytotoxicity to be measured (V3YAC, 2 Gy; PARP-1 +/+, 2 Gy; V3, 0.1, Gy; PARP-1 -/-, 0.14 Gy). Exponentially growing cells were pre-incubated with

inhibitor for 1 hr and post-incubated for 16 hrs at 37°C before seeding for colony formation in drug-free medium.

NU7026, alone, did not significantly reduce survival up to a concentration of 20 μ M. However, the highest achievable concentration of 50 μ M decreased survival by approximately 20 % in all cell lines. For example, (Figure 4.3 (a) A), the plating efficiency (which is a measure of a cells ability to form colonies) of the V3YAC cells was 67 ± 3 in the presence of 1 % DMSO and this was significantly reduced to 55 ± 2 in the presence of 50 μ M NU7026 alone ($p = 0.02$). This is consistent with the growth inhibitory data described in the previous Chapter, where NU7026 inhibited cell growth in a concentration dependent manner. Doses up to 20 μ M had no significant effect on growth, however, doses above 20 μ M gave a modest inhibition of growth of approximately 20 % in all cell lines. As discussed in Chapter 3, it is possible that this is due to an effect on PI3- kinase since the survival and growth effects of NU7026 also occur in the DNA-PK deficient V3 cell line.

Radiosensitisation of V3YAC, PARP-1 ++ and PARP-1 -/- cells by NU7026 was concentration dependent, with concentrations ≥ 5 μ M giving maximal potentiation (Figure 4.3 (a) and 4.3 (b)). At these doses, there was a 2.5-3-fold potentiation of 2 Gy IR in V3YAC and PARP-1 +/- cells and a 2-fold potentiation of 0.1 Gy in PARP-1 -/- cells. As expected, no significant radiopotential with NU7026 was seen in the V3 cells, $p = > 0.05$ (Table 4.1). Analysis of the survival curves gave the concentration of NU7026 required to reduce cell survival of irradiated cells by 50 % (LC_{50} value). These were; 3.4 ± 0.1 μ M (V3YAC); > 50 μ M (V3); 1.3 ± 0.01 μ M (PARP-1 +/-); 1.9 ± 0.03 μ M (PARP-1 -/-).

10 μ M was selected as the concentration of NU7026 for use in subsequent studies described in this thesis, unless otherwise stated, as it should allow maximal potentiation of IR without being growth inhibitory or cytotoxic per se. As described in Chapter 3, 0.4 μ M AG14361 was used as a standard dose as it is 100 x K_i and <5 % of the GI_{50} and is sufficient for maximum chemosensitisation (Calabrese *et al.*, in press).

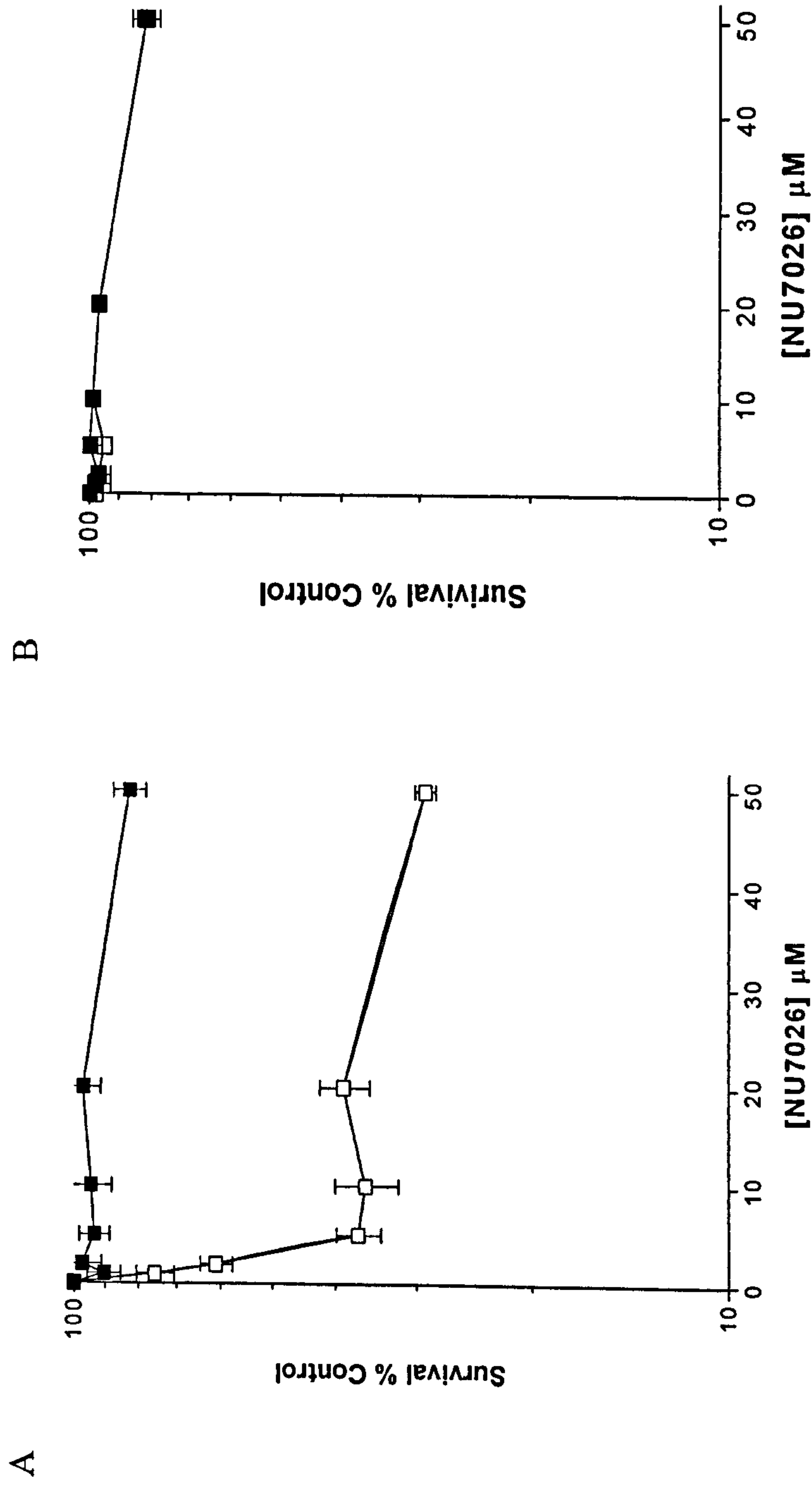


Figure 4.3 (a) Cytotoxicity of NU7026 alone and in combination with IR in exponentially growing cells:- A, V3YAC ; B, V3 ■ IR alone; □ IR + NU7026. Cells were exposed to increasing concentrations of NU7026 in the presence or absence of equitoxic doses of IR (2 GY, V3YAC; 0.1 GY, V3) and post-incubated for 16 h before seeding for colony formation. Data are normalised to 1 % v/v DMSO or IR alone controls and are the mean \pm SEM of at least 3 independent experiments.

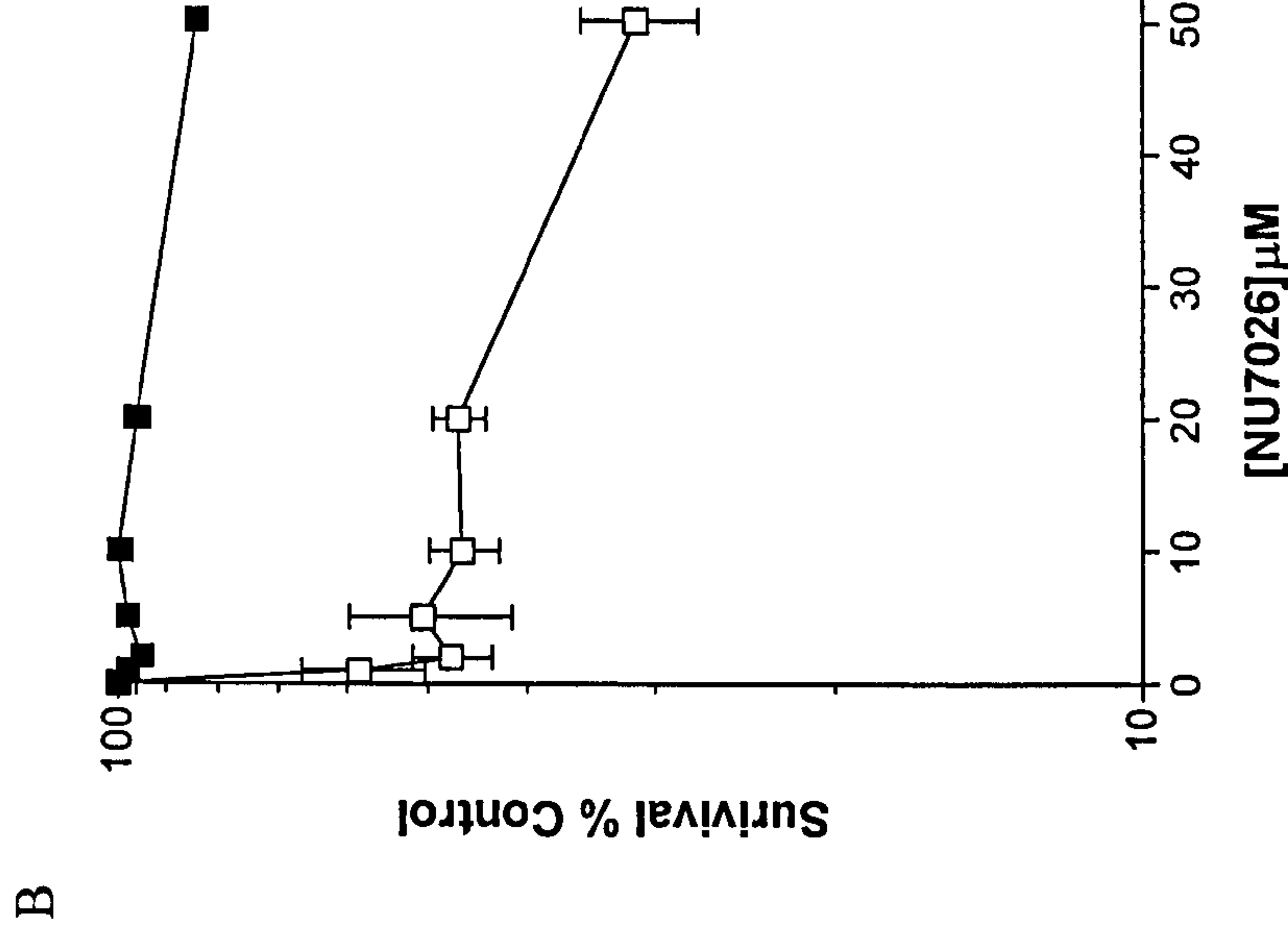
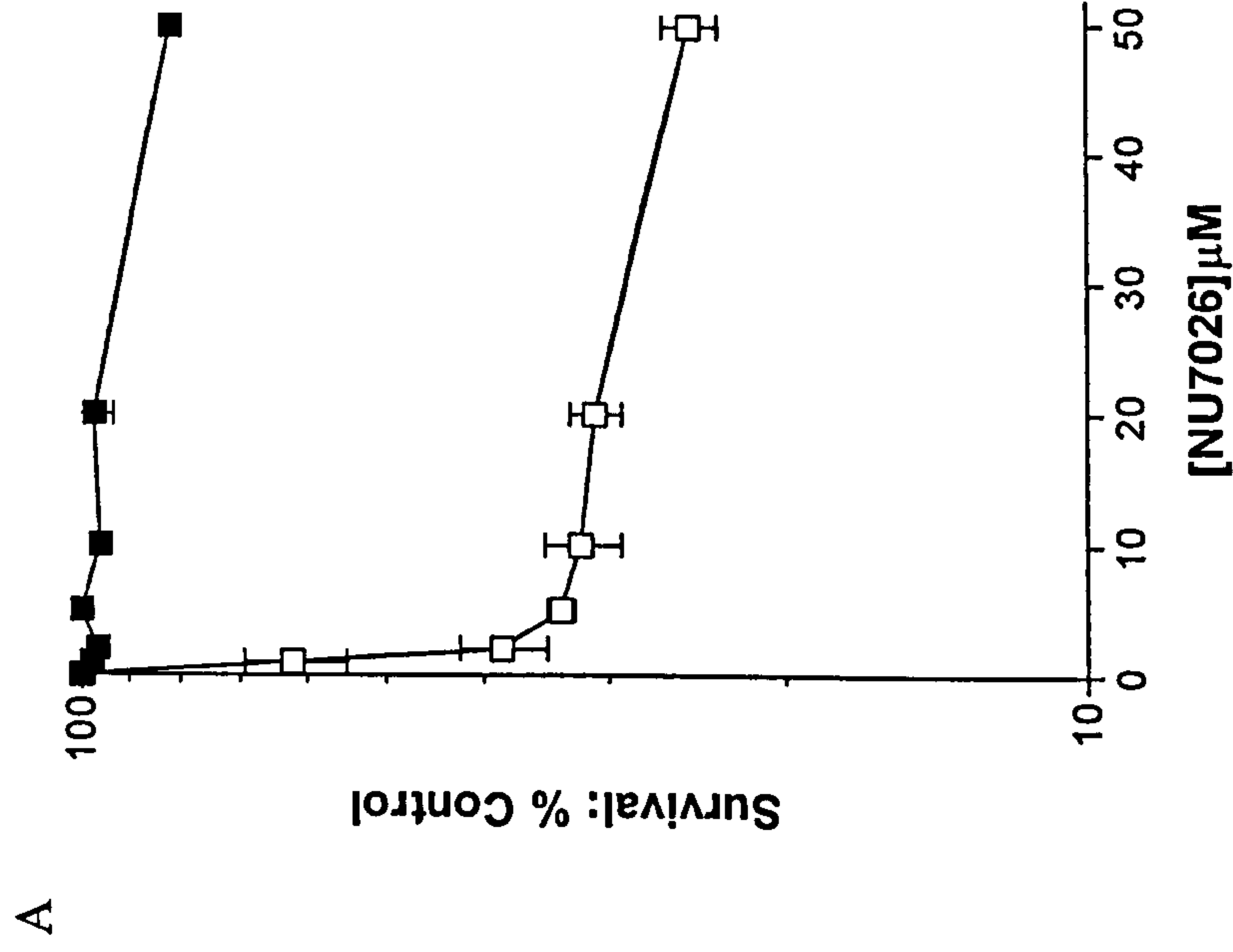


Figure 4.3(b) Cytotoxicity of NU7026 alone and in combination with IR in exponentially growing cells :- A, PARP-1++ ; B, PARP-1 -/- ; ■ IR alone; □ IR + NU7026. Cells were exposed to increasing concentrations of NU7026 in the presence or absence of equitoxic doses of IR (2 Gy, PARP-1 +/+; 0.14 Gy, PARP-1 -/-). and post-incubated for 16 h before seeding for colony formation. Data are normalised to 1% v/v DMSO or IR alone controls and are the mean \pm SEM of at least 3 independent experiments.

Cell line /Dose NU7026 (μM)	V3YAC	V3	PARP-1 +/+	PARP-1 -/-
1	1.2 ± 0.20	1.0 ± 0.04	1.6 ± 0.20	1.7 ± 0.06
2	1.6 ± 0.13	1.1 ± 0.01	2.6 ± 0.15	2.0 ± 0.04
5	2.5 ± 0.32	1.0 ± 0.03	3.0 ± 0.11	1.9 ± 0.02
10	2.6 ± 0.21	0.9 ± 0.04	3.1 ± 0.15	2.2 ± 0.03
20	2.4 ± 0.24	1.2 ± 0.04	3.0 ± 0.22	2.3 ± 0.08
50	2.8 ± 0.22	0.9 ± 0.02	3.2 ± 0.18	2.4 ± 1.1

Table 4.1 Comparison of the effect of increasing concentrations of NU7026 on the cytotoxicity of IR

Figures represent the killing enhancement factor for each dose of NU6026 (survival in the presence of NU7026 alone / survival NU7026 + IR). Data are derived from the experiments shown in Figure 4.3 and are the mean ± SEM from at least 3 independent experiments.

4.3.1.2 The cytotoxic effect of increasing doses of IR in the presence or absence of a fixed concentration of AG14361 and/or NU7026

As described in Chapter 3 (see Figure 3.7), there was a dose dependent reduction in cell survival following treatment with IR. Cell lines deficient in PARP-1 or DNA-PK were approximately 4-fold more sensitive to IR than their proficient counterparts. The effect of fixed concentrations of AG14361 or NU7026 (alone or in combination) on the cytotoxicity of increasing doses of IR were studied in exponentially growing cells. Briefly, cells were pre-incubated with inhibitor(s) for 1 hr before irradiation and then post-incubated in the presence of inhibitor(s) for a further 16 hrs at 37°C before seeding for colony formation in drug free medium.

Figures 4.4(a)A and 4.4(b)A show that in the presence of fixed concentrations of AG14361 and NU7026 (alone or in combination) the dose-dependent reduction in survival induced by IR was enhanced in exponentially growing V3YAC and PARP-1 +/- cells. In both cell lines, AG14361 reduced survival by at least 1.5-fold for all doses of IR whilst NU7026 reduced survival by 2-fold for all doses of IR. Combination of the inhibitors reduced survival by at least 5-fold for all doses of IR. The magnitude of the potentiation was observed to increase as the dose of IR was increased. For example, in the V3YAC cell line, survival following 2 Gy IR was reduced 5-fold when AG14361 and NU7026 were used in combination. However, survival following 6 Gy IR was reduced 34-fold in the presence of both AG14361 and NU7026.

Potentiation of IR cytotoxicity by the inhibitors, alone and in combination, was quantified utilising 'potentiation factor' values calculated at a survival of 10 % (PF₉₀). PF₉₀ values were defined as a ratio of the dose of IR alone that reduced cell survival to 10 % divided by the dose of IR that reduced survival to 10 % when co-incubated with a fixed dose of PARP-1 or DNA-PK inhibitor. The values are summarised in Table 4.2. Both inhibitors used alone potentiated the cytotoxicity of IR in the enzyme proficient V3YAC and PARP-1 +/- cells and similar PF₉₀ values were obtained for these cell lines. Greater than additive effects were observed when the inhibitors were co-incubated. For example, the mean PF₉₀ values for AG14361

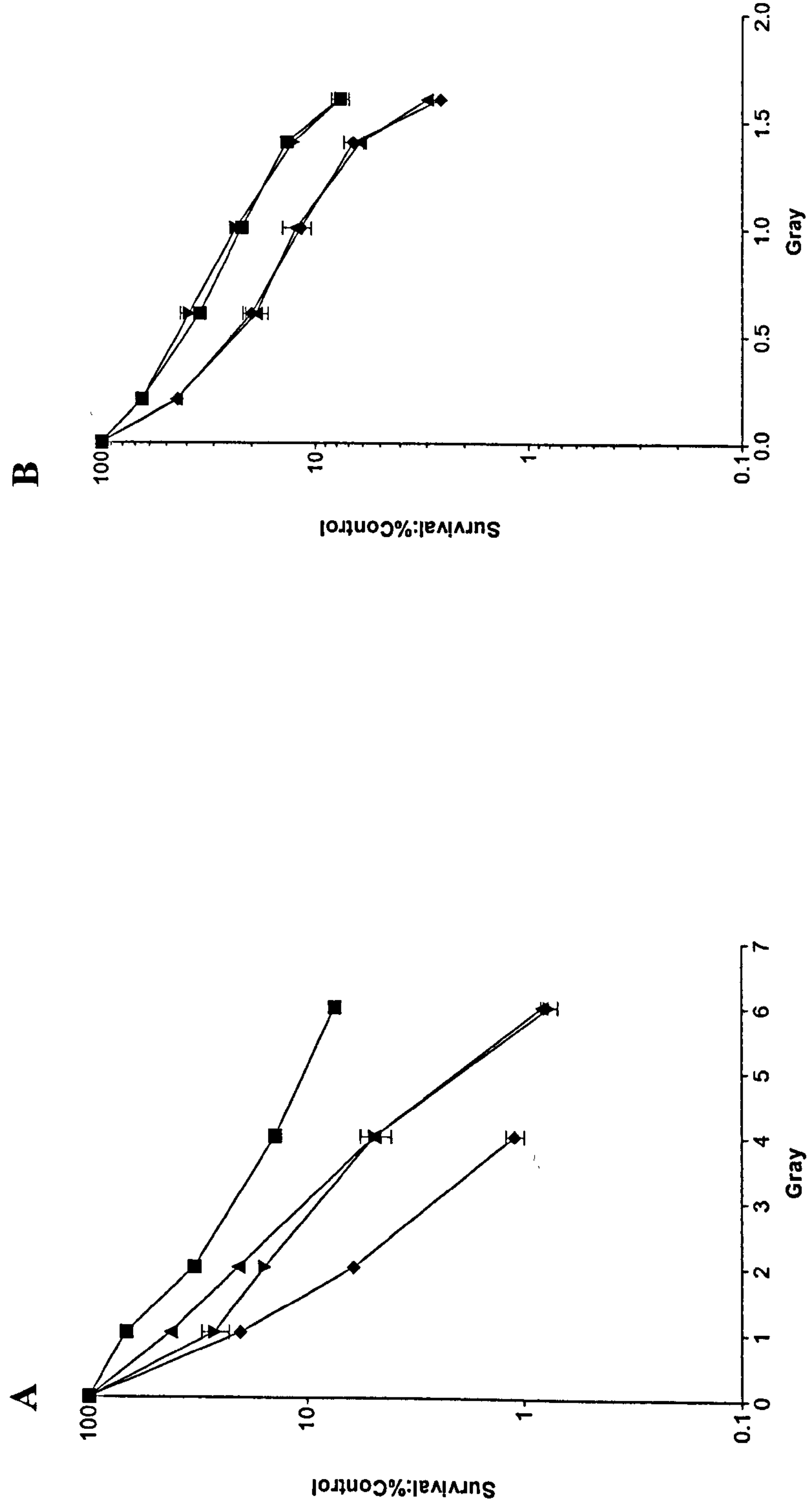


Figure 4.4 (a) Effects of increasing doses of IR in the presence or absence of AG14361 and NU7026 on the survival of exponentially growing cells A, V3YAC; B, V3; ■ IR alone; ▲ IR + 0.4 μ M AG14361; ▼ IR + 10 μ M NU7026; ◆ IR + AG14361 + NU7026. Cells were pre-incubated with inhibitor(s) for 1 h before irradiation and then post-incubated in the presence of inhibitor(s) for 16 h before seeding for colony formation in the absence of inhibitor(s). Data are normalised to 1 % (v/v) DMSO alone or inhibitor alone controls and are the mean of at least 3 independent experiments \pm SEM

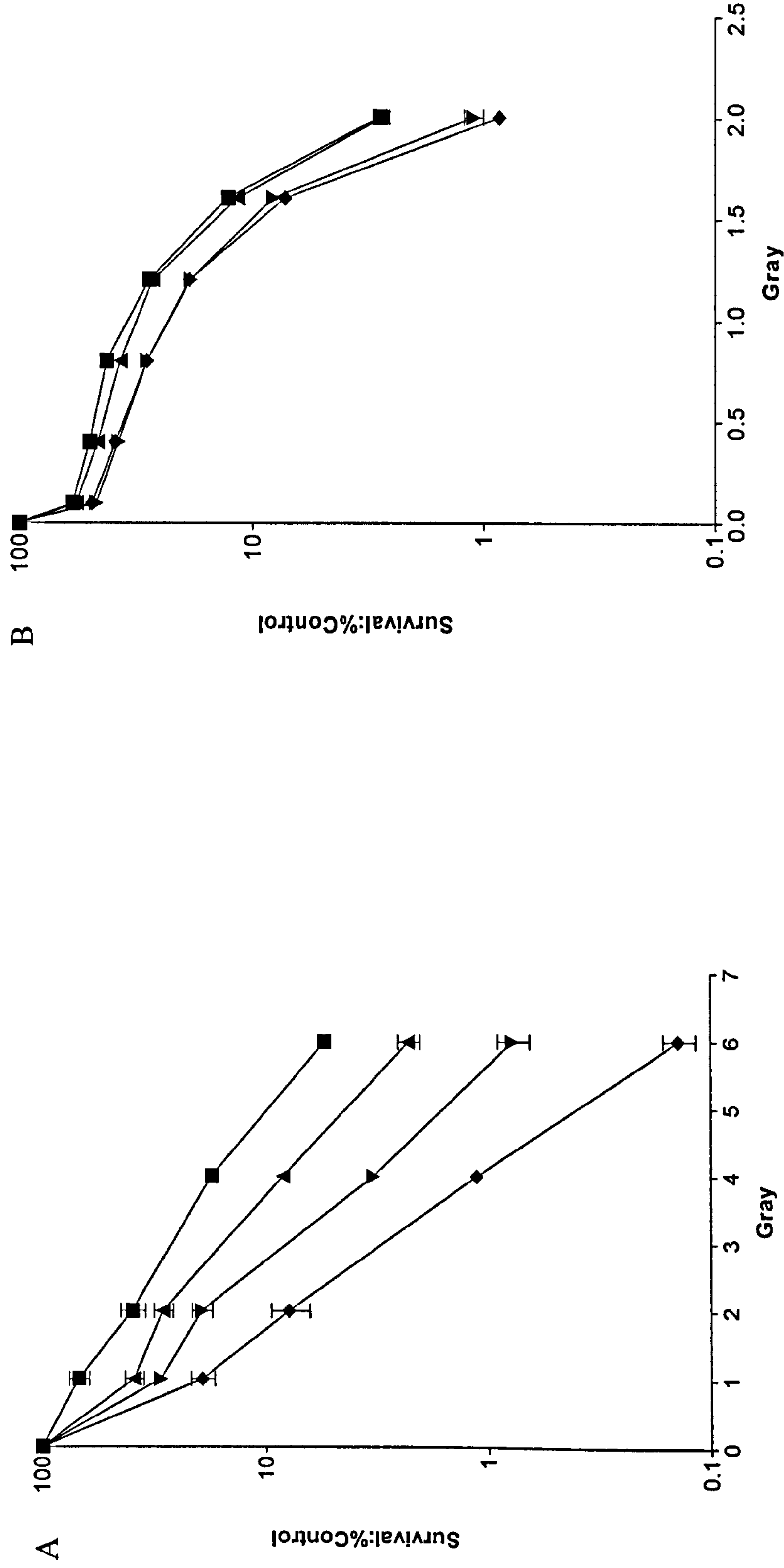


Figure 4.4 (b) Effects of increasing doses of IR in the presence or absence of AG14361 and NU7026 on the survival of exponentially growing cells A, PARP- +/+; B, PARP-1 -/- ; ■ IR alone; ▲ IR + 0.4 μM AG14361; ▼ IR + 10 μM NU7026; ◆ IR + AG14361 + NU7026. Cells were pre-incubated with inhibitor(s) for 1 h before irradiation and then post-incubated in the presence of inhibitor(s) for 16 h before seeding for colony formation in the absence of inhibitor(s). Data are normalised to 1 % (v/v) DMSO alone or inhibitor alone controls and are the mean of at least 3 independent experiments ± SEM

Treatment/ Cell line	IR + AG14361	IR + NU7026	IR + AG14361 + NU7026
V3YAC	1.40 ± 0.00	1.51 ± 0.04	2.78 ± 0.04.
V3	1.30 ± 0.01	1.0 ± 0.00	1.3 ± 0.03
PARP-1 +/+	1.37 ± 0.00	1.69 ± 0.03	2.81 ± 0.19
PARP-1 -/-	1.03 ± 0.01	1.12 ± 0.01	1.13 ± 0.00

Table 4.2 Comparison of the PF₉₀ values derived from the IR survival curves in Figures 4.3 a and b

All mean PF₉₀ values (± SEM) were calculated from the ratio of the individual LD₉₀ values. The mean LD₉₀ values for IR + inhibitor(s) were all significantly different from the mean LD₉₀ values for IR alone in the same cell line ($p \leq 0.05$, $n = \geq 3$, two-tailed Student's t-test), excluding the LD₉₀ values for IR + inhibitor in the cell line lacking its corresponding target enzyme (see below).

² The bold PF₉₀ values, which are not significantly different from unity, demonstrate the lack of potentiation by the inhibitor in the cell line which lacks its corresponding target enzyme.

and NU7026 in the V3YAC cell line were 1.4 and 1.5, respectively but when used in combination, the PF₉₀ value was 2.8 as opposed to the expected additive value of 2.1.

Whilst significant radiosensitisation by AG14361 was observed in all cell lines with PARP-1 activity (PF₉₀ values 1.3-1.4), there was no significant radiosensitisation of the PARP-1^{-/-} cells (PF₉₀ = 1.03, $p = 0.12$). Similarly, NU7026 caused significant radiosensitisation in the DNA-PK proficient cell lines (PF₉₀ values 1.1-1.7) but it did not significantly potentiate the cytotoxicity of IR in the DNA-PK deficient V3 cells (PF₉₀ = 1, $p = 0.8$). These data support the hypothesis that AG14361 and NU7026 are mediating their potentiating effects specifically by the inhibition of their target enzymes, PARP-1 and DNA-PK, respectively.

AG14361 significantly potentiated IR cytotoxicity in the DNA-PK deficient V3 cell line (PF₉₀ = 1.3). However, the radiosensitivity of PARP-1^{-/-} cells was only slightly reduced by treatment with 10 μ M NU7026 (PF₉₀ = 1.12) although this is significant by a two tailed Student's t test ($p = 0.04$).

4.3.1.3 Determination of exposure time required to achieve maximal potentiation of IR with AG14361

Experiments were carried out to determine the length of exposure time required to obtain maximum potentiation of IR-induced cytotoxicity in parental CHOK1 cells. Briefly, cells were irradiated with 4 Gy IR (which causes approximately 75 % cell kill) in the presence or absence of 0.4 μ M AG14361 and post-incubated for different periods of time before being seeded for colony formation in drug-free medium. Potentiation was similar (2-2.5-fold) for all time points and extending the incubation period with AG14361 beyond 30 minutes did not result in any further potentiation (Figure 4.5).

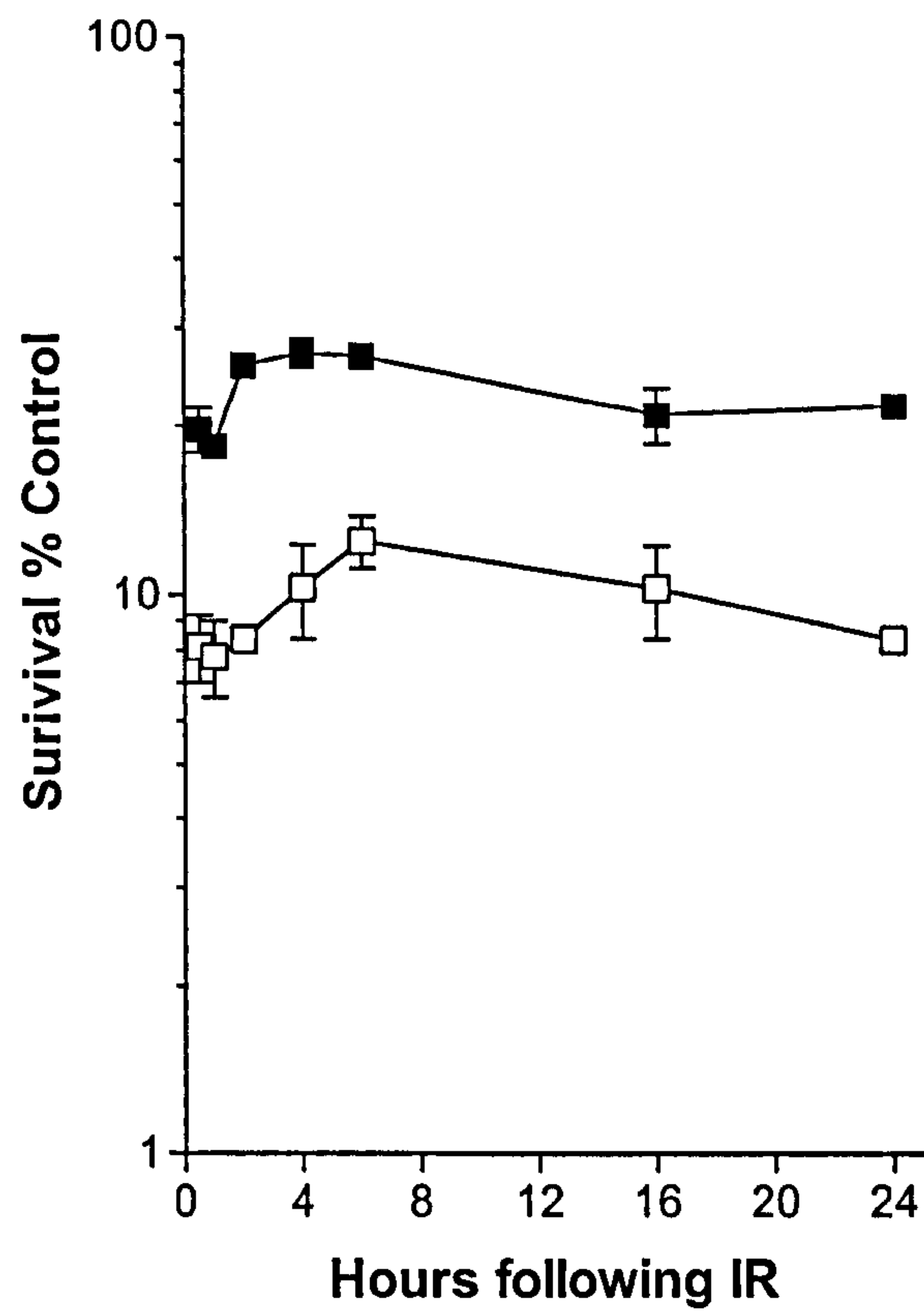


Figure 4.5 Sensitisation of CHOK1 cells to 4 Gy IR by exposure to AG14361 for varying times ; ■ IR alone; □ IR + 0.4 μ M AG14361. Data are normalised to DMSO alone or drug alone controls and are the mean \pm SEM of at least 3 independent experiments.

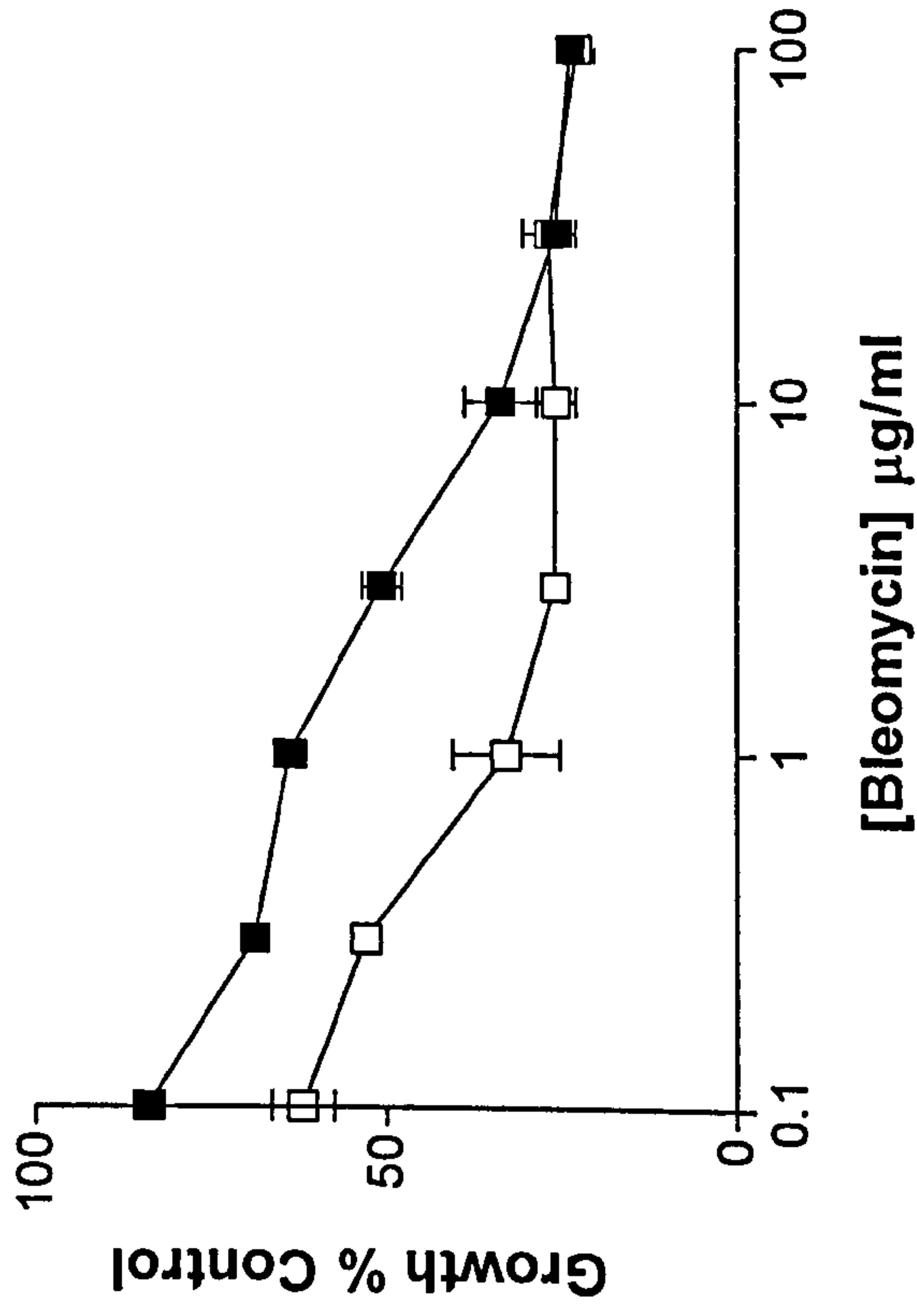
4.3.2 Chemopotential by NU7026

Studies were carried out to identify other cytotoxic agents for use in combination with DNA-PK inhibitors. Damaging agents that produce DNA DSBs either directly, or as repair intermediates, and which therefore may require DNA-PK mediated repair were chosen. The direct cellular effects of the clinically useful chemotherapeutic agents, bleomycin and etoposide and the effect of a fixed concentration of NU7026 were determined by means of growth inhibition studies carried out using the SRB assay in the DNA-PKcs deficient cell line V3 and its proficient counterpart, V3YAC. The studies with etoposide were carried out as part of an undergraduate project carried out by Gillian Newton, under my supervision.

4.3.2.1 The growth inhibitory effects of bleomycin in the presence or absence of NU7026

Bleomycin is a 'radiomimetic' drug and therefore produces similar lesions to IR which include both single and double strand breaks and base release. The active biological species of bleomycin are oxygen free radicals that cause scission of DNA through an oxidative process (section 1.3.4.2). As such, bleomycin would be expected to activate DNA-PK. A 48 hour continuous exposure of V3YAC and V3 cells to an increasing concentration of bleomycin resulted in a concentration-dependent inhibition of cell growth (Figure 4.6) with GI_{50} values of 3 $\mu\text{g/ml}$ and 0.3 $\mu\text{g/ml}$, respectively (Table 4.3). V3 cells were therefore 10-fold more sensitive to bleomycin than their proficient counterparts, indicating a major role for DNA-PK in the repair of bleomycin-induced DNA damage. The ability of NU7026 to potentiate this growth inhibition was investigated. In the V3YAC cells the GI_{50} value was reduced to 0.34 $\mu\text{g/ml}$ in the presence of 10 μM NU7026. This gave a corresponding fold decrease in GI_{50} (PF_{50}) of 8.8, which was significant ($p < 0.05$). The GI_{50} value in the V3 cells obtained for bleomycin co-incubated with 10 μM NU7026 (0.3 $\mu\text{g/ml}$) was the same as that obtained with bleomycin alone.

A



B

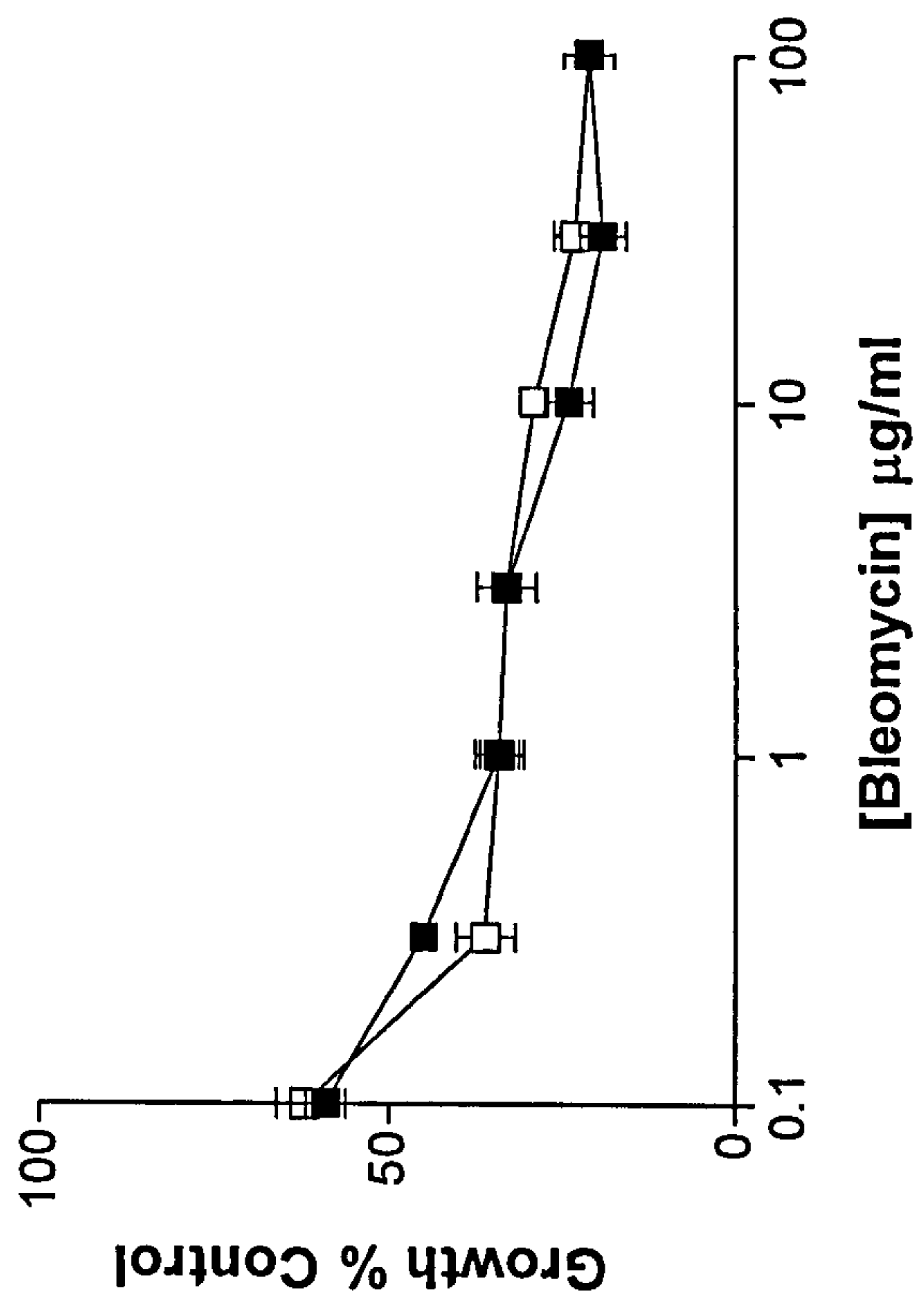


Figure 4.6 Growth inhibition of bleomycin alone and in combination with NU7026 in exponentially growing cells:-

A, V3YAC; B, V3; ■ bleomycin alone; □ Bleomycin + NU7026. Cells were continuously exposed to increasing concentration of bleomycin in the presence or absence of 10 µM NU7026 for 48 h. Data are normalised to 1 % v/v DMSO and inhibitor alone controls and are the mean \pm SEM of at least 3 independent experiments.

4.3.2.2 The growth inhibitory effect of Etoposide in the presence or absence of NU7026

Etoposide is a topoisomerase II (topo II) poison that acts by ‘trapping’ topo II as a cleavable complex at an intermediate step following DNA strand scission but before religation thereby giving rise to DNA DSBs (section 1.3.4.2). Western blot analyses were carried out to assess the topo II α protein levels in the V3YAC and V3 cell lines as sensitivity to topo II poisons is related to topo II protein expression (Asano *et al.*, 1996; Stacey *et al.*, 2000). As can be seen in Figure 4.7 A, topo II α levels were the same in both cell lines and this was verified by probing for actin as a loading control. The concentration-dependent effects of a 48 h continuous exposure to etoposide in the presence or absence of 10 μ M NU7026 on the growth of V3YAC and V3 cells were evaluated. Cell growth in the presence of etoposide alone was inhibited in a concentration-dependent manner in both cell lines (Figure 4.7 B and C). NU7026 potentiated the growth inhibitory effect of etoposide in the V3YAC cells (Table 4.3), reducing the GI₅₀ value of 2.3 μ M for etoposide alone to 0.95 μ M and this gave a PF₅₀ value of 2.4. The V3 cells were 8 -fold more sensitive than V3YAC cells to etoposide alone (GI₅₀ = 0.3 μ M) and no significant potentiation of growth inhibition was obtained in the presence of 10 μ M NU7026 (GI₅₀ = 0.31 μ M, p = 0.15).

In order to ascertain whether the potentiation of growth inhibition by NU7026 reflected a potentiation in the cytotoxicity of etoposide, these studies were extended by investigating the dose dependent effects of a 24 h continuous exposure to etoposide on cell survival in these cell lines by clonogenic assay. Figure 4.8 shows that in both cell lines, etoposide treatment resulted in a concentration-dependent decrease in survival. The LD₅₀ was 4.7 μ M and 6.8 μ M for the V3 and the V3YAC cell lines, respectively. In the presence of 10 μ M NU7026, the LD₅₀ of the V3YAC cells was reduced to 2.7 μ M giving a PF₅₀ value of 2.5. No additional significant effect on survival with the co-incubation of NU7026 was seen in the V3 cells (LD₅₀ = 4.4 μ M, p = 0.16).

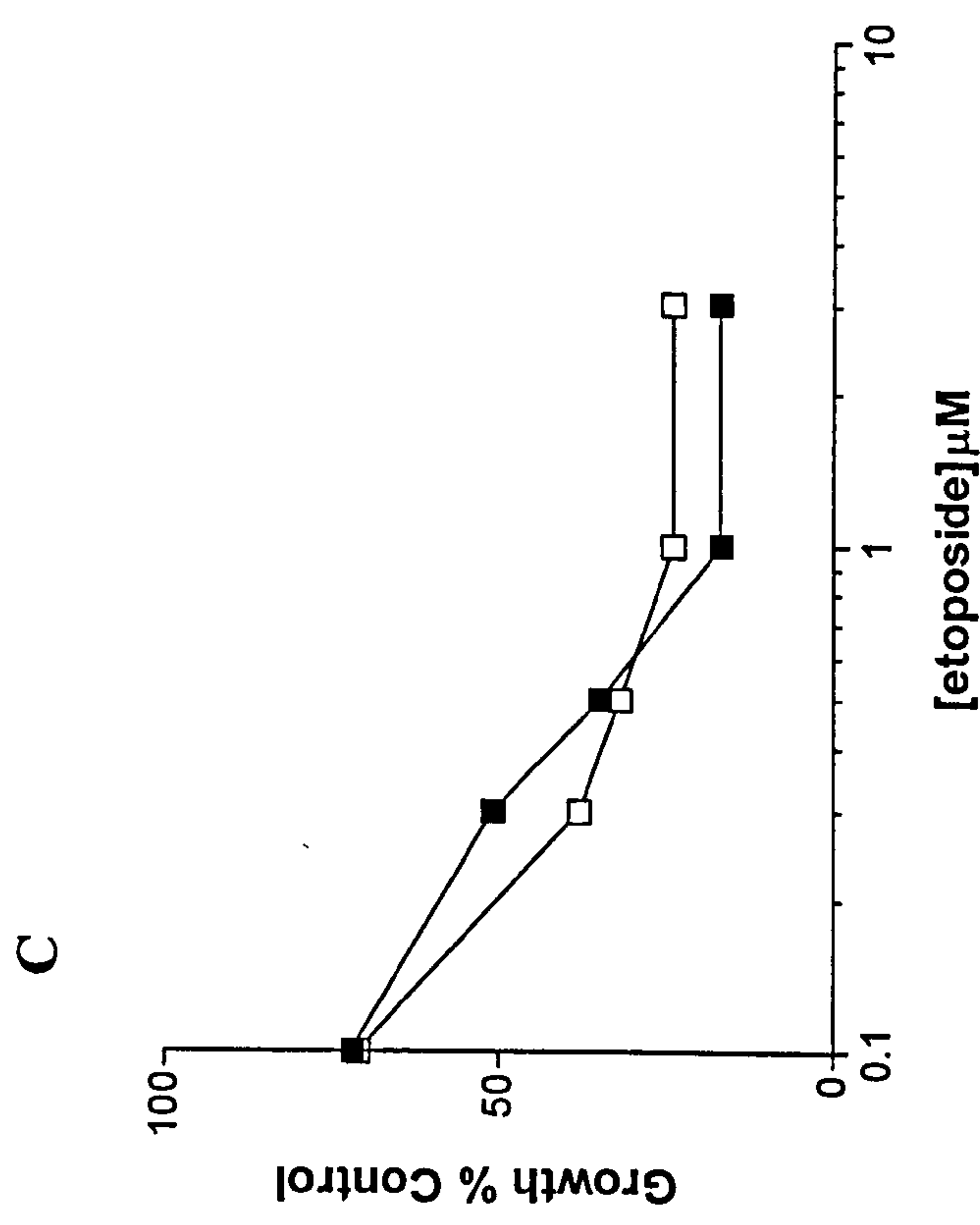
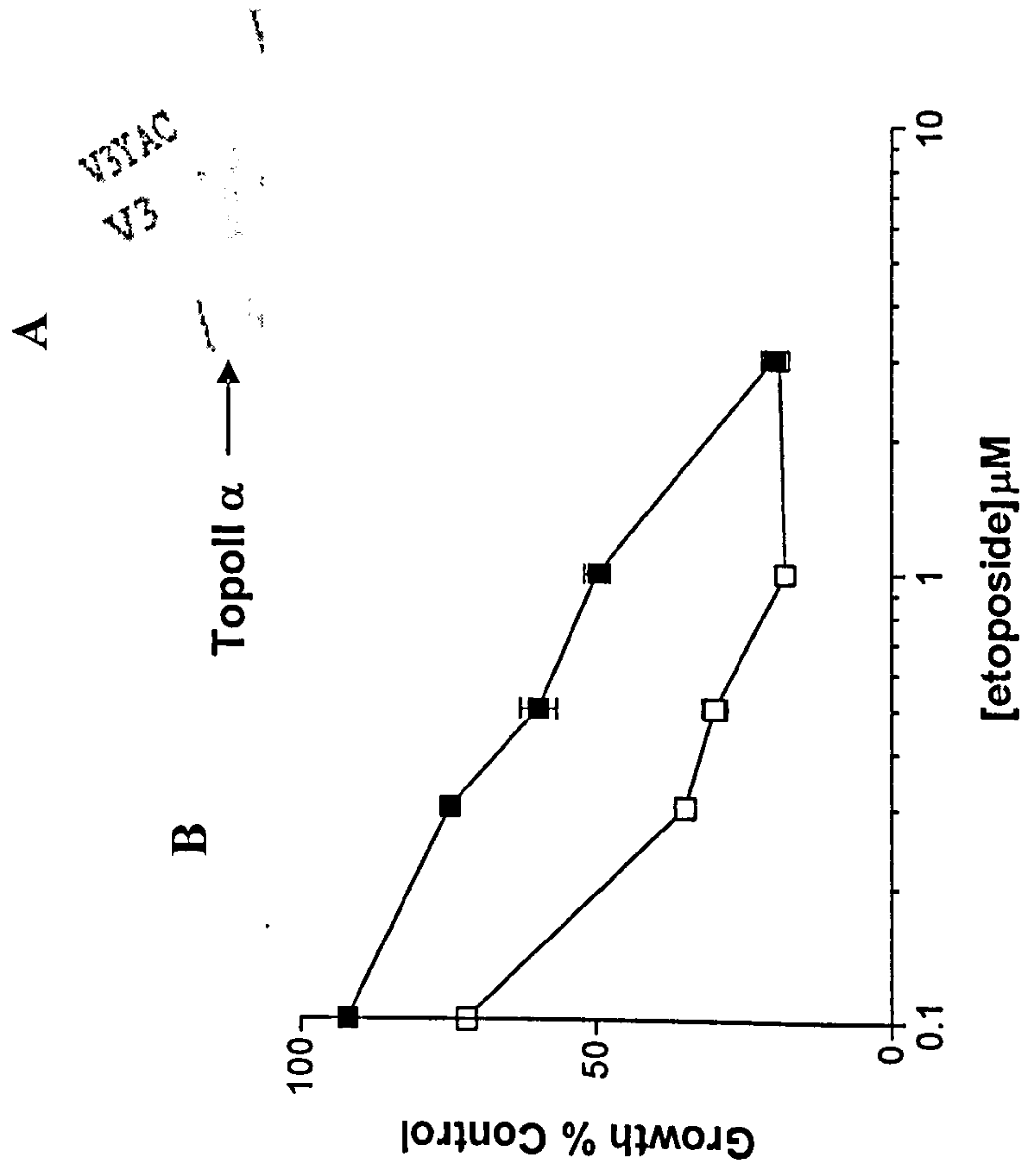


Figure 4.7 Comparison of topoisomerase II- α protein levels in V3 and V3YAC cells as assessed by Western blotting analysis (A) and Growth inhibition of etoposide alone and in combination with NU7026 in exponentially growing cells :-

B, V3YAC ; C, V3 ; ■ Etoposide + NU7026. Cells were exposed to increasing concentrations of etoposide in the presence or absence of 10 μM NU7026 for 48 h. Data are normalised to 1 % v/v DMSO or inhibitor alone controls and are the mean \pm SEM of at least 3 independent experiments.

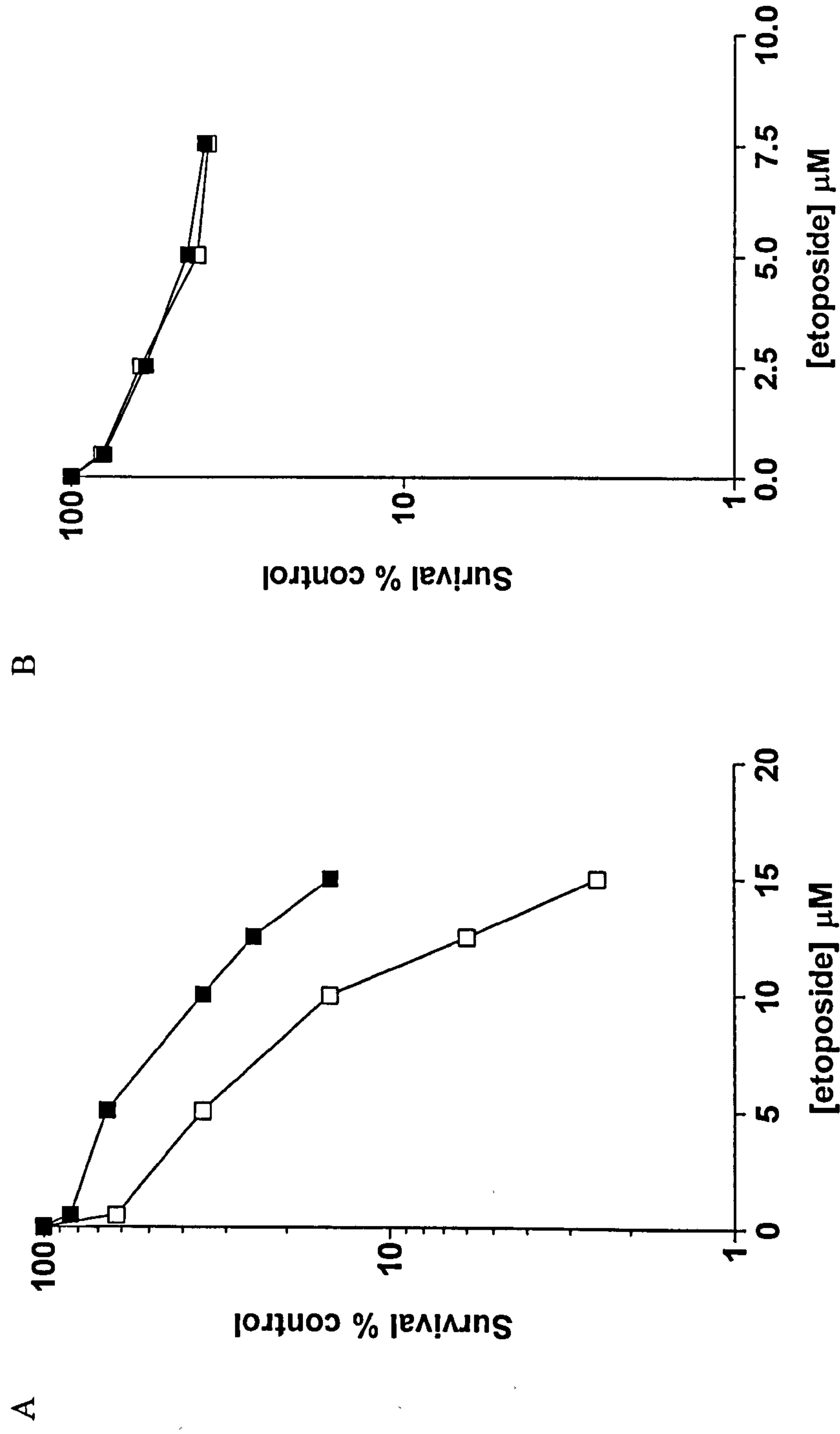


Figure 4.8 Effect of increasing doses of etoposide in the presence of absence of NU7026 on the survival of exponentially growing cells A, V3YAC; B, V3; \blacksquare , etoposide + NU7026. Cells were pre-incubated with 10 μM NU7026 for 1 h before addition of etoposide and then post-incubated for 24 h in the presence of inhibitor before being seeded for colony formation in the absence of drug(s). Data are the mean \pm SEM from a single experiment.

Cell line	V3YAC				V3		
Drug	Drug alone	Drug + NU7026	PF ₅₀	Drug alone	Drug + NU7026	PF ₅₀	
Bleomycin GI ₅₀ (μg/ml)	2.98 ± 0.02	0.34 ± 0.02	8.8 ± 0.03	0.29 ± 0.01	0.29 ± 0.09	1 ± 0.00	
Etoposide GI ₅₀ (μM)	2.29 ± 0.03	0.95 ± 0.03	2.40 ± 0.01	0.29 ± 0.07	0.31 ± 0.09	0.8 ± 0.08	
Etoposide LD ₅₀ (μM) (*1)	6.8	2.7	2.5	4.7	4.4	1.1	

Table 4.3 Chemopotentialiation by NU7026

Comparison of the GI₅₀ values derived from the growth curves shown in Figures 4.6 and 4.7. GI₅₀ values represent the dose required to cause 50 % inhibition of growth. The LD₅₀ values were derived from the survival curves in Figure 4.8. All mean PF₅₀ values (± SEM) were calculated from the ratio of the individual GI₅₀/LD₅₀ values (dose which causes 50 % growth inhibition or cell kill, respectively) for bleomycin or etoposide alone / GI₅₀/LD₅₀ for bleomycin or etoposide + NU7026. Data are normalised to 1 % v/v DMSO alone or Drug alone controls and are the mean ± SEM of at least 3 experiments, unless otherwise stated in parentheses.

4.3.3 Potentially lethal damage recovery

The degree of PLDR following IR treatment was assessed in all cell lines and this was correlated with their inherent radiosensitivity. In addition the effects of AG14361 and NU7026 on PLDR were examined.

4.3.3.1 Cell cycle arrest by using conditioned or serum free medium

In order to compare the effects of IR and the additional effects of AG14361 and NU7026 in non-cycling cells, various methods of non-drug induced cell cycle arrest were investigated. Previous data suggest that serum-free medium (Chou and Langan, 2003) or conditioned medium (that which has been obtained from confluent cells) (Davis *et al.*, 2001) can be used. The suitability of these methods for both inducing and maintaining cell cycle arrest without cell death was therefore investigated in the parental CHOK1 cell line. Briefly, confluent or sub-confluent cells were counted at time '0' and their medium replaced with either conditioned or serum-free medium. Triplicate cell counts for each sample were then taken at 24 h, 48 h and 96 h thereafter and plotted on an x-y graph against time (Figure 4.9). Samples were also taken at 0, 24 and 96 h for cell cycle analysis by flow cytometric analysis, as described in section 2.7.

In all cells, the rate of cell growth was dramatically slowed down by both treatments. Indeed, the growth of the cells in serum-free medium almost completely arrested, having only undergone 1-2 doublings between 24 and 96 h as opposed to the expected 6-8 doublings. Likewise, the growth of cells in conditioned medium underwent far fewer doublings over the 96 h than expected (around 2-3 rather than 8). In addition, by 96 h some cell death had occurred in both the serum-free and conditioned medium as floating cells were present in the medium.

Corresponding cell cycle profiles (Figures 4.10a and 4.10b) show that the sub confluent cells in serum-free medium arrested in G1 at 24 h but that there was a large sub-G1 (apoptotic) peak by 96 h accounting for 54 % of the cell cycle phase distribution. In conditioned medium, the sub-confluent cells demonstrated an increase in G1 by 24 h from 28 % to 47 % with a corresponding decrease in G2/M

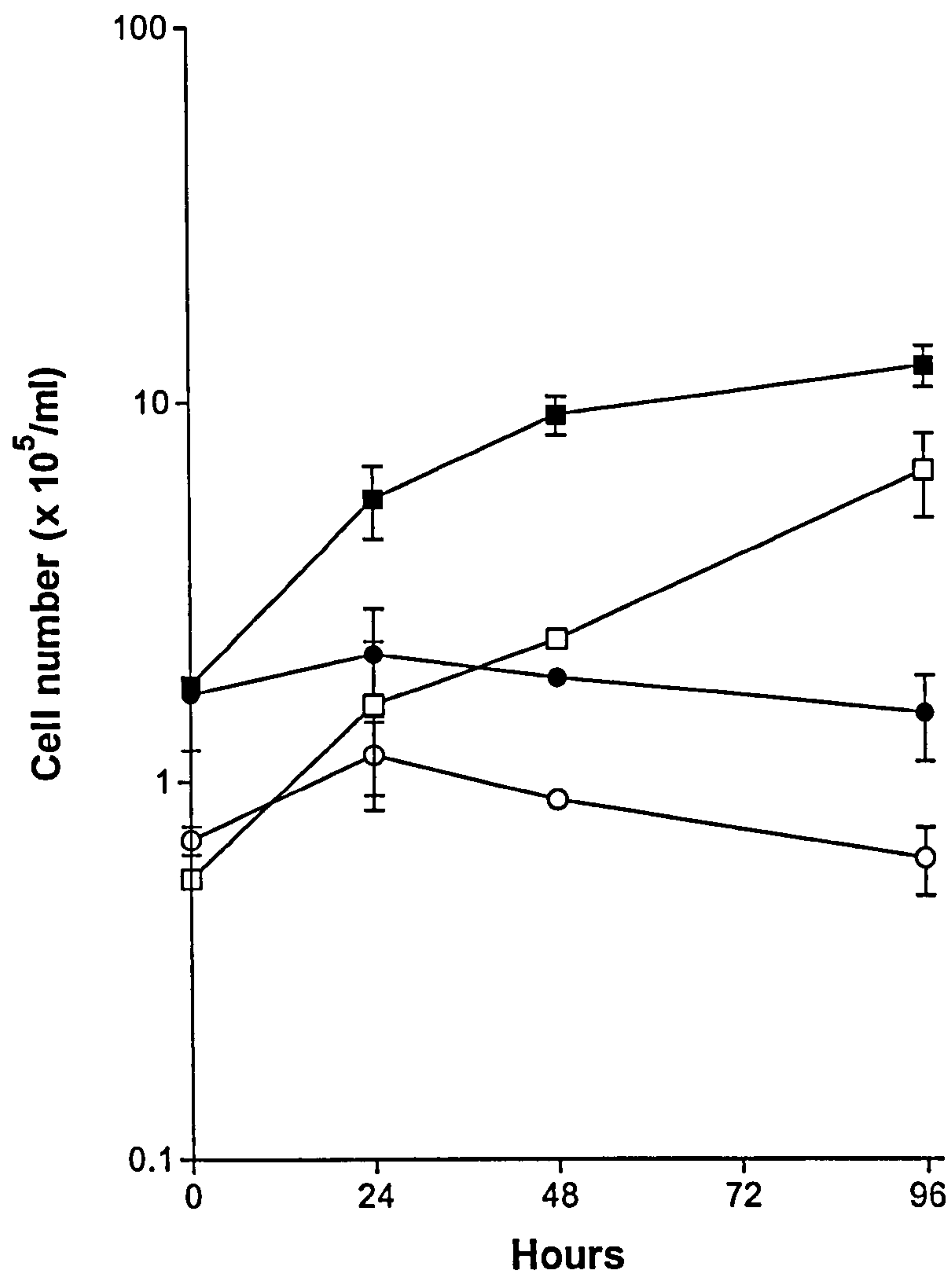
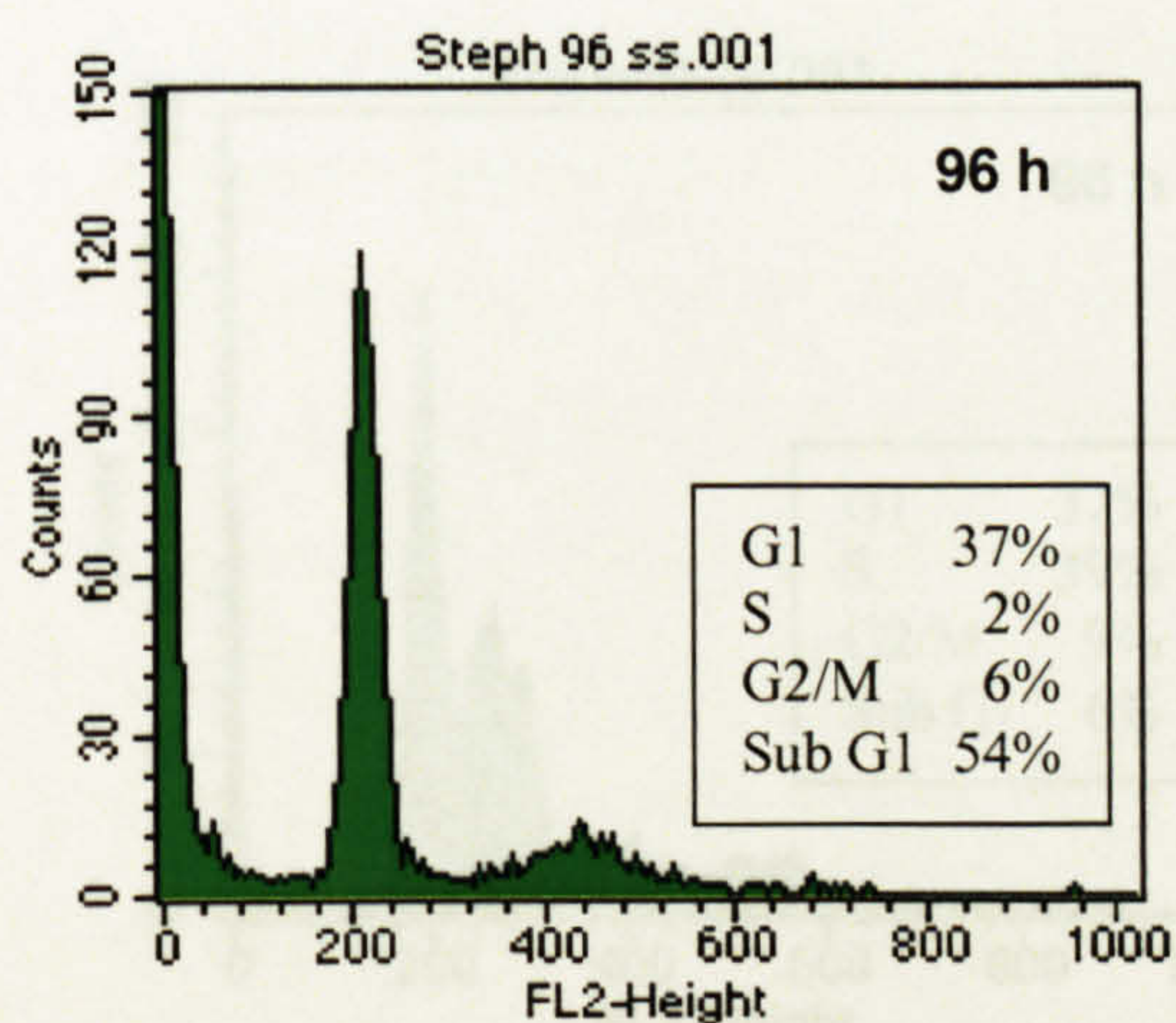
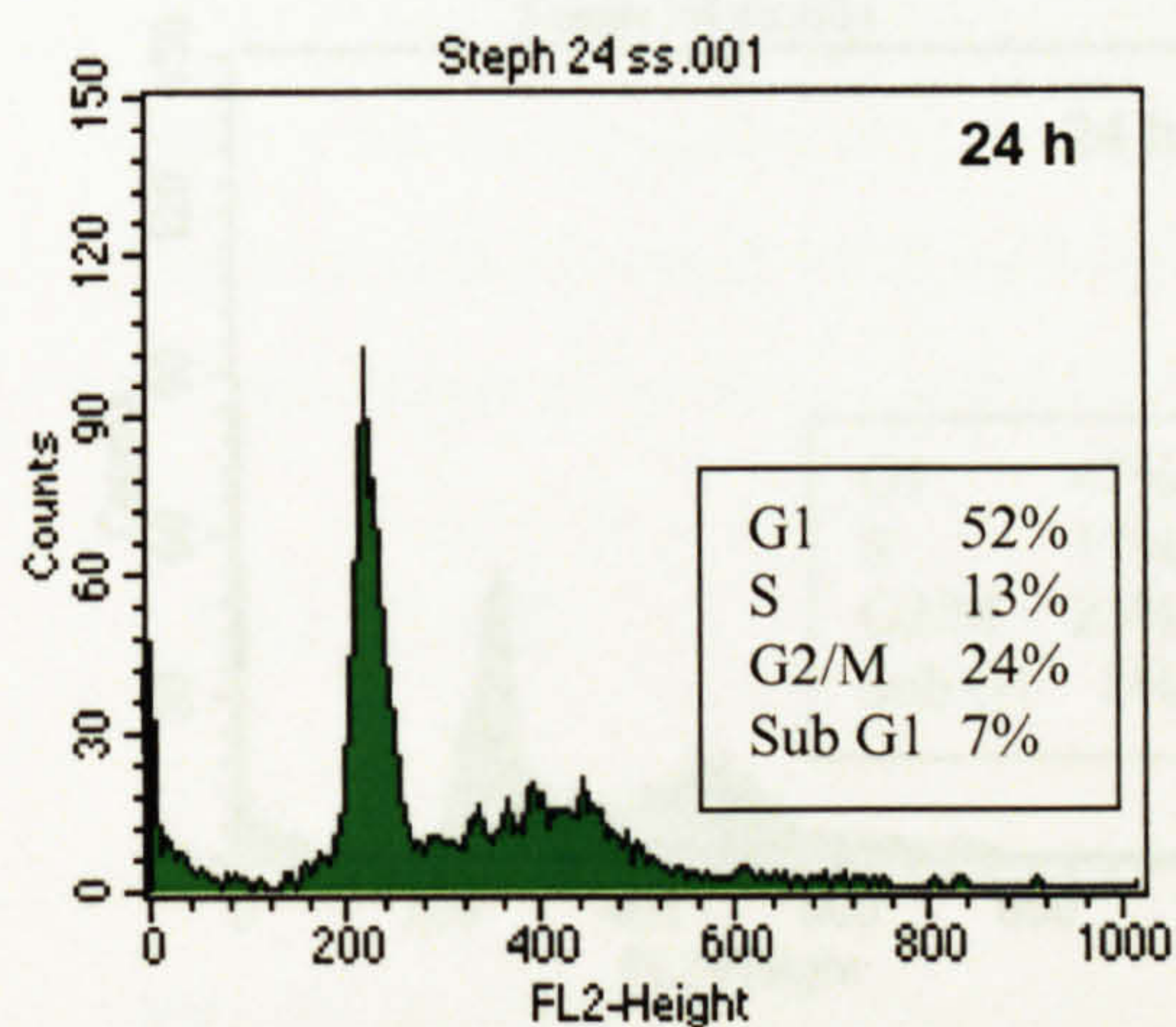
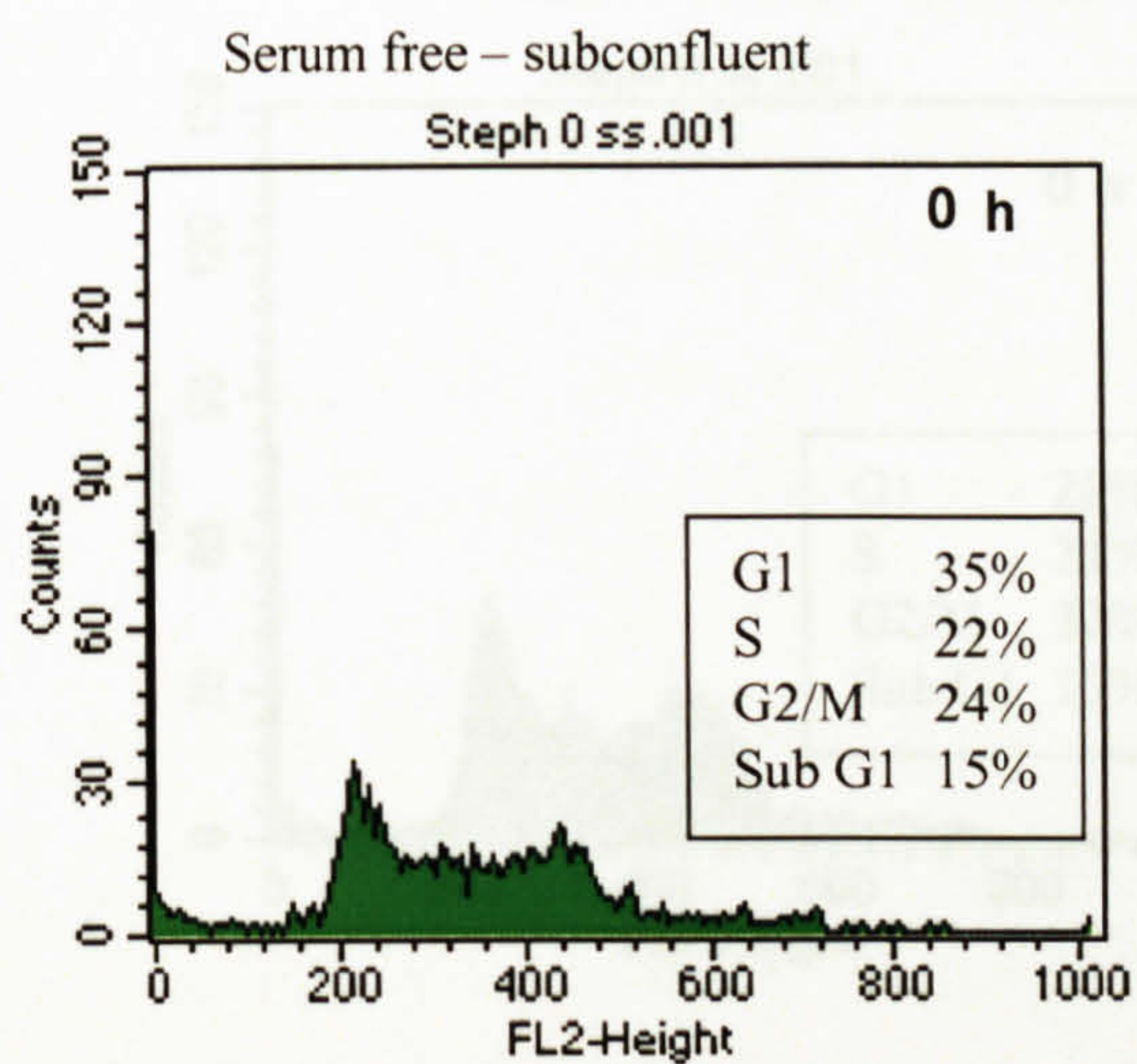


Figure 4.9 Growth of CHOK1 cells when placed in conditioned or serum free medium:- Growth of confluent (filled symbols) and subconfluent (open symbols) when exposed to conditioned (squares) or serum-free medium (circles). Data are the mean \pm SEM of triplicate cell counts taken at 24, 48 and 96 h, from a single representative experiment.



Serum free - confluent

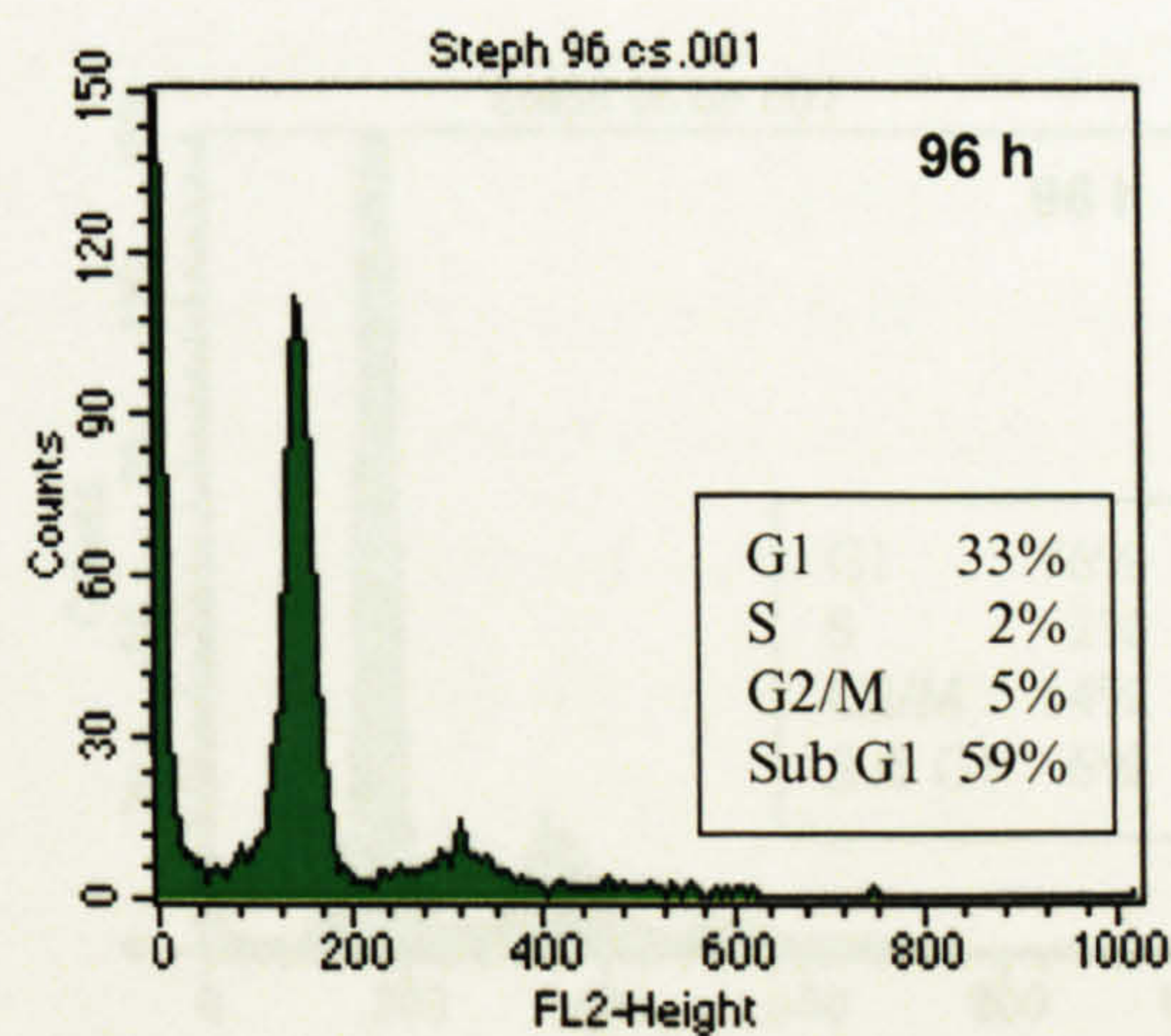


Figure 4.10 (a) Cell cycle analysis of CHOK1 cells exposed to serum free-medium

Cell cycle phase distribution was analysed using flow cytometric analysis as described in section 2.7. Treatment schedules are indicated on individual plots.

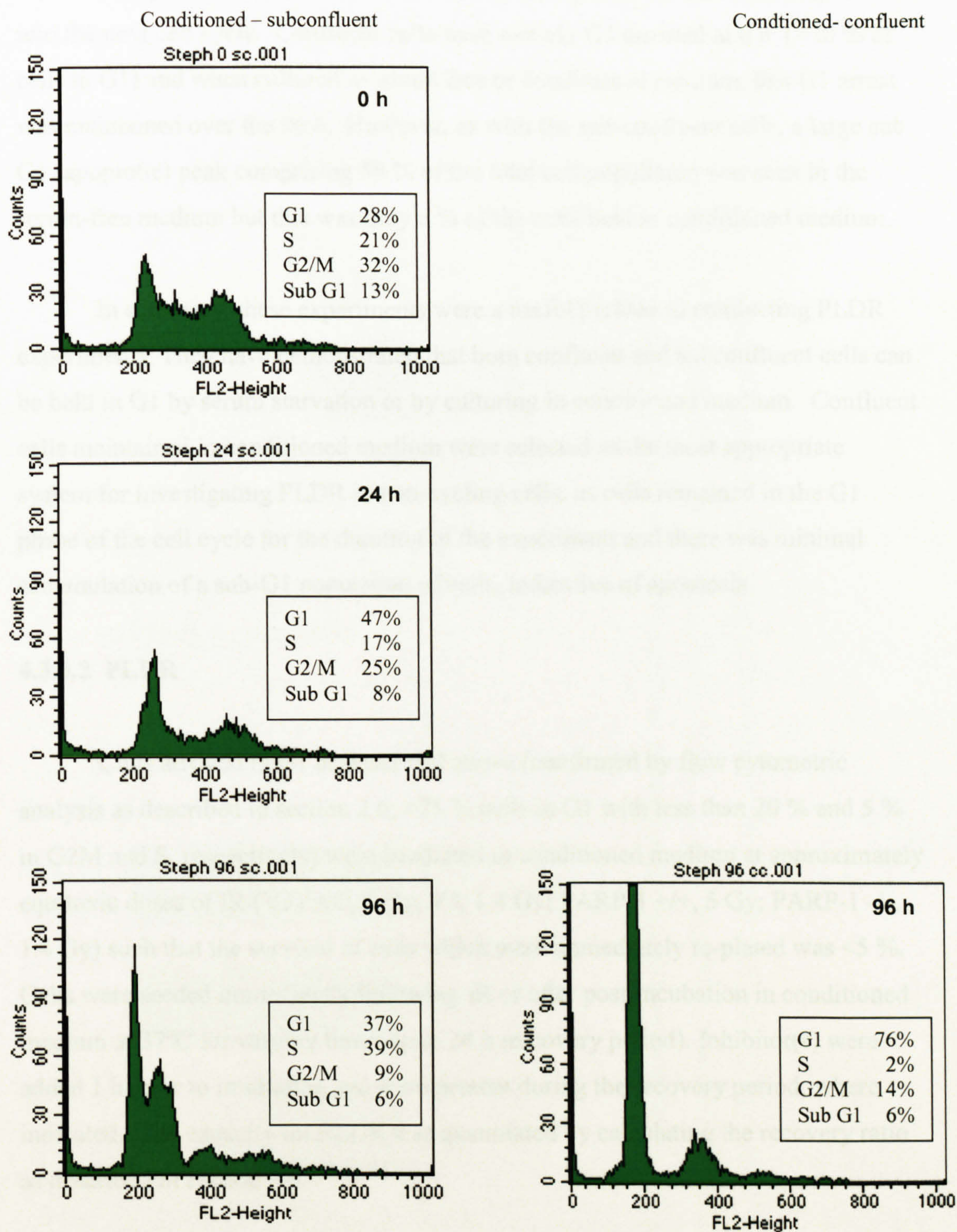


Figure 4.10 (b) Cell cycle analysis of CHOK1 cells exposed to conditioned medium Cell cycle phase distribution was analysed using flow cytometric analysis as described in section 2.7. Treatment schedules are indicated on individual plots.

and S phases and, notably, only a very small sub G1 peak was observed by 96 h (6 %) at which point the cells can be seen to be escaping from G1 into S as they move into the next cell cycle. Confluent cells were already G1 arrested at 0 h (>70 % of cells in G1) and when cultured in serum free or conditioned medium, this G1 arrest was maintained over the 96 h. However, as with the sub-confluent cells, a large sub G1 (apoptotic) peak comprising 59 % of the total cell population was seen in the serum-free medium but this was only 6 % of the cells held in conditioned medium.

In summary, these experiments were a useful prelude to conducting PLDR experiments. They have demonstrated that both confluent and subconfluent cells can be held in G1 by serum starvation or by culturing in conditioned medium. Confluent cells maintained in conditioned medium were selected as the most appropriate system for investigating PLDR in non-cycling cells, as cells remained in the G1 phase of the cell cycle for the duration of the experiment and there was minimal accumulation of a sub-G1 population of cells, indicative of apoptosis.

4.3.3.2 PLDR

Cells arrested in G1 as described above (confirmed by flow cytometric analysis as described in section 2.6; >75 % cells in G1 with less than 20 % and 5 % in G2M and S, respectively) were irradiated in conditioned medium at approximately equitoxic doses of IR (V3YAC, 6 Gy; V3, 1.4 Gy; PARP-1 +/+, 5 Gy; PARP-1 -/-, 1.4 Gy) such that the survival of cells which were immediately re-plated was <5 %. Cells were seeded immediately following IR or after post-incubation in conditioned medium at 37°C for varying times up to 24 h (recovery period). Inhibitor(s) were added 1 h prior to irradiation and were present during the recovery period, where indicated. The capacity for PLDR was quantitated by calculating the recovery ratio as described in section 2.6.

Initially, the PLDR capacity of V3YAC and V3 cells as a function of post-incubation time before subculture (0, 4, 6 and 24 h) was examined (Figure 4.11). The delay in subculture, which allows quiescent cells to repair PLD, resulted in a clear increase in cell survival. In both cell lines, PLDR was observed at 4 h (V3YAC, 6.0-fold; V3, 1.4-fold) and it was maximal by 6 h. (V3YAC, 8.3-fold; V3,

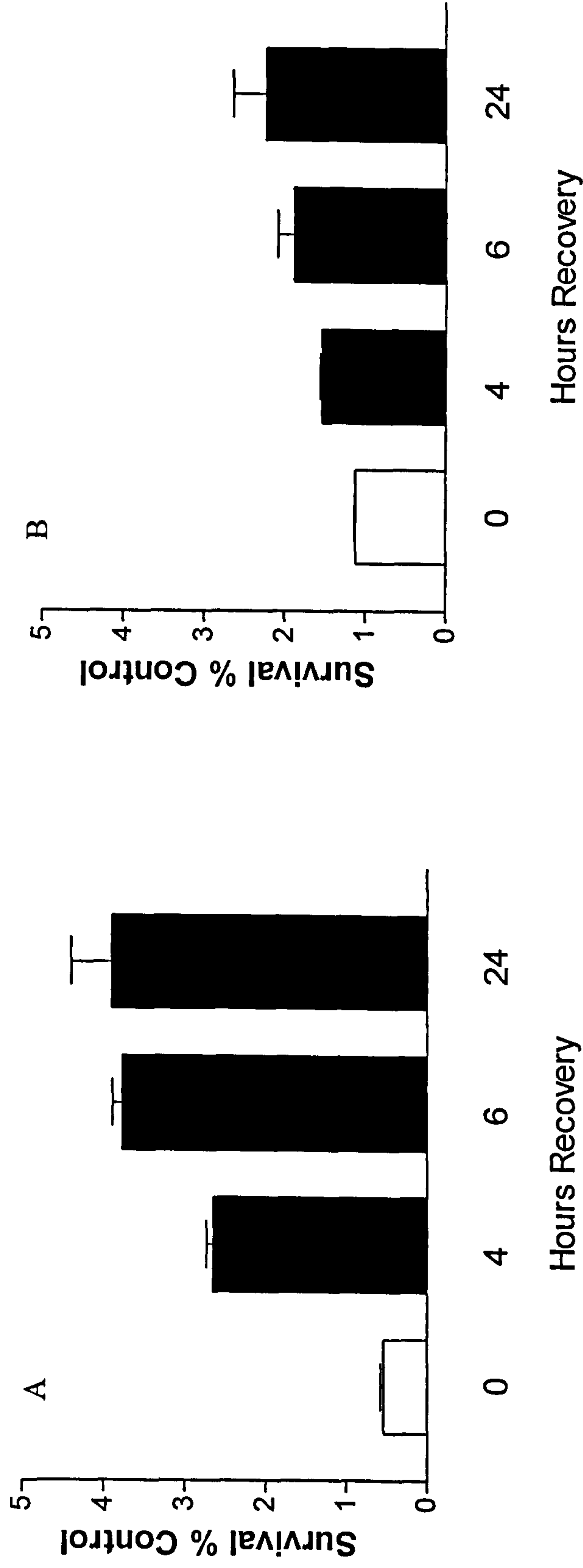


Figure 4.11 Recovery from IR-induced potentially lethal damage in growth arrested cells as a function of time:- A, V3YAC cells; B, V3 cells. Cells were irradiated with equitoxic doses of IR (6 Gy, V3YAC; 1.4 Gy, V3) and either re-plated immediately (white bars) or following 4, 6 or 24 h post-incubation to allow for PLDR (black bars). Data are the mean \pm SEM of at least 3 independent experiments.

1.75-fold). No further significant enhancement in survival was seen in either cell line by extending the post-IR incubation time to 24 h. The reduced PLDR in the V3 cells compared to the V3YAC cells indicates a requirement for DNA-PK function in PLDR.

PLDR in all cell lines was compared and the effects of AG14361 and NU7026 on PLDR were investigated. Following a 24 h delay in subculture, the surviving fraction of V3YAC and PARP-1 +/+ cells (recovery ratio) was increased 8.7 ± 0.4 -fold and 6.7 ± 0.5 -fold, respectively, $p = <0.05$, (Figure 4.12 A, C). There was no significant difference in the surviving fraction of inhibitor treated cells compared to control cells when seeded for colony formation immediately following IR (data not shown). PLDR was significantly reduced in both cell lines by AG14361 (71 % and 74 %, for V3YAC and PARP-1 +/+ cells, respectively) as shown in Table 4.4 (% inhibition was calculated as described in section 2.6). 10 μ M NU7026 fully abolished recovery in both cell lines. Whilst both inhibitors alone substantially prevented PLDR, more dramatically, when used in combination, AG14361 and NU7026 not only completely abolished PLDR, but reduced survival significantly below that observed in cells exposed to IR-alone and re-plated immediately. For example, (Figure 4.12 A), when growth arrested V3YAC cells were irradiated, survival was 0.38 % if the cells were re-plated immediately. Survival was increased to 3.3 % by a 24 h delay in re-plating, but the presence of AG14361 and NU7026, in combination, during the recovery period reduced survival to 0.064 %, approximately 6-fold lower than the cells which were re-plated immediately post-IR. This represents a 50-fold radiosensitisation.

4.3.3.3 Recovery from potentially lethal damage in cells deficient in PARP-1 or DNA-PK

PLDR was reduced approximately 5-fold in the V3 cells (recovery = 1.70 ± 0.02 -fold) and approximately 2.5-fold in the PARP-1 -/- cells (recovery = 2.70 ± 0.3 - fold), compared to their proficient counterparts (Figure 4.12 B and D), consistent with the differential in radiosensitivity of exponentially growing cells (Figure 3.7). This modest recovery was fully inhibited by AG14361 or NU7026,

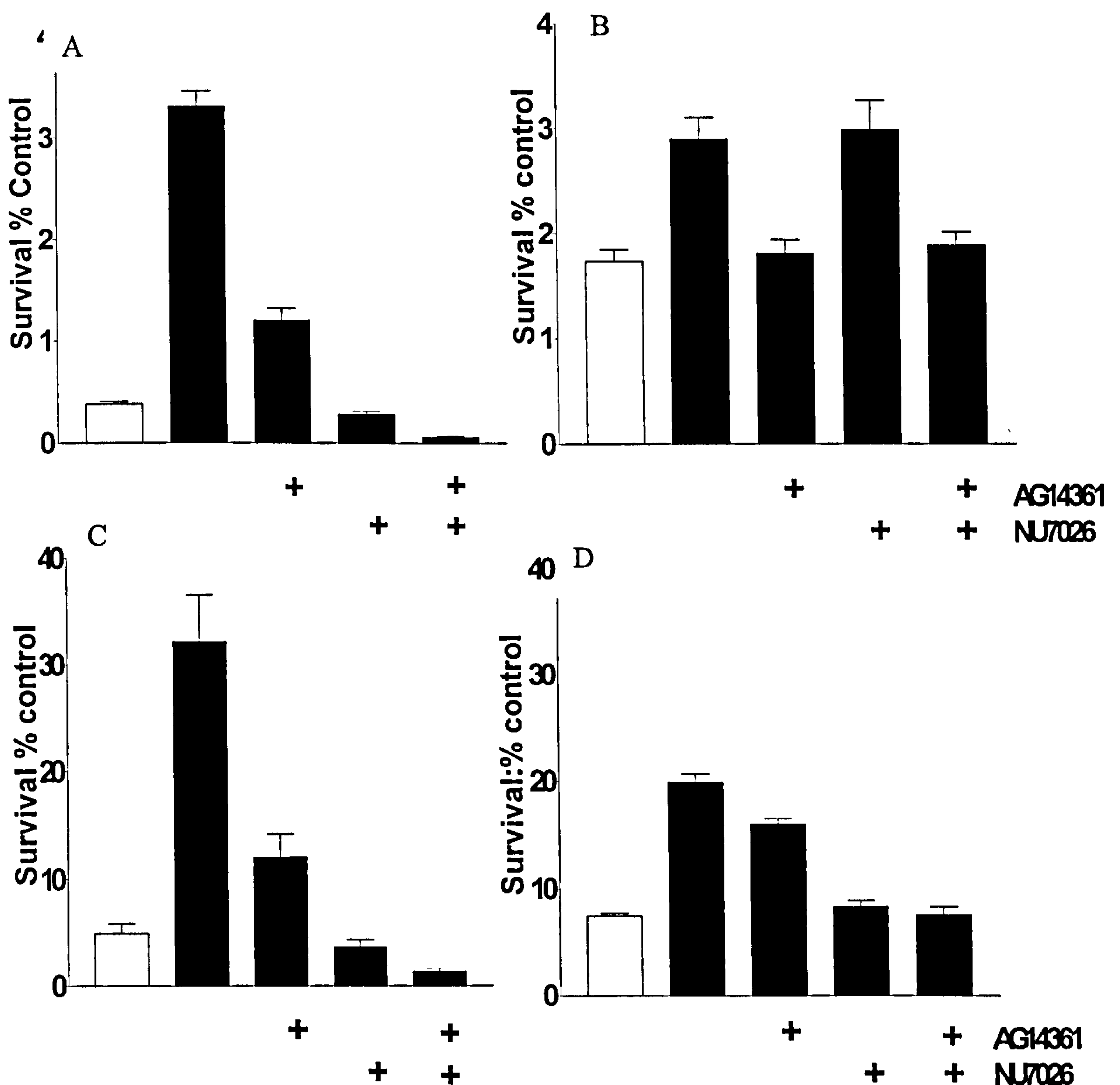


Figure 4.12 Effects of AG14361 and NU7026 on recovery from IR-induced potentially lethal damage in growth arrested cells:- A, V3YAC ; B, V3; C, PARP-1 +/+ ; D, PARP-1 -/- . Cells were treated with approximately equitoxic doses of IR in the presence or absence of 10 μ M NU7026 or 0.4 μ M AG14361 (6 Gy, V3YAC; 1.4 Gy, V3; 5 Gy, PARP-1 +/+; 1.4 Gy, PARP-1 -/-) and either plated immediately (white bars) or following a 24 h post-incubation to allow for PLDR (black bars). Cells were replated in the absence of inhibitors. Data are the mean \pm SEM of at least 3 independent experiments.

Treatment/ Cell line	IR + AG14361	IR + NU7026	IR + AG14361 + NU7026
V3YAC	71 ± 2.2	110 ± 0.8	122 ± 0.55
V3	92 ± 3.2	0±0.00	90 ± 2.1
PARP-1 +/+	73.5 ± 3.87	104 ± 1.89	112 ± 2.01
PARP-1 -/-	6.0 ± 2.2	93 ± 2.17	95 ± 3.00

Table 4.4 Effect of AG14361 and NU7026 on IR- induced potentially lethal damage. Figures represent the % inhibition of recovery for each inhibitor in each cell line. Inhibition of recovery of > 100 % arises when the survival of drug-treated cells after 24 h PLDR is below the survival of cells seeded immediately. Figures are calculated as described in section 2.6. Data are the mean ± SEM of at least 3 independent experiments

respectively. As expected, no significant inhibition of PLDR was obtained by the use of NU7026 in the V3 cell line ($p=0.8$). However, when AG14361 was used in the PARP-1 $-/-$ cell line there was a small, but significant inhibition of PLDR (6 %, $p=0.04$).

PLDR was also assessed in the xrs-6 and xrs-6 HamKu80 cell lines (Figure 4.13). The cells were irradiated with approximately equitoxic doses of IR (xrs-6 HamKu80, 5 Gy; xrs-6, 1.2 Gy) and either re-plated immediately or following 24 h recovery at 37°C. Strikingly, the xrs-6 cells showed no significant PLDR (fold recovery = 1.11 ± 0.13). However, this deficiency was rescued by the transfection of Ku80 which gave a PLDR of 6.4 ± 0.9 -fold, comparable with the V3YAC cells (8.7-fold).

4.3.3.4 Analysis of cell cycle progression following subculture

Given that radiation influences the cell cycle checkpoints and induces G1 and G2 arrest, it was speculated that effects on cell cycle progression may be involved in the mechanism of inhibition of PLDR by the inhibitors. Indeed, members of the PI3-kinase family (i.e. ATM and ATR) function in both cell cycle progression and DNA damage-induced cell cycle checkpoints (section 1.13). Moreover, earlier PLDR studies with wortmannin (WM) (data not shown) demonstrated a failure to produce visible colonies following subculture into drug-free medium, indicative of a permanent G1 block. Collado *et al.*, 2001, showed that following arrest in G1, the kinase inhibitors WM and LY294002 induced a permanent arrest in cell growth resulting in a senescent-like phenotype, at least partially through the inhibition of PI3-K.

The effect of AG14361 and NU7026 on cell cycle progression was investigated in the V3YAC and V3 cells by flow cytometric analysis. Briefly, growth arrested G1 phase V3YAC and V3 cells were pre-incubated with inhibitor for 1 h, irradiated with equitoxic doses of IR (see section 2.6) in the presence or absence of inhibitor and subcultured in drug free medium following 24 h recovery at 37°C. The cell cycle distribution was examined immediately and 24, 48 and 72 h thereafter (Figures 4.14 a, b, c).

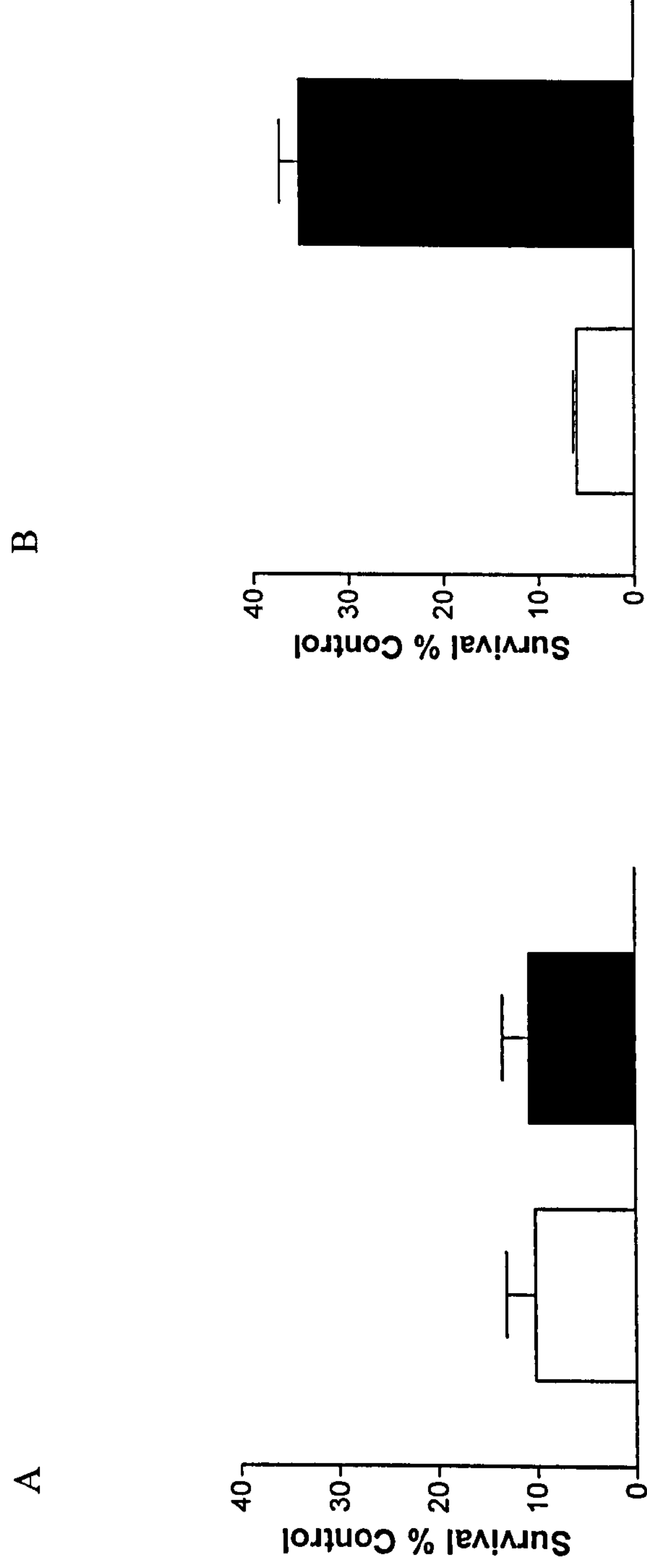


Figure 4.13. Comparison of recovery from IR-induced potentially lethal damage in growth arrested cells:- A, xrs-6 cells; B, xrs-6 HamKu80 cells. Cells were irradiated with equitoxic doses of IR (5 Gy, xrs6 hamKu80; 1.2 Gy, xrs6) and either re-plated in the absence of inhibitors either immediately (white bars) or following a 24 h post-incubation to allow PLDR (black bars). Data are the mean \pm SEM of at least 3 independent experiments.

Following release from G1, V3YAC cells progressed into S and G2M phase with a reduction of G1 from 81 % to 50 %, with a corresponding increase in S, from 9 % to 25 %, and G2/M, from 10 % to 25 % over 72 hrs (Figure 4.14(a),(b) and (c) A), corresponding to a near normal phase distribution (Figure 3.13). Similarly, in the V3 cells, following release from G1 arrest, the G1 fraction decreased from 69 % to 35 % and the S and G2/M phases increased from 12 % to 20 % and 19 % to 45 %, respectively, over the next 72 hrs (Figure 4.14 (a), (b) and (c) B). This represents a slight accumulation in G2/M compared to untreated exponentially growing V3 cells (Figure 3.13B).

Neither NU7026 nor AG14361 affected the cell cycle distribution of exponentially growing cells (Figure 3.13). However, NU7026 caused a progressive accumulation of irradiated V3YAC cells in G2/M following release from G1, consistent with the observed G2 accumulation in the V3 cell line. NU7026 did not affect the cell cycle progression of irradiated V3 cells. It was apparent that the majority of cells treated with IR + NU7026 remained arrested at the initial G2 checkpoint and were unable to progress to the second cell cycle as even 72 hours after subculture 74 % cells remained in G2/M. In contrast, AG14361 had virtually no effect on the cell cycle phase distribution perturbations caused by irradiation of V3YAC cells following release from G1, but caused a transient increase in G2/M at 24 h (41 % compared to 30 % in cells irradiated alone) which was gradually lost over the next 48 h in the V3 cells.

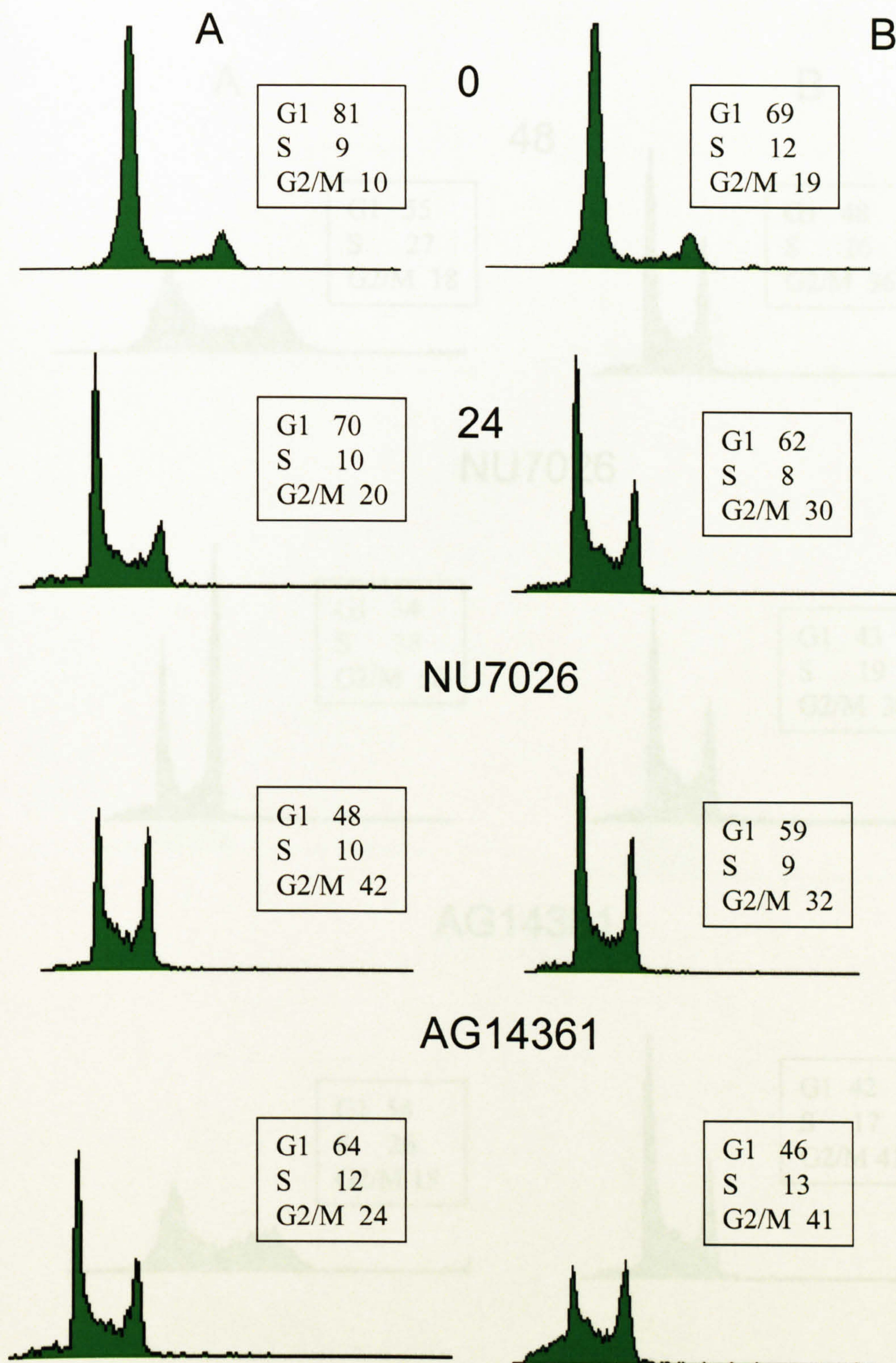


Figure 4.14(a) The effect of NU7026 and AG14361 on cell cycle progression 24 h post subculture; A, V3YAC cells; B, V3 cells
Cells were seeded and analysed by flow cytometry as described in section 2.7.

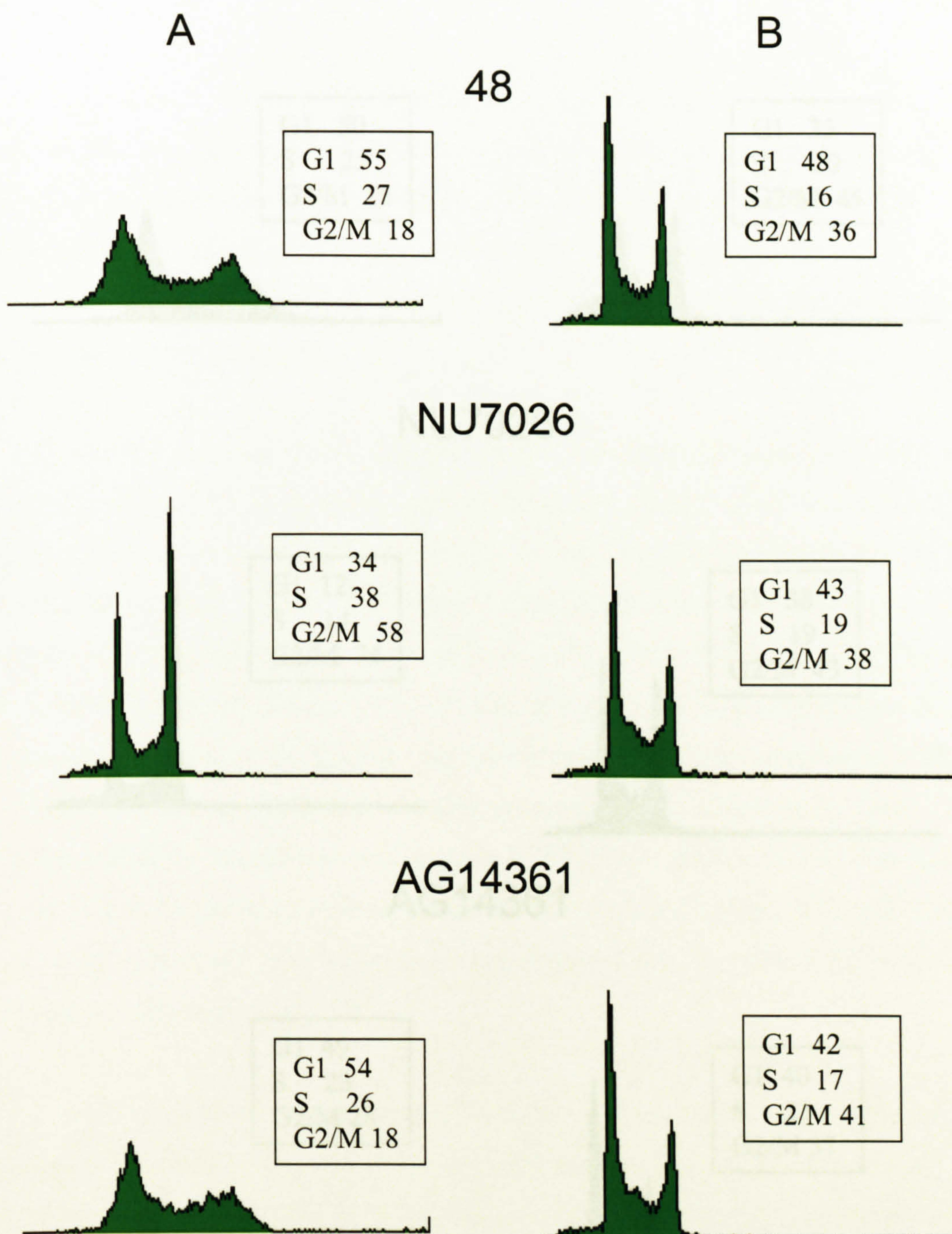


Figure 4.14(b) The effect of NU7026 and AG14361 on cell cycle progression 48 h post subculture; A, V3YAC cells; B, V3 cells
 Cells were seeded and analysed by flow cytometry as described in section 2.7

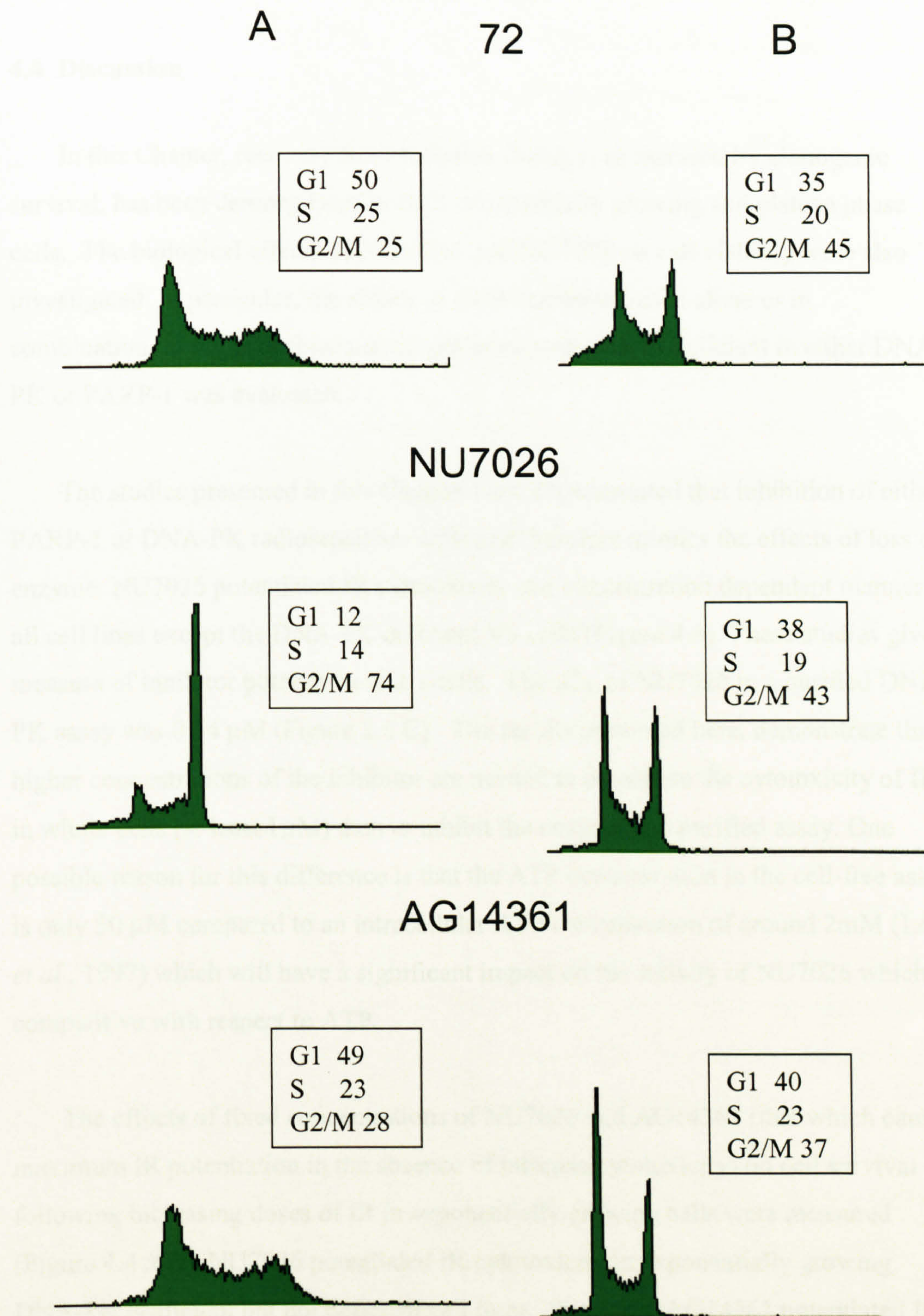


Figure 4.14(c) The effect of NU7026 and AG14361 on cell cycle progression 72 h post subculture; A, V3YAC cells; B, V3 cells

Cells were seeded and analysed by flow cytometry as described in section 2.7.

4.4 Discussion

In this Chapter, recovery from radiation damage, as assessed by clonogenic survival, has been demonstrated in both exponentially growing and plateau phase cells. The biological effects of AG14361 and NU7026 on cell viability were also investigated. In particular, the ability of these inhibitors, used alone or in combination, to radiosensitise paired cell lines proficient or deficient in either DNA-PK or PARP-1 was evaluated.

The studies presented in this Chapter have demonstrated that inhibition of either PARP-1 or DNA-PK radiosensitises cells and therefore mimics the effects of loss of enzyme. NU7026 potentiated IR cytotoxicity in a concentration dependent manner in all cell lines except the DNA-PK deficient V3 cells (Figure 4.3). These studies give a measure of inhibitor potency in intact cells. The IC_{50} of NU7026 in a purified DNA-PK assay was 0.44 μ M (Figure 3.8 C). The results presented here, demonstrate that higher concentrations of the inhibitor are needed to potentiate the cytotoxicity of IR in whole cells (at least 1 μ M) than to inhibit the enzyme in a purified assay. One possible reason for this difference is that the ATP concentration in the cell-free assay is only 50 μ M compared to an intracellular ATP concentration of around 2mM (Leist *et al.*, 1997) which will have a significant impact on the activity of NU7026 which is competitive with respect to ATP.

The effects of fixed concentrations of NU7026 and AG14361 (that which cause maximum IR potentiation in the absence of intrinsic cytotoxicity) on cell survival following increasing doses of IR in exponentially growing cells were measured (Figure 4.4 a, b). NU7026 potentiated IR cytotoxicity in exponentially growing DNA-PK proficient but not deficient cell lines. Similarly, AG14361 potentiated IR in PARP-1 +/+ but not PARP-1 -/- cell lines. These data are consistent with the observation that both PARP-1 and DNA-PK deficient cell lines were 4-fold more sensitive to IR alone than their proficient counterparts, and provide confirmation that the inhibitors mediate their effects on IR-induced cytotoxicity specifically via inhibition of their target enzymes. This work is supported by clonogenic survival

assays carried out in the DNA-PK deficient human glioma cell line, MO59J and their proficient counterpart, MO59K. In these studies 50 μ M NU7026 potentiated IR cytotoxicity in the M059K cells but did not significantly potentiate IR in the M059J cells (G.C.M Smith, KuDOS Pharmaceuticals; personal communication).

Inhibition of PARP-1 or DNA-PK therefore reduces the 'threshold dose' (dose at which IR becomes toxic) and increases cytotoxicity at higher doses. When used in combination, the inhibitors caused at least additive effects for the potentiation of cytotoxicity. These results are consistent with the findings of Boulton *et al.*, 1999, who showed that combined use of wortmannin and the PARP-1 inhibitor, NU1025, gave additive effects for the potentiation of IR cytotoxicity and suggest that the two enzymes do not function solely in a common pathway, because using both inhibitors has a greater effect than either one alone. It is generally assumed that the inhibition of BER and NHEJ is the cause of the enhanced cytotoxicity observed with PARP-1 and DNA-PK inhibitors, respectively. However, data presented in Chapter 5 (section 5.3.3) have shown that AG14361 also inhibits DNA DSB repair in cells exposed to IR and therefore this may contribute to the cytotoxic mechanism.

The studies presented in this Chapter are the first to evaluate radiosensitisation by a small molecule selective inhibitor of DNA-PK. The data are consistent with recent reports which have utilised several alternative strategies to selectively deplete or inhibit DNA-PK function in human tumour cell lines; antisense oligonucleotides, small interfering RNAs and a C terminal peptide which targets Ku80 and prevents DNA-PKcs binding to Ku (Sak *et al.*, 2002; Blenkov *et al.*, 2002; Kim *et al.*, 2002; Omori *et al.*, 2002). These have been previously described in section 1.12.1. In summary, in all reports, a loss of DNA-PK activity correlated with radiosensitisation and inhibition of DNA repair.

Maximum potentiation of 4 Gy IR cytotoxicity by AG14361 was achieved in CHOK1 cells by 30 minutes, with no further increases in potentiation being achieved by extending the exposure period to 24 h. (Figure 4.5). This is consistent with the previous findings of Ben Hur *et al.*, (1985) and Arundal-Suto and Scavone (1991), who showed that following exposure to IR, ≤ 2 hrs is sufficient to yield near maximal potentiation with the PARP-1 inhibitor, PD128763. There are two possible

explanations for this finding:- Either the majority of repair occurs within 30 minutes and therefore it is the initial time which is critical for the inhibition of PARP-1 and therefore potentiation, or because AG14361 is a very potent inhibitor, it binds PARP-1 so tightly that it is not removed following washing and plating into drug-free medium. That the majority of repair occurs by 30 minutes is supported by data presented in Chapter 5 where enzyme proficient cells were shown to repair at least 50 % of IR-induced SSBs and DSBs within 30 minutes (sections 5.3.5 and 5.3.2). One potential way to distinguish between these two possibilities would be to irradiate the cells and then delay the addition of AG14361 by 30 minutes to see if radiosensitisation can still be achieved.

Owing to the collaborative nature of this project, studies on the exposure time required to achieve maximal radiosensitisation with NU7026 were carried out by Lloyd Kelland and colleagues of the CRC centre for Cancer Therapeutics, Sutton. Studies were carried out to assess the clonogenic survival of CH1 cells following 3 Gy IR in the presence or absence of 10 μ M NU7026 and either a 3, 6 or 24 h post-incubation period. This group concluded that a 6 h post-incubation period was sufficient to achieve maximal radiosensitisation with NU7026 (personal communication).

Ideally, paired cell lines should be isogenic, differing only in the gene of interest. However, this is rarely the case, and as described in Chapter 3, it was established during the course of this research that in contrast to the PARP-1 $-/-$ MEFs which had retained wild type p53 following spontaneous immortalisation, the PARP-1 $++$ MEFS used in this study were shown to have acquired a mutation in p53 thus rendering the protein unable to act as a transcription factor. This was confirmed by the observation that the PARP-1 $+/+$ cells were unable to cause the induction of the p53 inducible genes, MDM2 and p21 following IR (Paul Jowsey, Northern Institute for Cancer Research, unpublished results). Nevertheless, both the DNA-PK and PARP-1 inhibitors, were able to radiosensitise the PARP-1 $+/+$ cell line, despite the lack of functional p53. This ability to radiopotentialise cells regardless of p53 status increases the range of tumours for which the use of the inhibitors may be effective since as many as 50 % of tumours carry a p53 mutation.

These observations are in general agreement with earlier data with the PARP-1 inhibitors, NU1025 and NU1085 which enhanced the growth inhibitory effects of temozolomide and topotecan in a panel of 12 human tumour cell lines regardless of p53 status (Delaney *et al.*, 2000). Similarly, AG14361 enhanced the growth inhibitory activity of the radiomimetic drug, bleomycin, with equal potency in the p53 wt HCT116 cell line and their HPV E6 degraded p53 null (HCT116 N7) pair; PF₅₀ values; HCT116 = 1.3 ± 0.4 , HCT116 N7 = 1.3 ± 0.8 (p= 0.4) (Gemma Swaisland, Northern Institute for cancer Research, unpublished results).

The correlation between DNA-PK function and IR-induced double strand break repair is well documented (section 1.12.3). However, few studies have addressed the involvement of DNA-PK in the repair of DNA lesions generated by antitumour agents and subsequent cell survival. Data presented in this Chapter assessed the ability of NU7026 to potentiate the effects of the clinically used chemotherapeutic agents, etoposide and bleomycin. Previous studies have demonstrated that all six members of the xrs complementation group (defective in Ku80 binding component of DNA-PK) are hypersensitive to topo II poisons (Jeggo and Kemp, 1983; Jeggo *et al.*, 1989). In addition, it has been demonstrated that these cells are defective in the rejoining of etoposide-induced DSBs (Caldecott *et al.*, 1990) and that etoposide resistance is restored by transfection of Ku80 cDNA into the mutant cell line (He *et al.*, 1996), thus implicating DNA-PK in the repair of topo II - induced lesions.

In this Chapter, results are presented which show that the growth inhibitory activity of etoposide differed between the DNA-PKcs deficient (V3) cell line and its proficient counterpart (V3YAC). The V3 cells were at least 8-fold more sensitive to etoposide (Figure 4.7), confirming studies comparing V3 and parental AA8 cells which showed that the V3 cells were more sensitive to etoposide (Jeggo *et al.*, 1989). Similarly, Eriksson *et al.*, 2002, demonstrated that in CLL tumour samples, DNA-PKcs protein expression correlated with sensitivity to topo II inhibitors.

NU7026 sensitised V3YAC cells to etoposide treatment bringing their sensitivity to approximately the same level as the V3 cell line. NU7026 failed to potentiate etoposide in V3 cells demonstrating that NU7026 potentiates etoposide specifically via DNA-PK inhibition. The most direct interpretation that is consistent with the results presented here is that loss of or inhibition of DNA-PK potentiates Topo II poison cytotoxicity by inhibiting DNA-PK mediated NHEJ of DSBs generated from cleavable complexes. However, the mechanisms may be distinct. NU7026 could bind and 'block', thus preventing topo II mediated resealing of breaks that would otherwise reverse normally whereas in cells deficient in DNA-PK activity, the mechanism might be an inability to mediate rejoining of frank double strand breaks that arise directly or indirectly from cleavable complexes. These data differ from those of Jin *et al.*, 1998, who reported that Ku, but not DNA-PKcs was implicated in the sensitivity of top II poisons. An explanation for the difference in results is apparent when we consider that these authors utilised murine SCID fibroblasts. It has been well documented that certain SCID cell lines have as much as 15 % residual DNA-PK activity (Blunt *et al.*, 1996). Notably, the V3 cell line used in these studies has no detectable DNA-PKcs protein or DNA-PK activity (Figures 3.4 and 3.6).

NU7026 also enhanced bleomycin induced growth inhibition 9-fold in V3YAC cells but had no effect in the V3 cells (Figure 4.6). Previous studies have demonstrated that xrs-6 and SCID cells, defective in Ku80 and DNA-PKcs, respectively, are sensitive to bleomycin and demonstrated that wortmannin sensitised two human tumour cell lines to bleomycin (Hosoi *et al.*, 1998). More recently, Oliviera *et al.*, 2002, demonstrated that wortmannin enhanced chromosomal damage induced by bleomycin. Wortmannin has also been shown to sensitise MDR cells when combined with bleomycin (Kim *et al.*, 2000). These data strengthen the argument that DNA-PK is involved in the repair of DNA lesions induced by bleomycin, and that NU7026 acts by inhibiting this repair.

For a compound to be an effective resistance modifier it should:-

- Enhance the cytotoxicity of the damaging agent with little or no inherent toxicity of its own.
- Allow selective killing of tumour cells and modulation of dose to obtain tumour control without enhancing the undesirable toxicity to normal tissues.

With respect to the inhibitors used here, a 16 hr exposure to 10 μ M NU7026 or 0.4 μ M AG14361 alone did not significantly reduce cell survival (Table 3.5) whereas survival in combination with IR at the LD₉₀ level was reduced by both inhibitors, alone and in combination. Thus, NU7026 and AG14361 fulfill the first requirement for a resistance modifier, described above.

DNA repair in tumour cells may be one cause of therapeutic failure. Where an enzyme is overexpressed in a tumour as a mechanism of resistance, the use of a selective inhibitor is predicted to allow treatment of these tumours which would otherwise become radio and chemo-resistant. Several groups have investigated whether DNA-PK activity or content correlate with IR sensitivity in different tumour types. Sirzen *et al.*, 1999, demonstrated that both DNA-PK content and activity correlate with intrinsic radiosensitivity in lung carcinoma. Similarly, Zhao *et al.*, 2000, showed that DNA-PK activity correlated with IR sensitivity in oesophageal cancer cell lines. Several groups have also demonstrated increased DNA-PK activity and a corresponding increase in resistance in cell lines treated with antitumour agents which induce DNA DSBs as part of their mechanism:- nitrogen mustards (Muller *et al.*, 1998), the topo II poison, doxorubicin (Shen *et al.*, 1998,) and the vinca alkaloid vincristine (Eriksson *et al.*, 2002). In addition, levels of DNA-PK activity appear to be closely associated with the development of chlorambucil drug resistance in chronic lymphocytic leukaemia (CLL) (Muller *et al.*, 1998 a, b). In a study of patients with relapsed CLL, DNA-PK activity was invariably elevated, and this correlated with chlorambucil resistance in ex vivo cytotoxicity assays conducted on patient peripheral blood lymphocytes (Muller *et al.*, 2000). Additionally, enhanced expression of DNA-PK participates in the development of multiple drug resistance (MDR); Kim *et al.*, 2000 demonstrated a 3-fold increase in DNA-PK activity in MDR variants compared to parental drug sensitive cells.

These studies demonstrate that overexpression of DNA-PK activity may be a novel cellular adaptation mechanistically contributing to the resistance of cancer cells to both IR and chemotherapeutic drugs. These tumour-specific alterations are likely to be important in terms of therapeutic exploitation.

Drug resistance can arise as a result of various biochemical mechanisms that are described in section 1.4. The precise mechanism by which DNA-PK mediates resistance to drugs is unknown although it is presumed to be due to an increase in the repair of cellular DNA damage induced by IR and chemotherapeutic drugs. Alternatively, DNA-PK may serve as a receiver and transmitter of signals that contribute to drug resistance by other mechanisms such as detoxification. From the studies presented in this thesis, inhibition of DNA-PK may be an important strategy to enable the preferential killing of tumour cells by radiotherapy and cancer agents, particularly where an overexpression of DNA-PK is implicated as a resistance mechanism.

Elevated expression of PARP-1 may be associated with malignant progression. For example, Yano *et al.*, 2002, found significantly higher PARP-1 mRNA in primary colorectal tumours in patients with liver metastasis and suggested that this could be related to an increased repair capacity of tumour cells which in turn enables cells with metastatic potential to survive. In this context, Bieche *et al.*, 1996, reported that PARP-1 mRNA expression was higher in human breast cancer tissue than in adjacent normal breast tissues. Nomura *et al.*, 2000, found that PARP-1 mRNA expression was elevated in hepatocellular carcinoma but not in normal liver. These data suggest an important role for enhanced PARP-1 expression in human cancers and could potentially allow selective treatment of tumour cells.

Alternatively, lower intracellular NAD^+ concentrations are observed in tumours compared to normal tissues (Jedeiken and Weinhouse, 1955; Glock and McLean, 1957). Furthermore, in hypoxic cells, NAD^+ levels are reduced by at least 3-fold (Wilson *et al.*, 1977) and consequently the NAD^+/NADH ratio is lowered and NADH is itself able to inhibit PARP-1 activity (Ueda and Hayaishi, 1982). As NAD^+ is the substrate for PARP-1, the potency of PARP-1 inhibitors which act in a

competitive manner will be dependent on the NAD^+ concentration. Therefore low NAD^+ levels and high NADH/NAD^+ ratios in tumour cells has the potential to increase the antitumour selectivity of PARP-1 inhibitor sensitisation to IR and chemotherapeutic agents.

There is some evidence to date for selective sensitisation of tumour cells in vivo. AG14361 caused significant in vivo radiosensitisation in mice bearing LoVo xenografts. Indeed, a local IR dose of 2 Gy for 5 days caused a 19 day growth delay which was extended to 37 days with the administration of 15 mg/kg AG14361. Furthermore, no local tissue toxicity was observed either with IR alone or in combination with AG14361 and AG14361 did not induce hypothermia which has been observed in mice treated with a dose of PD128763 required for radiopotentialisation (Calabrese *et al.*, in press).

The application of repair inhibitors would be particularly advantageous in tumour cells that have lost compensatory repair pathways. Loss of heterozygosity of ATM is a common event in lymphoid malignancy and frequent inactivating mutations of the ATM gene have been reported in patients with leukaemia (Stankovic *et al.*, 2002). ATM is considered to function in the DSB repair pathway of homologous recombination (HR). It has been proposed that non-homologous end-joining (NHEJ) may function as a back up or complementary pathway for DSB repair and vice-versa. DNA-PK is the major complex required for NHEJ and therefore inhibition of DNA-PK on an ATM deficient background may be an important means of selectively targeting tumour cells.

It is therefore fitting that 30-40 % of CLL patients have decreased ATM (Reviewed in Boulton *et al.*, 2001), and significantly 30-40 % of relapsed CLL patients have overexpressed DNA-PK (Muller *et al.*, 1998; Eriksson *et al.*, 2002) thus suggesting that DNA-PK might be a compensatory mechanism for the loss of ATM. In support of this hypothesis, there is synthetic lethality between mutation in ATM and DNA-PKcs during murine embryogenesis (Gurley *et al.*, 2001). Moreover, there is also embryonic lethality in PARP-1 and ATM double knockout mice (Menissier de Murcia *et al.*, 2001) and therefore there is potential for the development of selective therapeutic strategies via the inhibition of a complementary

non-defective pathway as this should significantly decrease the ability of these tumours to withstand therapy without a corresponding increase in normal tissue sensitivity.

In summary, the issue of antitumour selectivity is particularly important in chemo-potential where the anti-tumour selectivity of systemic therapy is limited. In radiotherapy, where sensitive tissues can be excluded from the radiation field, PARP-1 and DNA-PK inhibitors may have the greatest utility.

Ionising Radiation PLDR

The response of human tumours to radiotherapy varies considerably from patient to patient. An understanding of the molecular repair processes which lead to maximal tumour cell recovery following IR is important for radiotherapy since modification of these processes by biological modifiers may increase the therapeutic index.

Tumour cells have an exceptional ability to adapt to altered micro-environmental conditions such as reduced nutrients or oxygen availability and changes in pH. DNA repair mechanisms rank high amongst the defence mechanisms of cancer cells. As described in the introduction (section 4.1.2), non-proliferating cells exposed to IR are known to undergo PLDR, leading to substantial increases in survival compared to proliferating cells, or cells induced to start a proliferative cycle shortly after irradiation (Phillips and Tolmach, 1966; Little, 1969). Evidence for PLDR in the non-proliferating compartment of tumours is very sparse, however, experimental and clinical observations suggest that this radiation resistant cell fraction is capable of re-entering the proliferation cycle and repopulating the tumour following IR-induced killing of the proliferating tumour compartment (Weichselbaum and Little, 1982; Barendson *et al.*, 2001). Since radioresistant tumours are able to carry out high levels of PLDR compared to those tumours considered curable, PLDR is considered to be a significant factor in determining tumour responses to radiotherapy (Barendsen *et al.*, 2001). The precise molecular mechanisms underlying PLDR are at present unknown although it has been suggested that DNA is the target (Frankenberger-Schwager *et al.*, 1987).

Studies described in this Chapter have shown that increased radiosensitivity due to deficiency or inhibition of either DNA-PK or PARP-1 is not limited to proliferating cells but also occurs in G1 arrested cells (Figures 4.12 and 4.13). As described in section 4.1.2, early studies indicated that PARP-1 inhibitors could reduce the PLDR capacity of cells. More recently, the effect of loss of PARP-1 has been investigated. Rudat *et al.*, 2001, demonstrated that in C060 hamster cells, PLDR following IR treatment was completely abolished due to the transdominant inhibition of poly(ADP-ribosylation). Chatterjee and Berger, 2000, compared PLDR following 1-6 Gy and a 6 h recovery in growth arrested CHO V79 cells and the mutant derivatives ADPRT54 and ADPRT351. These mutant cell lines have been previously demonstrated to have 5-11 % PARP-1 activity compared to V79 parental cells (Chatterjee *et al.*, 1989). In marked contrast to the results obtained with inhibitors of PARP-1 and those obtained with transdominant inhibition, Chatterjee and Berger, 2000, demonstrated that mutant V79 cells deficient in PARP-1 activity were proficient in PLDR and therefore concluded that PARP-1 does not participate in PLDR. The results derived from these studies are not consistent and therefore the data presented in this Chapter compared two techniques for removing PARP-1 activity; gene knockout and a novel, specific inhibitor, to analyse the role of PARP-1 in PLDR.

As PLD is dose-dependent (Guichard *et al.*, 1984), the initial survival level needed to be similar in all cell lines, and therefore they were irradiated with equitoxic doses to allow for comparison of PLDR capacity. In all cell lines, with the exception of the Ku80 deficient xrs-6 cells, a delay in subculture resulted in a higher surviving fraction than immediate subculture (Figures 4.12 and 4.13). PLDR was significantly reduced in cell lines deficient in either DNA-PK or PARP-1 compared to their proficient counterparts, demonstrating a role for both PARP-1 and DNA-PK in PLDR and that the capacity for PLDR reflected the inherent radiosensitivity of the cell lines. For example, the V3 cells only recovered 2-fold following a 24 h delay in subculture compared to an 8-fold recovery in the V3YAC cells. This reflects the 4-fold higher radiosensitivity of exponentially growing V3 cells compared to V3YAC cells at the LD₉₀ level (Table 3.3). Similarly, Li *et al.*, 1998, reported that SCID cells demonstrated a lack of PLDR following delayed plating. Mice or cell lines deficient

in Ku have a more severe phenotype than those deficient in DNA-PKcs and therefore it was not surprising to find that the xrs-6 cells were unable to carry out any PLDR. Similar studies showed that delayed plating of Ku80 deficient xrs-5 cells, did not significantly modify cell survival suggesting a deficiency in PLDR (Illiakis *et al.*, 1990). These data suggest a possible role for Ku in the recovery of PLDR that is independent of DNA-PKcs. Possible independent functions for Ku in DNA repair are reviewed in Chapter 5 (see section 5.4.2).

The effect of inhibitors of PARP-1 and DNA-PK in the proficient cell lines was comparable with loss of the enzyme. Inhibition of both enzymes had a more profound effect on PLDR than inhibition of one enzyme combined with the lack of the other and this is consistent with the data where DNA DSB repair was investigated (Chapter 5). AG14361 slightly, but significantly inhibited PLDR in the PARP-1 $-/-$ cells. A possible explanation for this is that AG14361 may additionally inhibit PARP-2, which has also been implicated in DNA repair (Schreiber *et al.*, 2002).

When PLDR was measured in the V3YAC and V3 cell lines over time, the surviving fraction of cells increased up to a maximum at 6 hours with no further enhancement in survival being achieved by extending the recovery period to 24 h (Figure 4.11). The lower survival under conditions that promote growth may therefore be interpreted as a fixation of repairable DNA damage during progression of cells through the cell cycle under post-IR conditions (Figure 4.2). DNA-PK activity but not content is expressed in a cell cycle dependent manner (Lee *et al.*, 1997) and increases as cells approach G1/S phase transition. This might be connected to the fact that PLDR largely occurs in G1 and may explain both the reduced PLDR in DNA-PK deficient cells and the extremely potent inhibition of PLDR by NU7026.

Referring back to the studies carried out by Chatterjee and Berger, 2000, discussed earlier, which suggested that PARP-1 is not involved in PLDR, the difference in conclusions is likely to be due to the different cell models which have been utilised. The cells used by this group were grown in nicotinamide deficient medium for over 3 months in order to isolate clones with constitutively reduced NAD levels (Chatterjee *et al.*, 1989). In addition, this group utilised high

concentrations of the mutagen MNNG to select cells deficient in PARP-1 activity. The resulting mutant cell lines have slower doubling times and increased frequency of SCE compared to parental cell lines (Chatterjee *et al.*, 1987). Although this is consistent with compromised DNA repair due to PARP-1 deficiency, it is likely that this phenotype is due to other genetic abnormalities instead of or in addition to reduced PARP-1 activity. Indeed, treatment with high concentrations of MNNG would cause multiple mutations at different gene loci as opposed to a mutation that is related directly to PARP-1 deficiency.

Cell cycle arrest at either the G1 or G2 checkpoint is important to ensure proper DNA replication, DNA integrity and mitotic cell division. The magnitude of G2 delay has been shown to reflect the degree of IR-induced DNA damage and cell radiosensitivity (Nagasawa *et al.*, 1994). Cell cycle analysis following IR treatment, reported here, showed that DNA-PK deficient V3 cells had an increased and prolonged G2 arrest. Similarly, NU7026 in combination with IR caused a substantial and prolonged G2 arrest in V3YAC cells which was greater than that observed in the V3 cells. In Chapter 5, data is presented which demonstrates that more DSBs remain 60 minutes post-IR in the presence of NU7026 than in the absence of DNA-PK and therefore this re-inforces the notion that the G2 arrest is reflecting levels of DNA damage. Consistent with these findings, Lee *et al.*, 1997 concluded that permanent G2 arrest following DNA damage is a feature common to cell lines defective or impaired in DNA-PK activity. In addition, Rosenzweig *et al.*, 1997, demonstrated that WM treated SW480 cells had a prolonged G2/M delay with > 50 % of cells arrested 50 h post-IR. Alternatively, DNA-PK might perform a novel function and be involved in the exit from the G2 checkpoint control.

AG14361 had a small effect on the proportion of G2 cells following IR and this is consistent with the findings of Jacobson *et al.*, 1985, who reported that PARP-1 inhibitors enhanced G2 arrest following treatment with alkylating agents. As with the DNA-PK deficient or inhibited cells, this could reflect higher levels of damage compared to untreated cells or a cell cycle progression function for PARP-1. The mechanism of PARP-1 and DNA-PK in cell cycle arrest remains to be clarified.

In the context of PLDR and cell cycle progression, it is possible that inhibition or absence of either of the enzymes serves to delay the cells from re-entering the cell cycle thus preventing colony formation. In addition, there may be cellular intolerance to concurrent blockade at these two distinct cell cycle checkpoints. Both DNA-PK and PARP-1 are induced when cells enter the cell cycle from a quiescent state (Cai *et al.*, 1994) and therefore this could account for the enhanced survival of enzyme proficient cells compared to deficient cells. In the case of a tumour this could enhance the repopulation of a tumour and therefore inhibitors of PARP-1 and DNA-PK should prevent this.

In conclusion, the data presented here suggest that pharmacological inhibition of DNA-PK and PARP-1, both alone and in conjunction, represents a promising strategy for tumour sensitisation. The mechanism by which these inhibitors of PARP-1 and DNA-PK sensitise cells have been further investigated in the following Chapters by evaluating the inhibition of DNA repair (Chapter 5) and the interaction of the enzymes (Chapter 6).

4.5 Summary

The studies presented in this Chapter have shown that;

- Inhibition of PARP-1 or DNA-PK has potent radiosensitising effects in both exponentially growing and radioresistant non-cycling cells
- Capacity for PLDR reflects radiosensitivity
- Both PARP-1 and DNA-PK are involved in the capacity of cells to recover from PLD

CHAPTER 5

INHIBITION OF DNA REPAIR BY INHIBITORS OF PARP-1 AND DNA-PK IN CELL LINES PROFICIENT OR DEFICIENT IN PARP-1 OR DNA-PK

5.1 Introduction

DNA double strand breaks (DSBs) are the most cytotoxic lesion that are induced by IR and radio-mimetic chemicals, and can also be generated during certain site-specific recombination processes or when a DNA polymerase encounters damage on its template. DNA DSBs can result in the direct inactivation or mutation of a key gene, leading to cell death or impaired cell function. Alternatively, being highly recombinogenic, unrepaired DSBs can lead to chromosomal translocations that generate highly unstable dicentric chromosomes or acentric chromosome fragments leading to genome rearrangement.

Ionising radiation (IR) induces a spectrum of DNA damage lesions ranging from an isolated lesion (for example, base modifications, AP sites, SSBs and DSBs) to a 'cluster of lesions'. Such 'multiply damaged sites' (MDS) are a unique feature of DNA modifications caused by IR and some radiomimetic drugs and many different forms of MDS are generated due to the plethora of types of oxidative lesions produced by IR. MDS consist of two or more lesions, within a distance of 10 to 15 base pairs or occurring within one or two helical turns of the DNA. The lesions can be situated on the same strand or opposing strands. The simplest MDS is a DSB (Figure 5.1), however, as described in section 1.3.4.1, the frequency of IR-induced damage is in the order of base damage > SSB>DSB>crosslinks. Hence, 80 % of MDS consist of base damages or AP sites whilst only 20 % of the total consist of DSBs. Thus, whilst DSBs are the most recognisable clustered lesions, they may constitute a small fraction of the total.

The more complex a lesion, the less likely correct repair will occur. Importantly, clusters are difficult to repair and thus potentially lethal or mutagenic lesions can be formed. For example, when two base damages are situated in opposing strands and are more than three base pairs apart, partial repair of both base damages

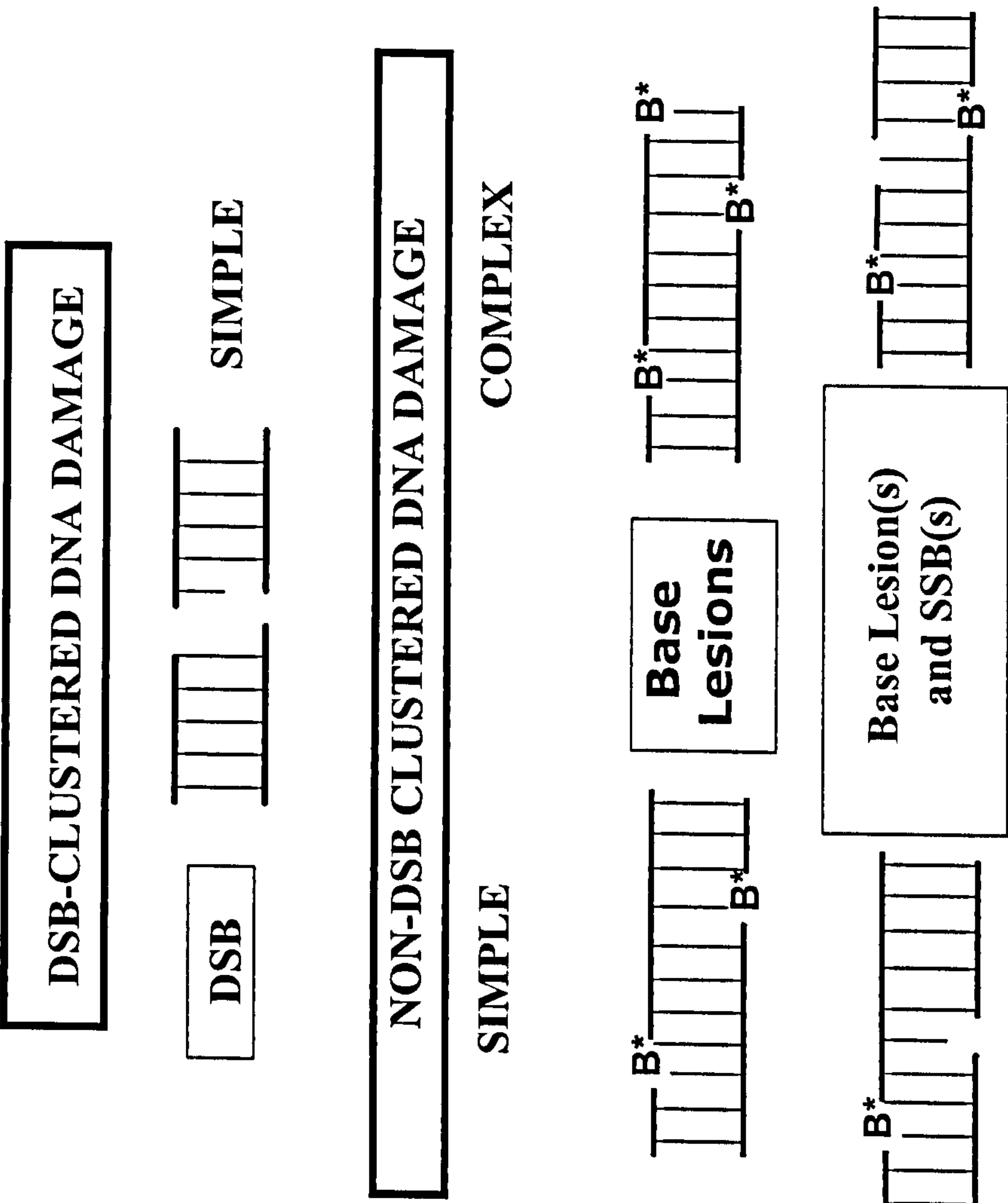


Figure 5.1 Types of clustered DNA damage

can occur at the same time, resulting in the production of a DSB. Since the formation of DSBs is of biological importance, the lethality of IR is therefore believed to be due to the production of these clusters of lesions.

DNA repair is known to occur via several pathways in living cells (section 1.5). Two fundamentally different pathways may be utilised to remove DSBs from the genome of eukaryotic cells; HR and NHEJ, of which NHEJ is thought to comprise at least 80 % of DSB repair in mammalian cells (Weaver, 1995). These pathways may compete, co-operate or act sequentially. In the past few years, major advances have been made in the identification of the factors required for DSB repair as well as in the analysis of the biochemical function of the individual proteins. The major factors involved in HR and NHEJ are described in sections 1.5.2.1 and 1.5.2.2 and shown in Figure 5.2.

The known NHEJ factors include Ku70, Ku80 and DNA-PKcs which comprise the DNA-PK holoenzyme but their precise function in repair and the critical target proteins are not known. The interaction between Ku protein and DNA-PKcs has been extensively investigated and recent studies have begun to map the sequences within the Ku heterodimer that are required for repair function (Singleton *et al.*, 1997; Jin and Weaver, 1997). In Ku70, all mutations that impair dimerization, DNA end binding and DNA-PK activation affect repair. Additional participants in the DNA-PK mediated NHEJ repair pathway include XRCC4 and ligase IV and the recently identified co-factor artemis (Reviewed in Jeggo and O'Neill, 2002 and references therein). XRCC4 and ligase IV form a tight and specific complex, with the interaction being mediated by the non-catalytic C-terminal region of ligase IV that contains two copies of the BRCT domain (Koonin *et al.*, 1996). Briefly, XRCC4 binds to DNA ends as a dimer and each monomer in this dimer binds, in turn, a monomer of ligase IV thus creating a tetrameric protein complex. This is ideally suited to allow ligation of two strands. Although the role that XRCC4 plays in repair is unknown, the activity of ligase IV is stimulated by its association with XRCC4 (Grawunder *et al.*, 1997). Taken together, XRCC4 might serve to activate ligase IV and target it to the DNA-PK containing DNA NHEJ complex.

Before DNA ligase IV can execute ligation, the ends need to be ‘tidied up’. A function for the RAD50-Mre11-NBS1 complex has been suggested. Artemis, a recently identified nuclease, interacts with DNA-PKcs and has been suggested to be required for efficient DSB repair (Moshous *et al.*, 2001). Indeed, DNA-PK phosphorylation specifically regulates the activity of artemis (Ma *et al.*, 2002). The exact function of artemis in DNA DSB repair is unknown, however, it is reasonable to suggest that this enzyme may be responsible for the endonucleotic activity that removes overhanging ends in preparation for repair (reviewed in Jeggo and O’Neill, 2002).

Deficiency in factors required for successful execution of NHEJ leads to defective V(D)J recombination, hypersensitivity to IR and defective DSB repair. Among the mutants, DNA-PKcs and artemis mutants are least severe, having no obvious phenotype other than the general defects described above. Interestingly, although the artemis mutants are hypersensitive to IR, they have been reported to be proficient in DSB repair (Nicolas *et al.*, 1996). Ku mutants have an intermediate severity, with prolonged doubling times due to a loss of proliferating cells and premature senescence. The XRCC4 and ligase IV mutants are the most severe, resulting in embryonic lethality. Intriguingly, it has been shown that deletion of Ku in these cells rescues the embryonic lethality (Karanjawala *et al.* 2002).

The mechanism of DSB repair by homologous recombination (HR) is only partially understood, though many models have been proposed. The loss of RAD54 leads to recombinational deficiencies and DSB repair defects (Bezzubova *et al.*, 1997). The absence of RAD51 causes the accumulation of chromosomal abnormalities and cell death (Sonoda *et al.*, 1998). Tsuzuki *et al.*, in 1996, suggested that HR is essential in vertebrate cells, however, the precise role is unsure. As shown in Figure 5.2, evidence suggests that the RAD50-MRE11-NBS1 complex participates in the repair of DNA DSBs via either NHEJ or HR (D’Amours and Jackson, 2002; Petrini, 1999). Indeed, in its absence, NHEJ is reduced 50-fold and HR is delayed.

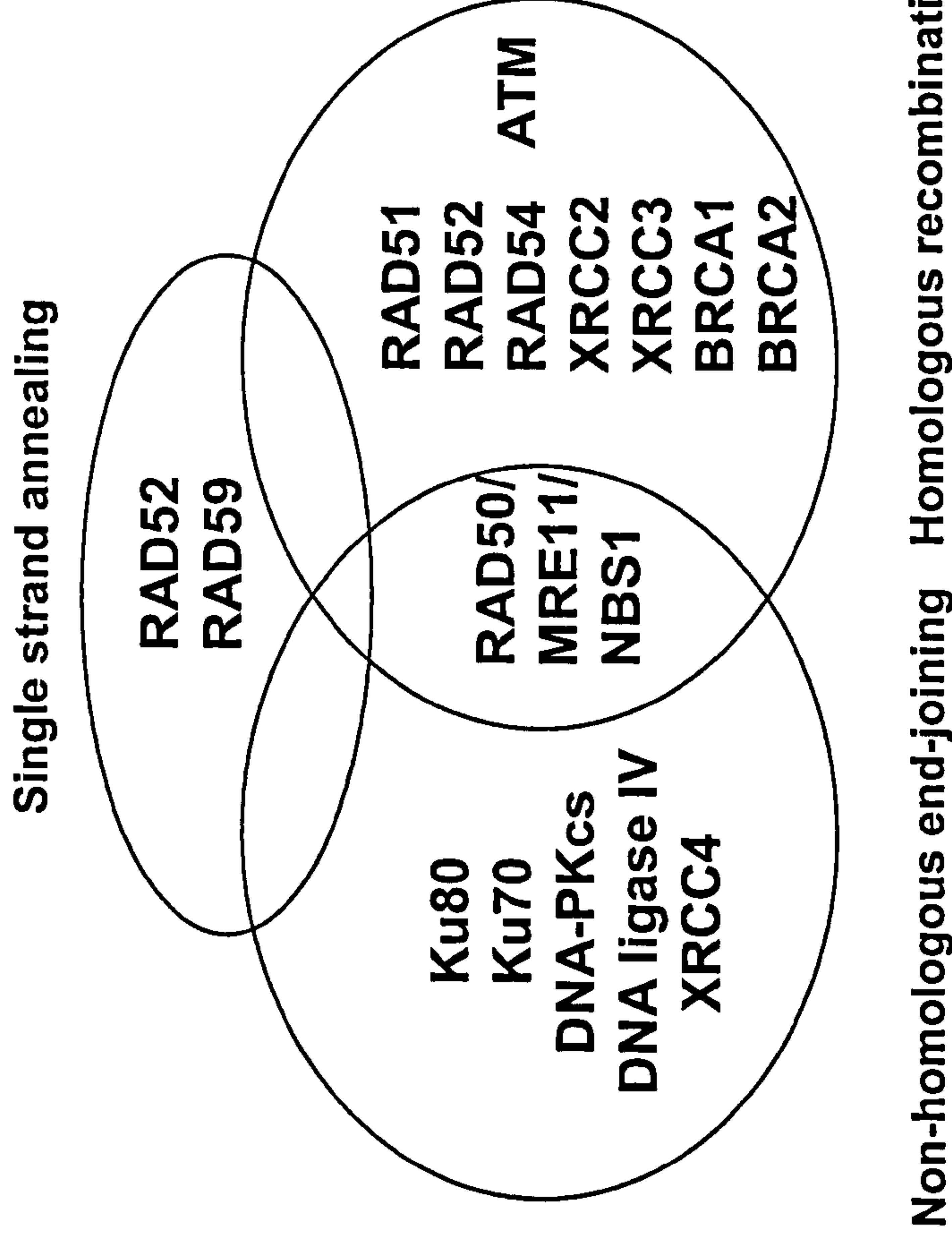


Figure 5.2 Proteins involved in DSB repair

The circle on the left contains a list of the known participants in NHEJ. Gene products involved in DSB repair by homology directed recombination are indicated in the large circle on the right. The SSA pathway which, like NHEJ, requires some degree of homology between joined termini is depicted as a sub-pathway of either NHEJ or HR. Possible overlaps are shown in the middle.

BER of MDS

One of the major pathways to repair base lesions, AP sites and SSBs is the BER pathway which is a multi-step process involving the sequential action of several proteins (reviewed in section 1.5.1). BER is therefore likely to be involved in the repair of clustered damage sites induced by IR. Importantly, MDS with a base damage situated close to and in the opposite strand to a SSB could be converted to a DSB. It has been proposed that repair of such MDS may enhance the number of DSBs in the DNA following treatment with IR (Ward, 1988). Since the induction of DSBs has been correlated with IR-induced cell death (Macmillan *et al.*, 1993), their production needs to be minimised. Several mechanisms have been proposed that are likely to prevent the conversion of non-lethal lesions or mutagenic lesions into lethal DSBs. Firstly, preferential repair of damage on one strand before the other has been suggested and secondly DSB formation may be prevented through the production of repair intermediates. The latter mechanism is particularly relevant when considering the role of PARP-1 in BER. A role for PARP-1 has been proposed as an antirecombinational factor and as a protection factor. In this regard, PARP-1 could ensure that SSB repair intermediates are never left unprotected and therefore remain unavailable to form part of a DSB during partial repair of MDS.

In summary, it is therefore likely that the NHEJ and BER machinery may interact to enhance the repair of complex lesions. There is good evidence for the function of PARP-1 and DNA-PK in BER and NHEJ, respectively (reviewed above and in sections 1.6.4.4. and 1.10.4) and therefore this is one way in which the two enzymes might interact in response to IR. Moreover, accumulating evidence has indicated a role for PARP-1 in DSB rejoining (reviewed in section 5.4.3).

5.2 Aims

The aim of this third results Chapter was to investigate the repair of DSBs and SSBs following treatment with IR, in paired cell lines proficient or deficient in PARP-1 or DNA-PK, in order to elucidate the mechanism underlying the increased radiosensitivity of PARP-1 and DNA-PK deficient cell lines. In addition, the effect of AG14361 and NU7026 on the repair of DSBs and SSBs was investigated. In order to allow comparison of these results with cytotoxicity data presented in Chapter 4, AG14361 and NU7026 were used at concentrations of 0.4 μ M and 10 μ M, respectively, in all experiments described below, unless otherwise stated.

5.3 Results

5.3.1 Induction of DSBs as a function of radiation dose in exponentially growing CHOK1 cells

The DSB levels following increasing doses of IR was investigated in the CHO-K1 cell line by neutral elution (Figure 5.3 A and B). This allowed a dose of IR to be chosen for subsequent DNA DSB repair assays that a) would enable a high level of DSBs to be induced such that the full extent of inhibition of DSB rejoining by the PARP-1 or DNA-PK inhibitors (alone and in combination) could be evaluated and b) was still within the sensitivity limit of the technique. A parental cell line was chosen since the initial level of damage induced by a particular dose of IR should be the same in all cell lines, regardless of their radiosensitivity status, as the initial yield of IR-induced DNA lesions mainly reflects physical and chemical processes rather than being dependent on the physiological state of the cells (Olive, 1998). The induction of DNA DSBs as a function of radiation dose is considered to be linear (Frankenberg-Schwager, 1989) and therefore a relationship exists between the rate of DNA eluted and the dose of irradiation. With increasing doses of IR, followed by immediate elution so that no repair would occur, a dose dependent increase in the rate of elution was observed (Figure 5.3B). Relative elution (RE) values were calculated from the elution profile (Figure 5.3A), as described in section 2.8. Figure

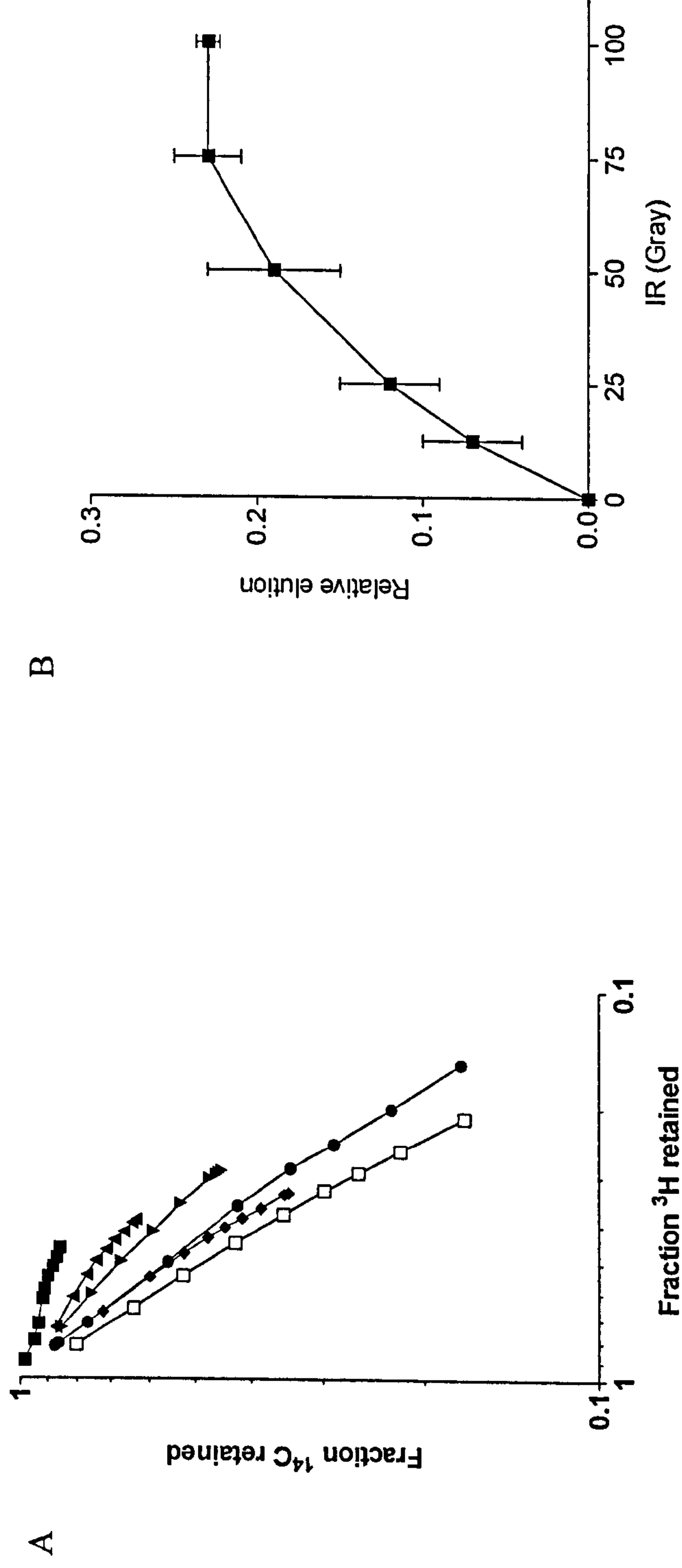


Figure 5.3 Relationship between dose of IR and elution in CHOK1 cells

Cells were exposed to increasing doses of IR and immediately harvested for neutral elution to determine levels of DNA DSBs. A; Representative elution profile (■, 0 GY ▲, 12.5 Gy; ▼, 25 Gy; ●, 50 Gy; ◆, 75 Gy; □, 100 Gy) Data are the representative elution profiles of duplicate samples from a single representative experiment. B; Relative Elution (RE) curve. Data are the mean \pm SEM of at least 3 independent experiments.

5.3B demonstrates that RE increased with the dose of IR up to 75 Gy and the level of strand breaks reached a plateau between 75 and 100 Gy, a reflection of the limit of sensitivity of the technique. A dose of 75 Gy was therefore used in all DNA DSB repair studies described in this Chapter.

5.3.2 Kinetics of DSB repair in cell lines proficient or deficient in PARP-1 or DNA-PK

The kinetics of DNA DSB repair following exposure to 75 Gy IR over a 60 min time period were compared in cell lines proficient or deficient in PARP-1 or DNA-PK (Figure 5.4). Briefly, DSB levels were measured by neutral elution either immediately after IR or following either a 10, 30 or 60 minute recovery period at 37°C in order to allow repair to occur. In order to quantitate the difference in the elution profile of irradiated cells compared to a non-irradiated control, and allow inter-experimental comparisons, RE values were calculated, and from these % repair and % inhibition of repair were calculated as described in section 2.8

In the PARP-1 proficient (PARP-1 +/+) and the DNA-PK proficient (V3YAC) cells, DNA DSBs were rejoined rapidly, with the majority of strand break rejoining (~60 %) occurring within the first 30 minutes and 81 % and 89 %, respectively, of DSBs rejoined by 60 min (Figure 5.4 A, B). By contrast, in the V3 (deficient in DNA-PKcs) and PARP-1 -/- cell lines, higher DNA DSB levels remained at 60 min post-IR with only 61 % and 58 % DNA DSB rejoined, respectively.

Interestingly, within the first 30 minutes, the V3 cells repaired DSBs with the same fast kinetics as the V3YAC cells with 50 % of the breaks being rejoined within 21 ± 3 and 17 ± 2 minutes, respectively. Furthermore, there was no significant difference in the amount of DSBs that had rejoined within the 30 minute period, 60 % (V3YAC) and 58 % (V3), $p=0.19$. However, in the V3 cells, no significant increase in rejoining occurred during the following 30 minutes. This data implies that initial repair up to 30 minutes of ~60 % of the lesions is not DNA-PKcs dependent but subsequent repair is DNA-PKcs dependent.

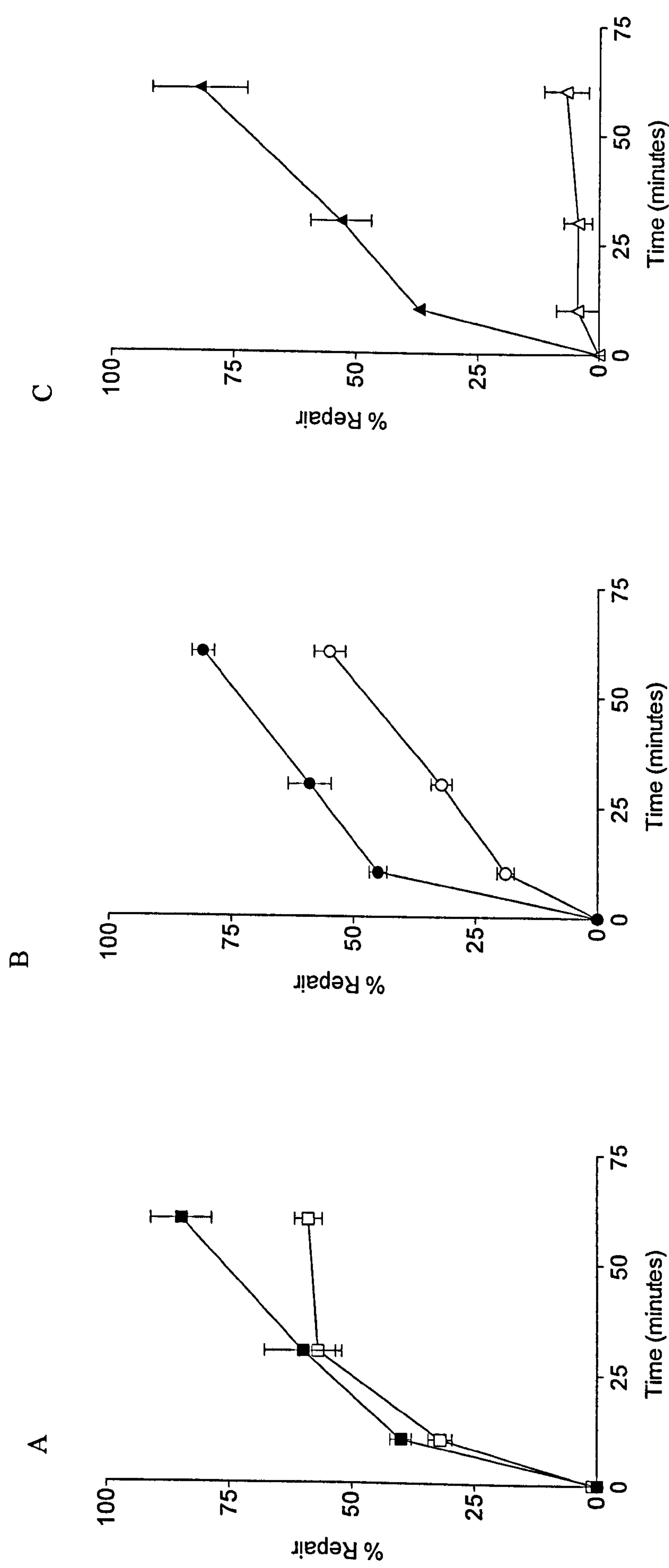


Figure 5.4 Kinetics of DNA DSB repair following a fixed dose of IR

A, ■ V3YAC cells; □ V3 cells; B, ● PARP-1 +/- cells; ○ PARP-1 +/- cells; C, ▲ xrs-6 cells; △ xrs-6 cells. Cells were exposed to 75 Gy IR and post-incubated for increasing amounts of time before harvesting for neutral elution. Data are the mean \pm SEM of at least 3 independent experiments.

In contrast, the PARP-1 $-/-$ cells were defective in rejoining DSBs at these early time points and were only able to repair 32 % of breaks within 30 minutes, taking 55 ± 4 minutes to repair 50 % of their DSBs compared to 16 ± 2 minutes in the PARP-1 $+/+$ cells. These data suggest that PARP-1 makes an important contribution to the very early stages of DSB repair.

Strikingly, in the Ku80 deficient xrs-6 cells, 99 % of DSBs remained unrejoined following 60 minute post-IR incubation, demonstrating that no significant repair ($p = 0.13$) had occurred during this time period (Figure 5.4C). This deficiency in repair was restored by complementation with Ku80 cDNA. Like the other enzyme proficient cell lines, the xrs-6 Ham Ku80 cells carried out the majority of rejoining within the first 30 minutes with 50 % of breaks rejoined within 26 minutes and 82 % of breaks rejoined within 60 min.

5.3.3 The effect of AG14361 and NU7026 on DNA double strand break repair.

To determine if the inhibitors also modulated DNA DSB repair and in order to investigate the mechanism underlying the single and combined effects of the inhibitors on cytotoxicity following IR, DNA DSB levels were assessed in the presence or absence of AG14361 and/or NU7026 at 10, 30 and 60 min post-IR. The percentage of inhibition of repair values are summarised in Table 5.1. The production of DSBs is not attributable to a direct effect of the inhibitors on the integrity of DNA since the elution profiles of DNA from cells exposed to AG14361 or NU7026 alone for 60 minutes was the same as that for untreated controls (data not shown).

As the greatest difference in repair between the enzyme deficient and proficient cell lines was seen at 60 min post-IR and the majority of DSBs had rejoined during this period (Figure 5.4 A,B and C), the % repair in the presence of inhibitor(s) 60 minutes post-IR are compared in Figure 5.6 and these are described in more detail below. Representative elution profiles for V3 and V3YAC cells are given in Figure 5.5. As expected, NU7026 had no significant effect on the limited repair

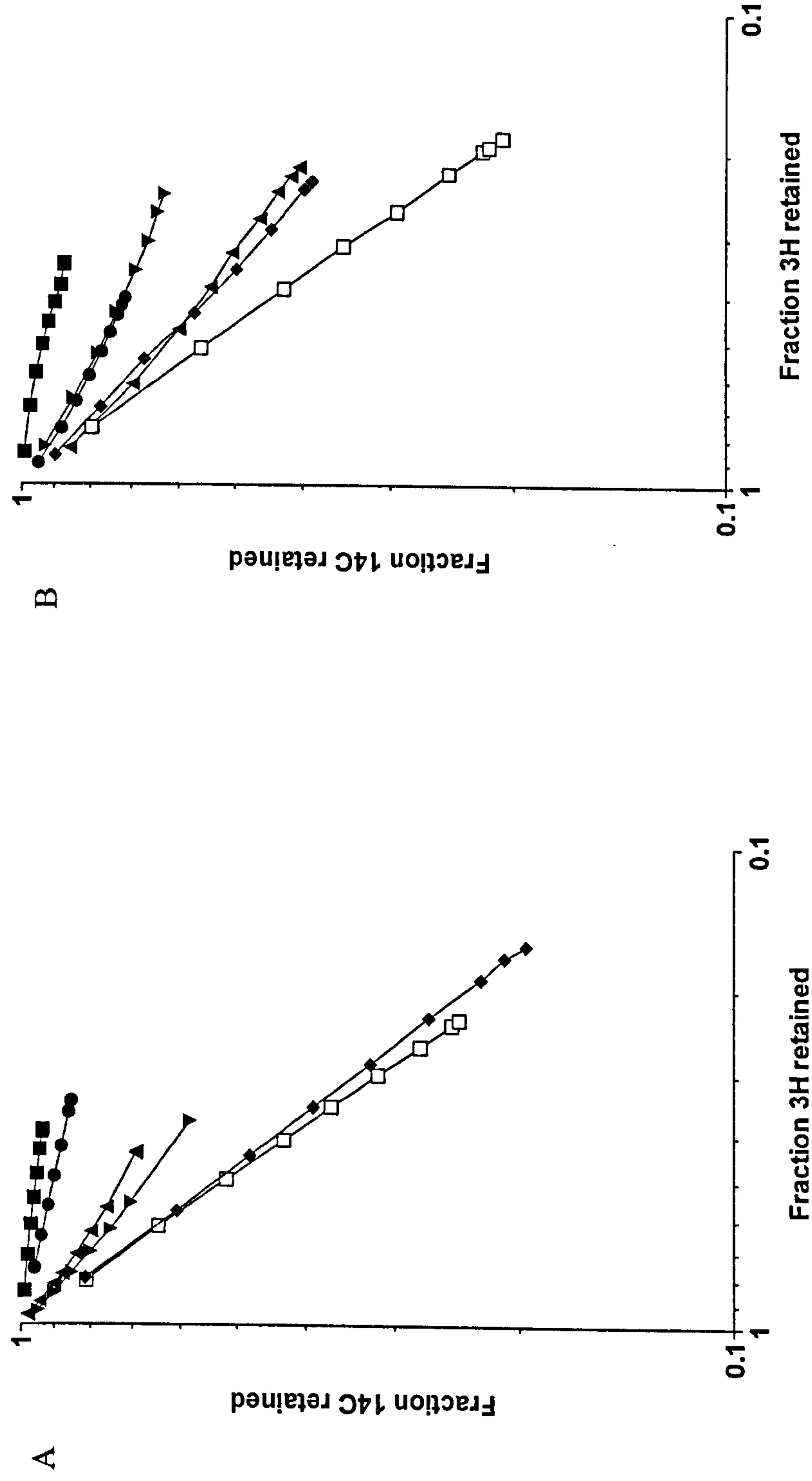


Figure 5.5 Representative elution profiles :Effect of AG14361 and NU7026 on DNA damage induced by 75 Gy IR

A, V3YAC cells; B, V3YAC cells \blacksquare , Control cells (Unirradiated); \bullet , IR alone; \blacktriangle , IR + AG14361; \blacktriangledown , IR + NU7026; \blacklozenge , IR + AG14361+NU7026; \square , IR + No repair. Control cells or cells irradiated with 75 Gy IR were analysed immediately or following 60 minutes repair at 37°C in the presence or absence of 0.4 μ M AG14361 or 10 μ M NU7026. Data are representative elution profiles from duplicate samples from a single representative experiment.

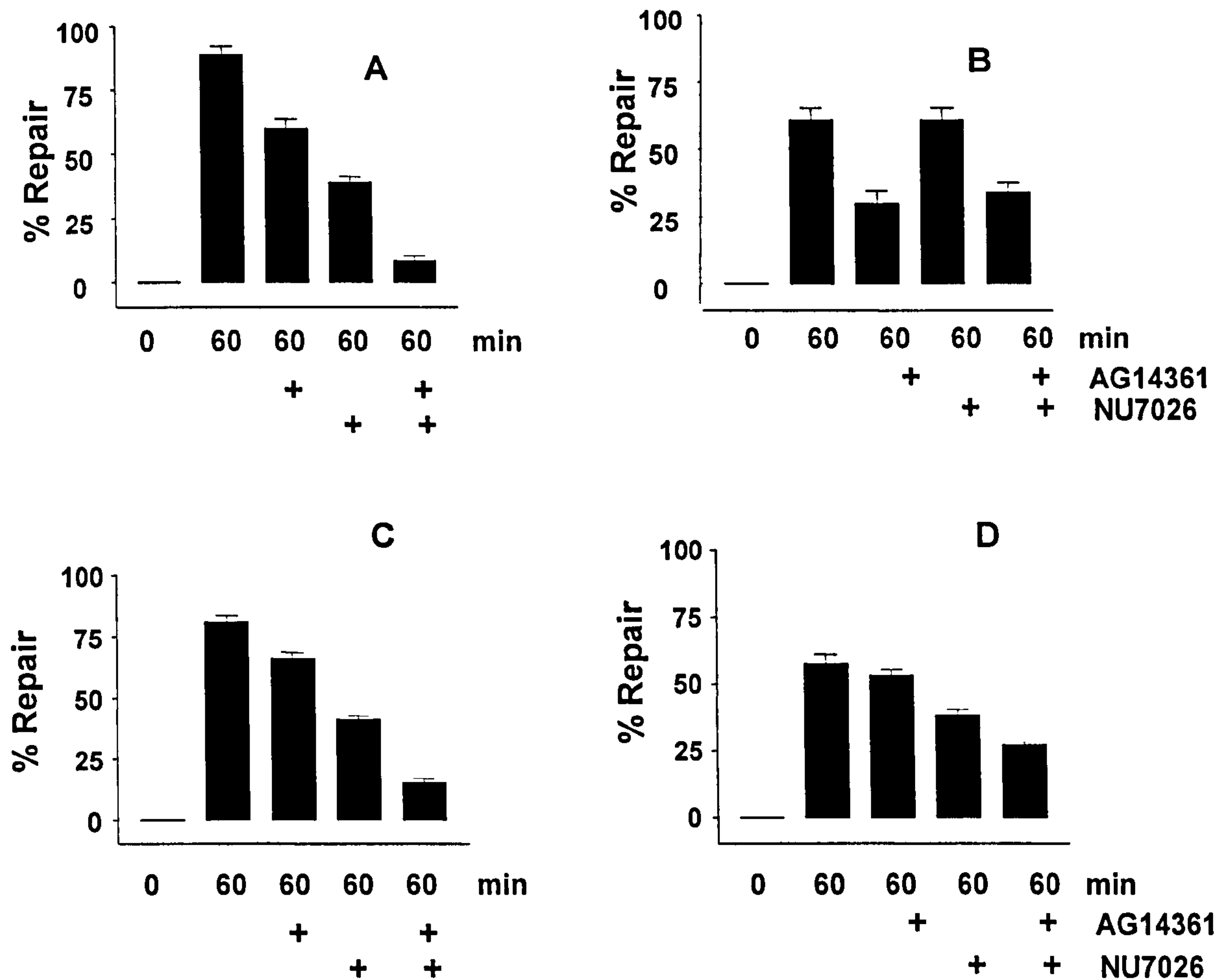


Figure 5.6 Effects of AG14361 and NU7026 on DNA DSB repair 60 minutes post-IR

A; V3YAC cells; B, V3 cells; C, PARP-1 +/+ cells; D, PARP-1 -/- cells. Cells were pre-incubated with inhibitor(s) for 60 min, exposed to 75 Gy IR and post-incubated for 60 min in the absence of inhibitor(s) before harvesting for neutral elution. Data are the mean \pm SEM of at least 3 independent experiments.

A

Cell line	V3YAC			V3		
Inhibitor (s)/Time (Min)	AG14361	NU7026	AG14361 + NU7026	AG14361	NU7026	AG14361 +NU7026
10	37±8	54±2	74±4	53±6	3±3	53±2
30	20±2	41±0.4	82±3	72±3	-3±2	71±4
60	40±6	55±3	95±4	55±3	0	50±1
4	42±2	65±4	99±3	49±3	0	47±2
24	17±3	25±2	89±3	40.1±2	0	39±2

B

Cell line	PARP-1 +/+			PARP-1 -/-		
Inhibitor (s)/Time (Min)	AG14361	NU7026	AG14361 + NU7026	AG14361	NU7026	AG14361 +NU7026
10	36±3	53±2	69±3	9.4±5	33±3.2	41±2.5
30	23±1	37±4	68±5	6.3±0.5	49.1±3.5	57±5
60	25±2.9	50±3.8	80±5.1	4.5±3.2	40±4.8	45±2.9

Table 5.1 Table of % Inhibition of repair by AG14361 and NU7026

A, V3YAC and V3 cells; B, PARP-1 +/+ and PARP-1 -/- cells. Values were calculated as described in section 2.8 and are the mean ± SEM of at least 3 independent experiments.

capacity of the V3 cells, $p = 0.16$, and similarly, AG14361 did not significantly inhibit repair in the PARP-1 $-/-$ cells, $p = 0.8$ (Figure 5.6B and D). NU7026, did, however, inhibit repair in the PARP-1 $-/-$ cells by $40 \pm 5 \%$ ($p < 0.001$) and AG14361 inhibited repair in the V3 cells by $55 \pm 3 \%$ ($p < 0.001$). In the V3YAC cell line, (Figure 5.6A) AG14361 and NU7026 significantly inhibited DSB repair by $40 \pm 6 \%$ ($p < 0.001$) and $55 \pm 4 \%$, ($p < 0.001$) respectively, compared to the drug free control. Repair in the V3YAC cells in the presence of NU7026 was below that of the V3 cells. Very similar results were obtained in the PARP-1 $+/+$ cells (Figure 5.6C), with AG14361 and NU7026 inhibited the rejoining of DSBs by $25 \pm 3 \%$ ($p < 0.001$) and $50 \pm 4 \%$ ($p < 0.001$), respectively. Repair in the PARP-1 $+/+$ cells in the presence of AG14361 was not significantly different from repair in the PARP-1 $-/-$ cells.

Consistent with the radiosensitisation data with the inhibitors, described in Chapter 4, at least additive effects on DSB repair inhibition were observed when the inhibitors were co-incubated in enzyme-proficient cells. The combined effect of the inhibitors in V3YAC cells led to an almost total inhibition of repair ($95 \pm 4 \%$) but the V3 cells were able to achieve approximately 60 % repair in the presence of both AG14361 and NU7026. In the PARP-1 $+/+$ cells, AG14361 and NU7026, in combination, inhibited repair by $80 \pm 5 \%$ whereas the same combination only inhibited repair in the PARP-1 $-/-$ cells by $45 \pm 3 \%$. These data suggest that inhibition of both enzymes has a more profound effect than inhibition of one enzyme, combined with a lack of the other.

The effects of the inhibitors at the earlier time points of 10 and 30 minutes were also assessed. The % repair at these time points are presented in Table 5.2. Repair in the V3YAC cells in the presence of NU7026 followed the same kinetics of repair as the DNA-PK deficient V3 cells, with an initial fast component followed by an insignificant amount of repair occurring between 30 and 60 minutes post-IR incubation (Figure 5.8 A and B). Interestingly, at all time points, repair in the V3YAC cells in the presence of NU7026 was lower than in the V3 cells indicating that inhibition of DNA-PK has a more profound effect on DSB repair than a lack of the enzyme.

	V3YAC	V3	PARP-1 +/+	PARP-1 -/-
10 min	40.0 ± 2.1	32.2 ± 2.4	45.0 ± 1.8	18.8 ± 1.7
10 min +AG14361	27.8 ± 2.2	16.3 ± 2.2	27.0 ± 2.5	16.9 ± 0.96
10 min +NU7026	20.4 ± 3.1	34.0 ± 3.5	22.3 ± 1.6	12.7 ± 1.45
10 min +AG14361 +NU7026	11.0 ± 2.1	12.5 ± 2.2	14.3 ± 2.1	11.2 ± 3.1
30 min	60.0 ± 7.9	57.0 ± 3.6	59.0 ± 4.4	32.0 ± 2.1
30 min +AG14361	49.7 ± 1.8	17.0 ± 2.1	44.0 ± 3.0	29.2 ± 3.7
30 min +NU7026	36.7 ± 2.5	61.7 ± 3.8	38.9 ± 1.4	16.3 ± 3.2
30 min +AG14361 + NU7026	11.6 ± 2.4	34.0 ± 2.6	11.2 ± 2.9	13.7 ± 2.2

Table 5.2 Table of % repair values at 10 and 30 min post-IR

Values were calculated as described in section 2.8 and are the mean ± SEM from at least 3 independent experiments.

DNA DSB repair in the PARP-1^{+/+} cells in the presence of AG14361 followed the same kinetics as observed in the PARP-1^{-/-} cells, however, in contrast to the effect of NU7026 in the V3YAC cells, at 10 and 30 minutes, repair was lower in the absence of PARP-1 rather than in the presence of AG14361 (Table 5.2).

5.3.4 The effects of AG14361 and NU7026 on double strand break (DSB) repair at 4 and 24 hours

The longer term repair and the effects of the inhibitors on DNA DSB repair were investigated by assessing DNA DSB levels in the V3YAC and V3 cells 4 and 24 h post-IR (Figure 5.7). DNA DSB repair was complete by 4 h in the V3YAC cells but 33 % of DSBs still remained at this time in the V3 cells, although by 24 h, only 5 % of DSBs remained such that there was no significant difference between the number of breaks rejoined in the V3YAC and the V3 cells at 24 h ($p = 0.84$). These data demonstrate that the DNA-PK deficient V3 cells are able to repair DNA DSBs nearly completely but with slower kinetics than the V3YAC cells. Incubation of V3YAC cells with AG14361 retarded DSB repair by $42 \pm 2 \%$ and $17 \pm 3 \%$ at 4 and 24 h, respectively. NU7026 hindered repair of DSBs by $65 \pm 4 \%$ and $25 \pm 2 \%$, at 4 and 24 h, respectively. The combination of inhibitors completely inhibited repair at 4 h and by approximately 90 % at 24 h ($p < 0.05$ for all values). These results are summarised in Figure 5.8 A. NU7026 exerted no significant additional effect on the repair in the V3 cells ($p = 0.09$), however, inclusion of AG14361 inhibited DSB repair by $49 \pm 3 \%$ ($p = < 0.001$) and $40 \pm 2 \%$ ($p = < 0.001$) at 4 and 24 h, respectively. Due to time constraints, similar experiments in the PARP-1^{+/+} and PARP-1^{-/-} cells were not carried out.

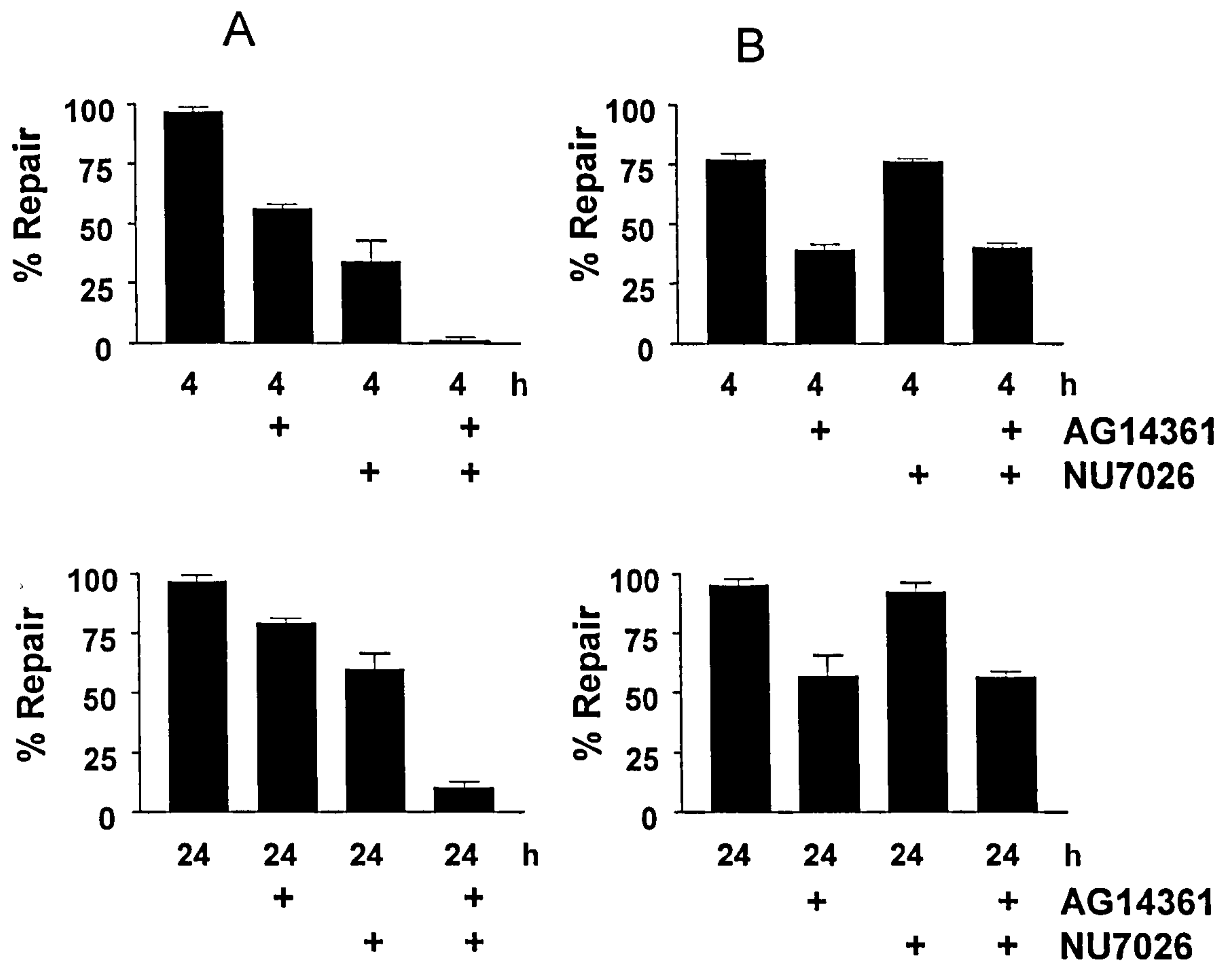


Figure 5.7 Effects of AG14361 and NU7026 on DNA DSB repair at 4 and 24 h post-IR

A, V3YAC cells; B, V3 cells. Cells were pre-incubated with inhibitor(s) for 60 min, exposed to 75 Gy IR and post-incubated in the presence of inhibitor(s) for 4 or 24 h before harvesting for neutral elution. Data are the mean \pm SEM of at least 3 independent experiments.

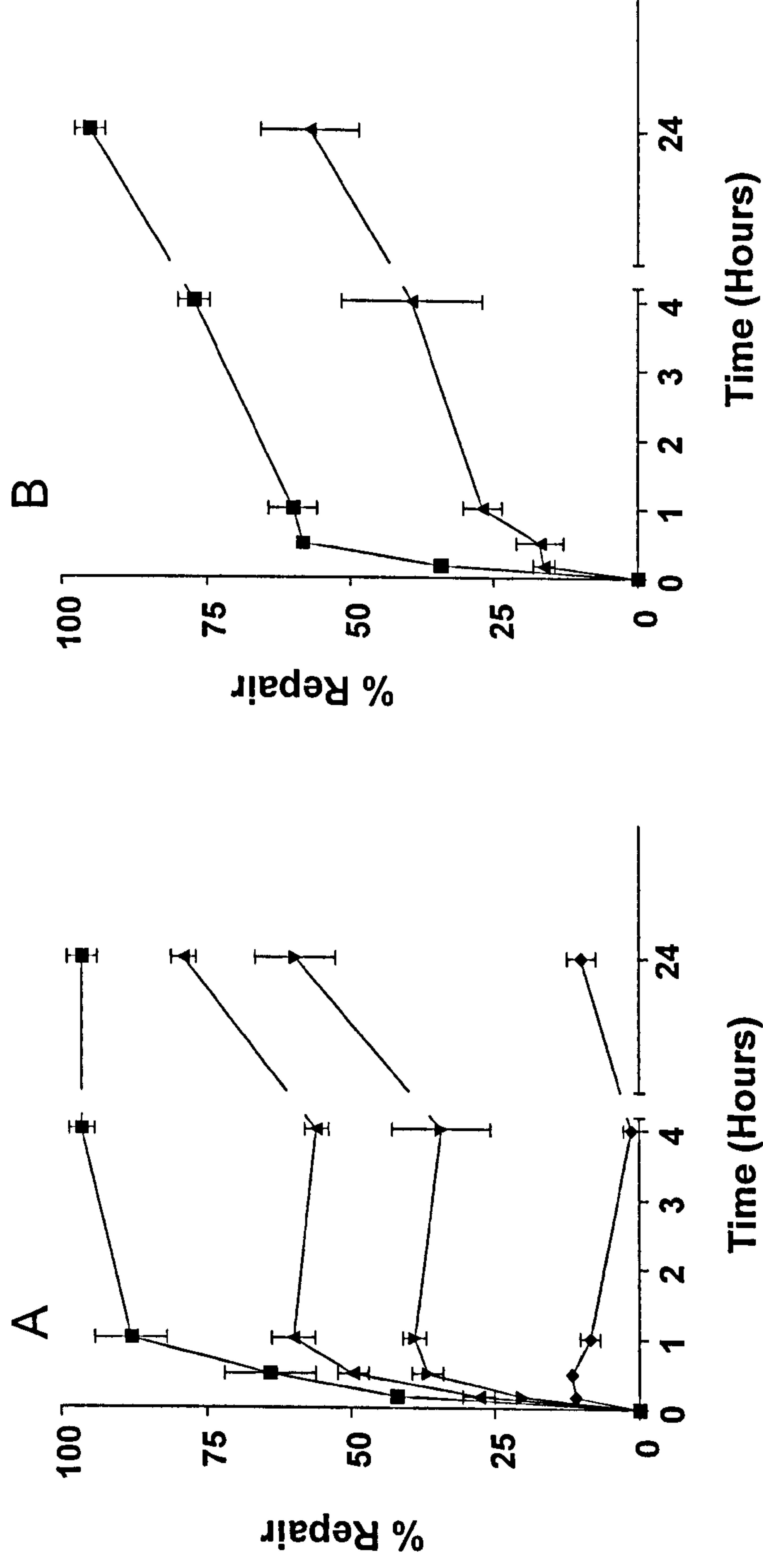


Figure 5.8 Effect of AG14361 and NU7026 on the kinetics of DNA DSB repair following a fixed dose of IR
A; V3YAC cells; B, V3 cells. Cells were pre-incubated in the presence or absence of inhibitor(s) for 60 min, exposed to 75 GY IR and post-incubated in the presence or absence of inhibitor(s) for increasing amounts of time before being harvested for neutral elution. ■, control; ▲, + AG14361; ▼, + AG14361 + NU7026; ◆, + AG14361 + NU7026. Results are the mean of at least 3 independent experiments \pm S.E.M

5.3.5 The effects of AG14361 and NU7026 on single strand break (SSB) repair

The effects of AG14361 and NU7026 on the repair of DNA SSBs induced by 6 Gy IR (which caused approximately 90 % cell kill in parental AA8 cells, see Figure 3.7) were investigated 60 min post-IR in the V3 and V3YAC cells by alkaline elution. As would be predicted, SSB repair in the absence of inhibitors was equally effective in both the V3YAC and V3 cell lines (Figure 5.9A and 5.9B, respectively), with 85 % of breaks rejoined within 60 minutes. Whilst AG14361 inhibited SSB repair by $38 \pm 2 \%$ ($p = <0.05$) and $39 \pm 3 \%$ ($p = <0.05$) in both the V3YAC and V3 cells, respectively. NU7026 had no significant effect in either cell line ($p = >0.05$).

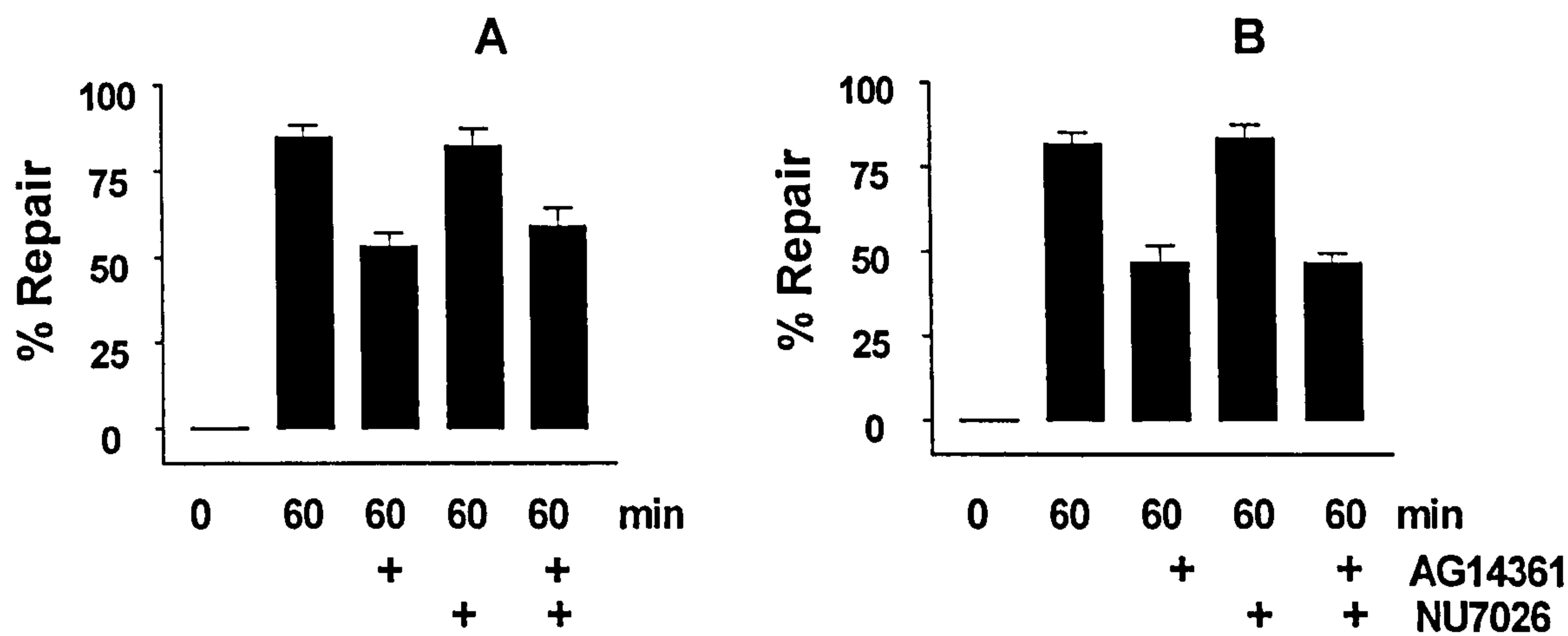


Figure 5.9. Effects of AG14361 and NU7026 on DNA SSB repair

A, V3YAC cells; B, V3 cells. Cells were pre-incubated in the presence or absence of inhibitor(s) for 60 min, exposed to 6 Gy IR and post-incubated for 60 min before harvesting for alkaline elution. Data are the mean \pm SEM of at least 3 independent experiments.

5.4 Discussion

The most important mechanism promoting survival of a tumour following IR is the faithful repair of DNA DSBs. Eukaryotic cells have evolved specific pathways for processing DNA DSBs by either homology dependent or non-homologous repair pathways (reviewed in section 1.5).

The techniques of neutral and alkaline filter elution were used to evaluate the repair capacity of paired cell lines proficient or deficient in PARP-1 or DNA-PK. The effect AG14361 and NU7026 on the repair of DNA DSBs and DNA SSBs in these cell lines were also investigated.

5.4.1 Repair of DSBs in cells deficient in DNA-PK

Data presented in this Chapter have demonstrated that whilst extensive DNA damage was observed immediately after irradiation in all cell lines, DSBs were rejoined rapidly in the parental V3YAC cells (80 % complete by 60 minutes). In the DNA-PKcs deficient V3 cells, DSBs were initially repaired rapidly with about half of the DSBs rejoined within 30 minutes but subsequent repair was slow (Figure 5.4). The deficiency in DSB repair was even more pronounced in the Ku80 deficient xrs-6 cells, with no significant rejoining observed within the 60 min post-IR incubation. However, the Ku80 transfectant cell line was able to repair the DSBs proficiently.

These data are consistent with many studies that have demonstrated a cessation of strand break rejoining following an initial short period of repair in DNA-PK deficient cells (Lees-Miller *et al.*, 1995; Biedermann *et al.*, 1991; Allalunis-Turner *et al.*, 1995; Chang *et al.*, 1993). However, these groups utilised relatively short periods of post-IR incubation. Rejoining in the V3 cells was further studied by allowing prolonged post-IR incubation times (up to 24 hours) to assess the ultimate fate of IR-induced DSBs in the genome of these DNA-PK deficient cells (Figure 5.8). Surprisingly, despite lacking DNA-PK activity, repair in the V3 cells nevertheless approached completion by 24 h, demonstrating that the V3 cells are only slightly less proficient than the V3YAC cells at rejoining DNA DSBs by 24 h.

These results are consistent with data published by Diabase *et al.*, 2000, who compared the rejoining of DNA DSBs in the DNA-PK proficient human glioma cell line M059K and the isogenic but DNA-PK deficient M059J cell line (Allalunis-Turner, 1995) using pulse field gel electrophoresis (PFGE). PFGE, like neutral elution detects DSBs by physically measuring the changes in the average size of the cellular DNA (as shown by the migration rate of DNA through an agarose gel) where the average size of DNA molecules is inversely proportional to the number of DSBs. Consistent with the data presented in this Chapter, rejoining of DSBs in both these cell lines was biphasic (fast and slow component) and was nearly complete following 30 h post-IR. Moreover, as with the data presented here with the V3 cells, the authors demonstrated that the slow component of repair removed the majority of DNA DSBs in cells lacking DNA-PK activity. Similarly, a study by Nevalidine *et al.*, 1997, also showed that the SCID defect results in much slower rate of repair of DSBs, but not high levels of residual breaks.

These data are therefore indicative of a DSB repair pathway that remains active in the absence of DNA-PKcs. A number of lines of evidence indicate that although DNA-PKcs highly stimulates the NHEJ pathway, in its absence a slower repair pathway still operates (Diabase *et al.*, 2000). The slower component of repair is probably mediated by DNA-PKcs-independent NHEJ, but may also involve HR which requires a complex of DNA repair proteins including RAD51, BRCA1, BRCA2, XRCC2 and ATM (Jackson, 2002).

NHEJ and HR probably represent alternative or overlapping pathways for the repair of the same types of lesion. Genetic evidence in the literature suggests that these pathways, whilst distinct, might compete (Roth and Wilson, 1985), co-operate (Richardson and Jasin, 2001) or, depending on the persistence or accumulation of DSBs, act sequentially for the repair of the same DSB (Saintigny *et al.*, 2001) and this may be a function of the cell cycle. For example, DNA-PK activity is highest in the G1/S phases of the cell cycle and SCID, V3 and xrs-6 cells are most sensitive to IR in the G1 phase of the cell cycle (Lee *et al.*, 1997), and therefore NHEJ is thought to act predominantly in these phases of the cell cycle. In contrast, HR is restricted to the S and G2 phases of the cell cycle when a sister chromatid is available to serve as a template (Takata *et al.*, 1998) and as such its primary function appears to be to

repair DSBs which arise frequently during DNA replication (Haber, 1999; Sonoda *et al.*, 1998). These data therefore suggest that the two pathways play distinct and independent roles.

If these two pathways are in competition for DSBs, eliminating one should increase the contribution of the other. Consistent with this hypothesis, Mateos *et al.*, 1994, observed elevated DSB rejoining in mammalian NHEJ mutants in late S/G2 that could be attributed to the HR pathway. Moreover, consistent with the theory that the inactivation of NHEJ shifts DSBs to the competing HR pathway, a number of groups have demonstrated increased HR in the absence of either Ku 70, Ku80 or DNA-PKcs (Pierce *et al.*, 2001; Allen *et al.*, 2002). Significantly, Fukushima *et al.*, 2001, demonstrated that abrogation of HR activity by targeting RAD54 in Ku70^{-/-} cells led to at least additive increases in IR sensitivity. Most recently, Allen *et al.*, 2002, sought to test the effects of DNA-PKcs on DSB-induced HR by comparing HR-mediated gene conversion in V3 cells and V3 cells complemented with human DNA-PKcs. The DNA-PKcs proficient cells had at least a 2-fold reduction in DSB-induced HR suggesting competition between DNA-PKcs and the HR apparatus. Delacote *et al.*, 2002, studied the impact of alteration of late NHEJ on HR. They demonstrated that IR-induced recombination was stimulated by both the XRCC4 mutation and wortmannin treatment, as assessed by the stimulation of Rad51 foci assembly which represent the assembly of the homologous recombination complex at the molecular level.

However, there is no consensus of opinion on the role of HR in NHEJ defective mutants. Liang *et al.*, 1996a, have reported that DSB-induced HR frequencies in mammalian cells are not affected by a defect in Ku80. In contrast, Fukushima *et al.*, 2001, demonstrated that the frequency of intrachromosomal HR induced by I-SceI restriction enzyme was increased in the absence of Ku but not in the absence of DNA-PKcs, an observation that contradicts the findings of Allen *et al.*, 2002. Moreover, earlier studies to those by Delacote *et al.*, 2002, suggested that a defect in XRCC4 shows very little effect on HR (Pierce *et al.*, 2001).

In support of a slow component of NHEJ, studies have shown that whilst HR is largely an error free process, the slow component of repair in DNA-PK deficient cells is largely error prone (Loeblich *et al.*, 1995). This is supported by the observation that loss of Ku is associated with impaired fidelity of repair in cell free systems (Liang *et al.*, 1996b). However, other authors have observed faithful and efficient repair in Ku and/ or XRCC4 deficient cells (Kabotyanski *et al.*, 1998). Similarly, Tzung *et al.*, 1998, found no decrease in the fidelity of DSB rejoining in Ku80 deficient xrs6 cells.

Single strand annealing (SSA) is another homology-dependent DSB repair mechanism (reviewed in section 1.5.2.3) that is not error-free and, as it does not require the presence of a sister chromatid, this repair pathway might also operate in the G1/ early S phases of the cell cycle. It is therefore possible that SSA accounts for the backup repair pathway in NHEJ deficient cells and generates the genomic rearrangements seen in these cells. Contrary to this, however, as described above, cells deficient in NHEJ have a marked defect in DSB rejoining in G1/early S (Jeggo, 1998a) which suggests that no efficient alternative pathway present in this cell cycle phase can fully compensate for the loss of NHEJ. Furthermore, given the much slower repair in the V3 cells compared to the V3YAC cells, if this system is capable of rejoining DSBs then it must be considerably less efficient than DNA-PKcs mediated repair.

As a consequence of the different mutant and knockout cell lines and assay end-points used by the various groups, current data therefore do not distinguish between the different DSB repair pathways as a mechanism for the slow repair observed in DNA-PK deficient cell lines. It is possible that both pathways may operate under certain conditions and that they are not necessarily mutually exclusive. Indeed, the Mre11/RAD50/NBS1 complexes have roles in both NHEJ and HR. Furthermore, evidence is accumulating regarding interactions between DNA-PKcs and proteins that influence HR such as ATM which is a potential target of DNA-PKcs phosphorylation.

It was intriguing that the V3 cells were able to repair DNA DSBs with the same kinetics as the parental V3YAC cells for the first 30 minutes, which accounted for as much as 60 % of the total breaks rejoined. In contrast, the PARP-1 $-/-$ cells only repaired 32 % of breaks within this time and with slower kinetics. In addition, the xrs-6 cells were unable to carry out any significant repair within this time. It is possible that the early and rapid repair in the V3 cells, which is absent in both the PARP-1 $-/-$ and xrs-6 cells, represents the repair of a specific subset of lesions of different complexity that do not require DNA-PKcs (although it obviously requires Ku and PARP-1). Indeed, as discussed in section 5.1, DSBs induced by IR can be exceptionally complex due to the proximity of base lesions and single strand breaks and as many as 10 lesions may be present in a complex DSB.

A number of groups have investigated the consequences of a lack of Ku80 on DNA DSB repair (Chen *et al.*, 1996; Wachsberger *et al.*, 1999; Liang *et al.*, 1996a). A previous study by Kemp *et al.*, 1984, demonstrated that whilst xrs-6 cells were defective in DSB rejoining following 90 Gy IR, as measured by neutral elution, they were still able to repair ~40 % of their breaks within 2 hrs. The data presented in this Chapter are more consistent with the results this group obtained with xrs-5 cells (independently isolated but also mutated in Ku80) which were only able to repair approximately 10 % of breaks even when time was extended to 4 h. The data presented in this Chapter, in agreement with others, demonstrated that transfection of Ku80 cDNA into the Ku80 defective cell line restores the DSB repair deficiency in xrs-6 cells (Ross *et al.*, 1995). Despite evidence that DNA-PKcs might function independently of Ku in DNA repair, as suggested by biochemical studies showing that DNA-PKcs has weak DNA binding kinase activity (Yaneva *et al.*, 1997) and suggestions that it plays a structural role as well as a signal transduction role by tethering the DNA ends and providing scaffolding for assembly of repair complexes (Jackson and Jeggo, 1995; Jeggo *et al.*, 1995; Roth *et al.*, 1995), the data presented in this Chapter indicate that Ku is required for efficient repair at least in the first 60 minutes, whereas both Ku and DNA-PKcs are required at later time points.

As described in section 5.1, the repair of clustered lesions formed following exposure to IR can lead to the formation of DSBs through the repair of closely associated lesions. For example, a DSB may be formed through the incision of an AP

site by AP endonuclease when the AP site is opposite or near to another AP site or SSB or when lesions within a clustered damage site are repaired simultaneously as opposed to sequentially. Hashimoto *et al.*, 2001, demonstrated that Ku could inhibit the nicking activity of the human BER enzyme, endonuclease III, thereby preventing the formation of a free DSB. It should therefore be considered that a fraction of the unrejoined DSBs in the xrs-6 cells may reflect a component of clustered lesions being converted to DSBs.

The relative repair capacities of the cell lines, correlates with the radiosensitivity data presented in Chapter 3, section 3.7, which showed that the DNA-PKcs deficient V3 cells were less sensitive to IR than the Ku80 deficient xrs-6 cells (Table 3.3). In support of these findings, Ku-deficient mice have a more severe phenotype compared to DNA-PKcs $-/-$ mice in terms of growth characteristics. In addition, Ku-deficient primary MEFs exhibit severe growth retardation and early senescence whilst DNA-PKcs $-/-$ primary MEFs are normal in these aspects. The more severe DNA repair deficiency of the Ku-inactivating mutation in the xrs-6 cells compared to the ability of the V3 cells to carry out the same amount of repair as parental V3YAC cells within the first 30 minutes could be due to DNA-PKcs independent functions for Ku in DSB repair or reflect residual DNA-PKcs protein in the V3 cells that is sufficient to carry out some (Ku-dependent) activities. Although Ku may be a component of HR, this is unlikely as the evidence reviewed above indicates that Ku competes for HR by preventing access of nucleases and other factors involved in this repair mechanism.

The exact nature of the mutation in the V3 cells has not been determined (see section 3.1.1) and these cells have not been shown definitively to represent a null-function mutation. Consequently, there is the potential existence of mutant DNA-PKcs protein which may retain sufficient activity to mediate some, albeit limited, DSB repair. However, it is unlikely that the initial fast repair in the V3 cells is due to residual DNA-PKcs, as Western blot analyses demonstrated an absence of detectable DNA-PKcs protein (Figure 3.4) and there was undetectable DNA-PK activity in V3 cell extracts (not significantly different from the minus oligonucleotide control) compared to 750 pmol ATP/ μ g protein in control V3YAC cells (Figure 3.6).

Furthermore, 10 μ M NU7026 had no additional inhibitory effect on the repair of DSBs or potentiation of cytotoxicity (sections 5.3.3 and 4.3.1.2) in the V3 cells.

In other systems, however, there may be residual DNA-PKcs activity. For example, SCID cells have been shown to have detectable DNA-PKcs transcripts and as much as 15 % residual DNA-PKcs protein (Blunt *et al.*, 1996). In addition, the presence of DNA-PKcs transcripts in M059J cells, albeit at 20-fold reduced levels compared to M059K cells, have been demonstrated (Galloway *et al.*, 1999; Lees-Miller *et al.*, 1995). In terms of the repair defect of these cell lines, mutant protein could possibly exert a dominant negative effect rather than simply reflecting a loss of DNA-PK activity (Jeggo, 1997).

The data presented here, and studies published elsewhere, point to a role for Ku in mediating the ligation of DNA ends independently of DNA-PKcs. Indeed, the molar ratio of Ku to DNA-PKcs in mammalian cells ranges from 3:1 to 15:1 (Chan *et al.*, 1996; Yumoto *et al.*, 1998) and therefore Ku is in excess if its function is restricted only to being the regulatory protein of DNA-PK.

In support of the suggestion that the Ku heterodimer may have roles in DNA repair that are independent of DNA-PKcs, Gao *et al.*, 1998, utilised a gene targeting study to generate embryonic stem (ES) cells with abrogated DNA-PKcs expression. Briefly, the authors inserted a neomycin resistance gene into exon 6 in the opposite transcriptional orientation from that of the DNA-PKcs gene. This prevented translation of a functional DNA-PKcs protein and thus represents a null mutation. The cells were further characterised for RNA protein and DNA-PK activity. Analysis of these cells demonstrated a phenotype very similar to the SCID phenotype. Surprisingly, these cells, unlike characterised Ku70-deficient ES cells, had the same radiosensitivity as their wild type or heterozygous mutant counterparts. The authors therefore concluded that these findings suggest that Ku may have DNA-PKcs-independent functions in DNA repair.

5.4.2 DNA-PK independent roles for Ku in DNA DSB repair

The role of Ku in DNA DSB repair is likely to be in the very early stages of DSB repair, most likely the initial recognition of the DNA end. Ku may act solely as a sensor of DNA damage by binding and signalling to other repair proteins such as DNA-PKcs or activating other repair pathways. Alternatively, one hypothesis to explain the hypersensitivity to DNA damaging agents and the inability to join DNA ends is that the absence of functional Ku proteins results in a lack of end protection, causing the broken DNA molecules to be susceptible to exonucleolytic degradation. This suggests that Ku may have a more direct role in DSB repair involving the protection of DNA ends from degradation and competing with the HR apparatus thereby enhancing the accuracy of NHEJ.

This hypothesis has been studied previously and with different outcomes. Many reports found that DNA ends are more degraded in Ku-defective mutants than wild type cells (Taccioli *et al.*, 1994; Roth *et al.*, 1995; Rathmell and Chu, 1994; Liang *et al.*, 1996; Getts and Stamato, 1994). However, Kabotyanski *et al.*, 1998 demonstrated that the degradation of linear plasmids was not significantly decreased in Ku80 deficient cells nor was there an increase in the frequency of deletions. These authors suggested an alternative role for Ku in aligning or stabilising intermediates in end joining rather than in protecting ends from degradation.

As originally proposed by Thode *et al.*, 1990, DNA ends must be held in juxtaposition by an 'alignment protein'. The Ku heterodimer has been put forward as a candidate for an alignment factor during NHEJ. The recent crystal structure of the Ku heterodimer (Walker *et al.*, 2001) has shown that Ku is well designed to structurally support broken DNA ends and bring the DNA helix into phase across the junction during end processing and ligation. Current models suggest that Ku can associate to form a bridging complex thus allowing DNA ends to be held in appropriate configuration for end processing and subsequent ligation (Yaneva *et al.*, 1997; Cary *et al.*, 1997; Pang *et al.*, 1997). Furthermore, Ku has the ability to translocate along DNA molecules in an ATP-independent manner (DeVries *et al.*, 1989). This 'sliding clamp' behaviour of Ku implies the existence of a channel in Ku that may well be suited to hold the ends of two molecules aligned in preparation

for ligation. The ability of Ku to self associate has been demonstrated both biochemically (Ramsden and Gelert, 1998) and by atomic force microscopy (Cary *et al.*, 1997; Pang *et al.*, 1997; Feldmann *et al.*, 2000).

To this end, Cary *et al.*, 1997, used a combination of electron and atomic force spectroscopy to visualise Ku in association with DNA. The authors showed that 2 molecules of DNA bound Ku can associate thus giving rise to the formation of DNA loops and therefore resulting in a physical tethering of the DNA ends. Consistent with this, direct evidence has been presented by Feldmann *et al.*, 2000, who used a plasmid rejoining assay to demonstrate that the capability to align DNA termini is lost in xrs-6 cells compared to parental CHOK1 cells, decreasing the stability of DNA ends and thereby compromising both the efficiency and accuracy of NHEJ.

In support of a direct role for Ku in mediating end ligation independent of DNA-PKcs, Ramsden and Gellert, 1998, demonstrated that purified eukaryotic ligases, I, III and IV are stimulated by Ku. Ligation of a 60 bp blunt ended substrate by recombinant DNA ligase 1 was stimulated > 100-fold by co-incubation with Ku in a concentration-dependent manner. DNA ligase IV, purified from HeLa cells, was also strongly stimulated by Ku. Notably, Ku stimulation of ligation was most efficient when the juxtaposed ends cohered poorly. These results are consistent with a role for Ku in bridging and stabilising two ends, yet maintaining accessibility of the ends to the ligase thus increasing the efficiency of NHEJ.

Several of the components of the NHEJ pathway which include ligase IV and XRCC4 may also be involved in DNA-PKcs independent rejoining of DNA DSBs and could fulfil such an alignment function (Wilson *et al.*, 1997; Baumann and West, 1998; Boulton and Jackson, 1996; Seide *et al.*, 1996). Furthermore, it is conceivable that Ku and XRCC4 may act together to allow assembly of DNA-protein complexes at sites of DNA damage to enable the rejoining of broken DNA ends. In fact Ku has recently been shown to recruit the XRCC4-ligase IV complex to DNA ends (Kabotyanski *et al.*, 1998). However, it has been shown more recently by Calsou *et al.*, 2003, that both Ku and DNA-PKcs are required for the recruitment of XRCC4 and ligase IV.

Other possible interrelated functions for Ku are helicase and ATPase activities. Ku translocation into the DNA away from the ends may also facilitate end joining by unwinding DNA ends through its intrinsic helicase activity thereby allowing microhomology alignments to occur (Tuteja *et al.*, 1994).

In summary, the roles of DNA-PKcs and Ku in end joining are currently unclear. The rapid, early repair in the DNA-PKcs deficient V3 cell line suggests that either multiple pathways of DSB rejoining are active in this early phase or that DNA-PKcs has a purely regulatory or damage sensory role. In contrast, Ku80 deficient cells were drastically defective in DSB repair suggesting critical roles for Ku which according to the published literature, may include the protection of ends from degradation, orientation of ends for ligation and the recruitment of DNA-PKcs and/or additional factors necessary for successful end-joining.

5.4.3 Repair of DSBs in cells deficient in PARP-1

It has been generally assumed for many years that PARP-1 has very little role, if any, in the repair of DSBs. In general, the literature is lacking an analysis of PARP-1 function in DSB repair although molecular evidence indicates that PARP-1 interacts with DSBs as well as SSBs. Using plasmid DNA digested with a variety of restriction enzymes and other endonucleases to produce defined fragments of DNA of known structure, Benjamin and Gill, 1980, measured the activation of PARP-1 by measuring poly(ADP-ribose) (PAR) synthesis. Blunt-ended DNA DSBs were 10 times more effective at activating PARP-1 and supporting PAR synthesis than SSBs. This was subsequently confirmed by (Weinfeld *et al.*, 1997) who showed using highly purified enzyme that DNA DSBs were more potent activators of PARP-1 than SSBs. Moreover, two early reports have shown that the rejoining of DSBs induced by the electroporation of restriction enzymes into cells was delayed by the classical PARP inhibitor 3-aminobenzamide (3-AB) (Chung *et al.*, 1991; Bryant and Johnston, 1993). These observations, implying an important role for PARP-1 in the processing of DSBs, have largely been overlooked among the burgeoning evidence for a role for PARP-1 in BER.

To the best of my knowledge this is the first description of a direct analysis of DNA DSB repair following exposure to IR in a PARP-1 knock out cell line. The data presented in this Chapter are consistent with PARP-1 activity being required for recovery from IR-induced DNA DSBs especially at early time points (< 60 minutes). However, these data do not prove a direct role for PARP-1 in NHEJ. Whilst PARP-1 could have a direct role in the DNA DSB repair process, in which DSBs arise due to a retardation in DSB rejoining, it may have a more indirect modulatory role. To this end, PARP-1 could serve as a backup function for the deficiency in DNA-PKcs described above, thus allowing ligation of breaks at early time points following initiation of damage in the absence of DNA-PKcs. Alternatively, a function for PARP-1 as an anti-recombinogenic factor has been proposed (Lindahl *et al.*, 1995) and therefore PARP-1 may function indirectly in end-joining by protecting ends from illegitimate homologous recombination thus promoting DNA-PK mediated NHEJ. This hypothesis is consistent with evidence showing that an additional loss of PARP-1 function in DNA-PK deficient SCID mice can rescue the block in V(D)J recombination that typifies the SCID phenotype (Morrison *et al.*, 1997).

The PARP-1^{-/-} cells were particularly defective in DSB repair at early time points and therefore this suggests that PARP-1 may be important for alterations in the chromatin structure that occur initially in strand break rejoining to allow access of multifactorial DNA-repair complexes. This could hold true for both BER and NHEJ. Alternatively, the increased DSBs measured in the absence of PARP-1 may arise from a subset of complex IR-induced lesions being converted to DSBs. As described above (section 5.1), one of the hallmarks of IR is the formation of multiply damaged or clustered sites (see section 5.1). These complex lesions include abasic (AP) sites, base lesions or SSBs that may be repaired by the BER pathway in which PARP-1 is intimately involved. Unrepaired or poor efficiency of repair of clustered lesions as a result of PARP-1 deficiency could result in the production of DSBs although in the studies described in this thesis there was no greater accumulation of DNA DSBs in PARP-1^{-/-} cells compared to those irradiated and eluted immediately. Studies by Lomax *et al.*, (Unpublished results, personal communication), have demonstrated that simple clustered lesions are repaired sequentially thus limiting the formation of DSBs to <2 %. For example, a SSB or AP site inhibits the excision of a base lesion on the opposite strand. However, in the experiments described in this

thesis, the possibility that in the absence of PARP-1, the kinetics of DNA DSB formation and repair could be altered, such that the slower net decrease in DSBs could be a function of an increase in DSB formation instead of or as well as reduced repair cannot be excluded.

In support of the suggestion that the effect of a loss of PARP-1 is actually due to a subset of lesions being converted to DSBs, a recent study by Noel *et al.*, 2003, investigated the rejoining kinetics of normal and PARP-1 knockout mouse 3T3 fibroblasts to neocarzinostatin (NCS) induced DSBs by PFGE. In comparison to IR, this drug does not induce a large spectrum of lesions but creates only frank DSBs. In contrast to the data presented here with IR, no significant difference in the rejoining kinetics of both cell lines was found and therefore the authors concluded that PARP-1 is unlikely to play a role in the repair of DSBs at least in the absence of other types of DNA damage.

A number of publications demonstrate an interaction and co-operation between PARP-1 and DNA-PK either functionally or physically (Ruscetti *et al.*, 1998; Ariumi *et al.*, 1999; Morrison *et al.*, 1997; Galande and Kohwi-Shughematsu., 1999). Significantly these enzymes have been shown to automodify one another in cell free extracts (Ariumi *et al.*, 1999; Ruscetti *et al.*, 1998). Specifically, Ruscetti *et al.*, 1998, showed that PARP-1 can poly(ADP-ribosylate) DNA-PK which leads to an activation of the modified protein. Whilst it is very unusual to find an example of poly(ADP-ribosyl)ation activating a DNA-binding enzyme it is relevant in this context as it could be postulated that a lack of PARP-1 protein would lead to reduced DSB rejoining as a reflection of a down-regulation of DNA-PK activity. However, in the studies described in this thesis, DNA-PK activity was shown to be the same in both the PARP-1+/+ and PARP-1 -/- cells (Figure 3.6). Furthermore, it is noteworthy that both AG14361 and NU7026 were able to further inhibit repair in the DNA-PK deficient and PARP-1 deficient cell lines, respectively, suggesting that although the two enzymes might inhibit DNA DSB repair indirectly by modulating the activity of the other enzyme, they also possess independent functions in DNA DSB repair.

5.4.4 Interactive effects of Ku and PARP-1 in DNA DSB repair

Notably, both PARP-1^{-/-} and Ku80 deficient cells were defective in DSB repair at early repair times in contrast to the normal early-phase repair in the V3 cells. Although possible DNA-PKcs independent roles for Ku in DSB repair have been discussed in section 5.4.2, this repair could be a function for PARP-1 or indeed a combined role for PARP-1 and Ku. An intimate association between PARP-1 and Ku has been demonstrated in co-immunoprecipitation studies (Ruscetti *et al.*, 1998;, Morrison *et al.*, 1997, Galande and Kohi-Shigamatsu., 1999). Ku70 possesses a high affinity motif for PAR binding (Pleshke *et al.*, 2000) and as described in section 3.3.3, Figure 3.5, Ku80-deficient xrs-6 cells had as much as 25 % less PARP activity than the isogenic xrs-6 HamKu80 cells. Moreover, PARP-1^{-/-} Ku80^{-/-} cells are embryonic lethal (Henrie *et al.*, 2002). Taken together, these data suggest that Ku and PARP-1 may have overlapping roles in the repair of DNA damage.

5.4.5 The effect of AG14361 and NU7026 on DNA DSB repair

It could be argued that the DSB rejoining capacity measured in the DNA-PKcs deficient V3 cells results from compensatory processes activated as a result of this defect, and for which the cells may have been selected during mutant isolation. Indeed, these cells may have adapted to this pathological state by altering their normal DNA damage responses and this could be true for any of the cell lines tested. Consequently, DSB rejoining in the presence of inhibitors of either DNA-PK or PARP-1 was evaluated.

It should be borne in mind, however, that a mutation in an enzyme and its inhibition are two distinct phenomena and therefore these two approaches could give very different outcomes. Nevertheless, chemical inactivation of either PARP-1 or DNA-PK caused changes in DSB rejoining which were very similar to those observed after genetic inactivation of the proteins i.e. drastic reduction in the level of strand breaks rejoined.

When the inhibitors were used at the concentrations required to potentiate cytotoxicity, inhibition of PARP-1 or DNA-PK did not completely block repair but slowed it down considerably (Figure 5.8). These results are consistent with the findings of Boulton *et al.*, 1999, who showed that both the PARP-1 inhibitor, NU1025, and the DNA-PK inhibitor, wortmannin increased net DSB levels induced by 100 Gy IR over a 3 h time period, as assessed by neutral elution. When used in combination, approximately additive effects were seen throughout the 3 h time period. Moreover, Rudat *et al.*, 2001 assessed the influence of overexpressing the DNA binding domain (DBD) of PARP-1 (under the control of the dexamethosone promoter) on the rejoining kinetics of IR-induced DNA DSBs using pulse field gel electrophoresis. This is an analogous situation to the use of PARP-1 inhibitors where it is the poly(ADP)ribosylation step that is inhibited rather than the ability to bind DNA strand breaks. Following treatment with 60 Gy IR, the fast component of DNA DSB rejoining was reduced from 60 % to 20 % when the PARP-1 DBD was overexpressed compared to control cells. The authors followed repair over 25 hrs and demonstrated that inhibition of PARP-1 activity induced a shift from rapid to slow DSB rejoining but less than 1 % of breaks remained following 25 h post-IR.

Inhibition of DNA-PK had a more pronounced effect on DSB rejoining than lack of enzyme at all time points measured (Table 3.2). As can be seen in Figure 5.8, DSB rejoining in the V3 cells approached completion by 24 h but only 70 % of the breaks, had rejoined at 24 h in the V3YAC cells treated with NU7026. In contrast, repair in the PARP-1 $-/-$ cells was not significantly different from repair in the PARP-1 $+/+$ cells treated with AG14361 at 60 min (Figure 5.6 C and D). It was particularly striking that combined inhibition of both enzymes resulted in a profound inhibition of DNA DSB repair. Exposure to both inhibitors for 60 min resulted in a complete inhibition of repair in the V3YAC cells and 80 % inhibition in the PARP-1 $+/+$ cells. Even at 4 h and 24 h, the majority of breaks remained unrejoined (99 % and 90 %, respectively) in the V3YAC cells exposed to both inhibitors. In contrast, repair was only inhibited by 50 % in the V3 and 45 % in the PARP-1 $-/-$ cells by co-incubation with both inhibitors and this was not significantly different from the inhibition observed with the appropriate inhibitor alone (i.e. V3+AG14361 and PARP-1 $-/-$ +NU7026). This suggests that the cells may have upregulated

compensatory repair mechanisms and/or that inhibition of repair enzymes can have a greater effect than the loss of the enzyme.

The dissociation kinetics of PARP-1 and DNA-PK from DNA ends will be changed dramatically by the presence of potent inhibitors of these enzymes. PARP-1 and DNA-PK undergo automodification when activated by DNA strand breaks and this reaction is essential to release PARP-1 and DNA-PKcs from DNA ends (Satoh and Lindahl, 1992; Merkle *et al.*, 2002). Wortmannin has been demonstrated to block DNA-PK at DNA ends and prevent their processing by DNA polymerisation, degradation or ligation (Calsou *et al.*, 1999). Satoh and Lindahl, 1992 also demonstrated, in a cell free-assay, that inactive PARP-1 sequestered DNA ends and prevented repair. Similarly AG14361 and NU7026 will block PARP-1 and DNA-PK, respectively, at DNA ends. Thus AG14361 could modulate DSB repair indirectly by binding and preventing access to DNA-PK rather than through an inhibition of PARP-1. However, the clear effect of an absence of PARP-1 on the rejoining kinetics of DSBs implicates PARP-1 directly in DSB repair. As well as interfering with the access of the other enzyme, these protein bound DNA termini could hinder assembly of the enzyme complexes required for successful execution of all modes of NHEJ and HR. This could easily explain the more pronounced effect of inhibited enzyme versus a lack of enzyme.

In support of this hypothesis, Sak *et al.*, 2002, compared the effect of an antisense oligodeoxynucleotide (As-ODN) specific for the translational start of DNA-PKcs mRNA with wortmannin on the kinetics of DSB repair over a 4 h period following exposure to 30 Gy. In both cases the fraction of DSBs rejoined was reduced 60 min post-IR compared to untreated control cells and the AS-ODN and wortmannin were equally as effective. However, whilst the cells treated with the SIR went on to repair the majority of their breaks within the following 4 h, wortmannin continued to significantly retard rejoining >4 h following IR. The authors suggest that the inhibitor renders the site inaccessible for repair by other pathways whilst, by contrast, the SIR physically removes DNA-PKcs and leaves DNA ends accessible to DNA-PK independent (slow repair) pathways.

Interestingly, in contrast to the inhibition of DNA-PK, at early time points (10 and 30 min), PARP-1 inhibition was not as deleterious as a lack of enzyme suggesting that PARP protein-protein interactions are important. Proximal SSBs on complementary strands could be stabilised and repaired by a two step process when PARP-1 is functioning. As described above, in the absence of PARP-1, this process could fail and therefore lead to the formation of DSBs. Inhibited PARP-1 on the otherhand would still bind these strands but would remain bound and therefore in effect could also stabilise DNA ends thus preventing their conversion to DSBs at early time points following IR. However, inhibited PARP-1 compared to lack of PARP-1 would prevent the access of other repair factors (as described above) leading to increased DSB formation at later time points. For example, DNA polymerases could stall on the DNA in the vicinity of an unrepaired nick occupied by PARP-1 (de Murcia *et al.*, 1994b).

The similar effects on DNA DSB repair seen with either lack of or inhibition of PARP-1 and DNA-PK favours the idea that the enzyme activities are required for processing of DNA ends, and that the effect of the inhibitor(s) are not solely due to a block of DNA breaks. These enzymes therefore probably regulate some aspects of repair such as the recruitment of repair factors, rather than functioning as mechanistic components of repair.

5.4.5 Neutral elution as a technique to measure DNA DSB breaks

Various experimental approaches have been developed for the measurement of DSBs in DNA. Neutral elution provides an accurate and quantitative evaluation of DNA DSB levels, however, the interpretation of elution data is controversial. It has been suggested by Flentje *et al.*, 1993, that neutral elution measurement of DSBs may be influenced by the presence of SSBs. Importantly, these authors suggested that the initial fast component of the biphasic repair kinetics measured by neutral elution may be attributable to the interference of SSBs rather than the measurement of DSBs. This is particularly relevant to the data presented in this Chapter, as if this were to be the case, the ability of V3 cells to initially repair the breaks induced by IR and the apparent inability of PARP-1 *-/-* cells to repair these breaks could actually be a reflection of an effect on the repair of SSBs and not DSBs in a PARP-1 proficient

and deficient background. This consideration has important implications for the interpretation of the data presented in this Chapter.

Clear evidence to the contrary, however, has been presented by Prise *et al.*, 1989, who showed that DNA from cells treated with hydrogen peroxide (which induces mainly SSBs) did not elute under neutral conditions at a significantly higher rate than DNA from untreated cells. Moreover, Johnston *et al.*, 1991, treated CHO cells with camptothecin (a topoisomerase I inhibitor) for 60 minutes and eluted the DNA under alkaline or neutral conditions. Under alkaline conditions, extensive elution was recorded. In contrast, very little elution occurred under neutral conditions. These results verify those of Prise *et al.*, (1989), demonstrating that the presence of SSBs in the DNA does not affect the rate nor the extent of elution of DNA containing DSBs under neutral conditions.

Neutral elution does not distinguish between DNA DSBs resulting from impaired rejoining of DNA ends and those induced during cell death. It could therefore be argued that rather than reflecting reduced rejoining, the high percentage of remaining DSBs in the presence of inhibitor(s) 24 h post-IR, may be due to the induction of apoptosis where endonuclease activity would give rise to DNA fragmentation. However, in the parental V3YAC cells, exposed to the same supralethal dose of IR, where < 1% of cells would be predicted to survive, all DSBs had nevertheless rejoined. Furthermore the data presented by Diabase *et al.*, 2000, (discussed in section 5.4.1.) who used PFGE, a technique that distinguishes between degradation and DNA repair, showed no evidence for apoptosis, expressed as DNA degradation in their experiments even at 30 h post-IR.

5.4.6 The effects of AG14361 and NU7026 on SSB repair

SSB repair in the absence of inhibitors was equally effective in both the V3YAC and V3 cell lines, with 85 % repair occurring within 60 min. AG14361 inhibited repair in both cell lines whilst NU7026 had no significant effect.

The initial observation that PARP-1 inhibition reduced SSB rejoining was demonstrated by Durkacz *et al.*, 1980, who showed that the PARP-1 inhibitor, 3AB, reduced the fraction of SSBs rejoined in L1210 cells following treatment with either DMS or MNNG. Other authors, using the comet assay, have shown that the rejoining of radiation-induced SSBs was dramatically reduced in PARP-1 knockout cells and significantly delayed by PARP-1 inhibitors in L1210 cells (Bowman *et al* 2001) and PARP-1 +/- 3T3 cells (Atorino *et al.*, 2001; Trucco *et al.*, 1998).

In contrast, Vodenicharov *et al.*, 2000, demonstrated that BER was efficient in cells lacking PARP-1 by measuring the repair of plasmid DNA following IR and proposed that the hypersensitivity of PARP-1 knock out cells to irradiation is not due to a defect in repair itself but rather results from greatly reduced poly(ADP-ribose) formation during base excision repair. Furthermore, depletion of PARP-1 by antisense RNA has been shown to result in a delay in strand break rejoining in HeLa cells at early time periods of recovery following MMS induced DNA SSBs, as measured by alkaline elution. However, consistent with the findings of Vodenicharov *et al.*, (2000), at later time points (90 minutes) the extent of repair in these cells did not differ from that of control cells (Ding *et al* 1992). It is therefore not possible to attribute the potentiating effects of AG14361 to a single repair pathway

A study by Weinfield *et al.*, 1997, showed that whilst PARP-1 could be activated by both DSBs and SSBs, DNA-PK could only be activated by DSBs, and it was not activated by nicks or SSBs and did not participate in BER. The ability of the DNA-PK deficient V3 cells to repair SSBs to the same extent as the parental V3YAC cells coupled with the lack of a significant effect of NU7026 on SSB repair was therefore predictable. The recent finding of a SSB binding site in DNA-PK (Hammersten *et al.*, 2000) is relevant, however, as DNA-PK could bind SSBs but only play a role in their recognition and/or signalling (to PARP-1 for example). If

this were the case, however, binding of DNA-PK in the presence of NU7026 would presumably block access to PARP-1 resulting in an apparent increase in SSBs. As this was not the case, either DNA-PK has no significant role in SSB repair or PARP-1 binds a SSB before DNA-PK.

5.4.7 Consequences of DSB repair for survival

The results described in this Chapter and the previous Chapter, demonstrate a correlation between the ability to recover from PLD in non-cycling cells and DSB repair capacity, i.e. those cells which were defective in PLDR were also defective in DNA DSB repair. Strikingly, there was no evidence of any PLDR within 24 h in the *xrs-6* cells which were incapable of carrying out any DNA DSB repair in 60 minutes. It has been shown previously that the increase in survival of yeast cells held after irradiation under non-growth conditions is accompanied by the disappearance of DSBs from DNA (Frankenberg-Schwager *et al.*, 1980). The level of damage remaining post-IR in cells may be critical for survival and therefore recovery processes manifested during the post-IR period may be a determinant of radiocurability. Thus, an enhancement in recovery seen following delayed plating of growth arrested cells could reflect repair under non-growth conditions. Conversely, the reduced survival seen in those cells forced to replicate immediately following IR could represent 'fixation' of DNA damage. These results are consistent with various studies which have attempted to elucidate the molecular mechanism underlying PLD recovery and concluded that it is a reflection of enhanced fidelity of repair under non growth conditions.

A further study by Frankenberg Schwager *et al.*, in 1987, studied DSB repair under growth and non-growth conditions in the *rad54* yeast mutant which has a temperature sensitive conditional mutation for DSB repair. The authors demonstrated that at the temperature permissive for DSB rejoining, recovery from PLD occurred after prolonged holding under non-growth conditions. In contrast, at the temperature restrictive for DSB rejoining, the extent of PLDR was negligible. They concluded that PLDR is due to the difference of DNA DSB repair under immediate and delayed plating conditions.

The almost complete and long term abrogation of DNA DSB repair observed with the combined use of inhibitors following 24 h post-IR incubation would easily explain their potent radiosensitising effects in both proliferating and growth arrested cells. Although the DNA-PK deficient V3 cells repaired to the same extent as the parental V3YAC cells following 24 h recovery, they were approximately 4-fold more sensitive to IR. Slower kinetics of repair would lead to longer-term persistence of DSBs that could cause cytotoxicity.

5.5 Summary

It is well established that PARP-1 mediates BER by binding to nicks but its higher affinity for DSBs, although first described by Benjamin and Gill in 1980 and more recently by D'silva *et al.*, 1999, has lacked a functional interpretation. The data presented in this Chapter, implicates PARP-1 in the processing and repair of DNA DSBs and provides a rationale for its affinity for DSBs. The data presented in this Chapter indicate that DNA-PK is not involved in SSB repair, and that whilst it is involved in DNA DSB repair, DNA-PKcs is not absolutely necessary. However, DNA-PKcs does increase the speed of the repair process very significantly.

In summary, the data presented in this Chapter have shown that;

- A lack of or inhibition of either DNA-PK or PARP-1 results in a deficiency in DSB rejoining
- DNA-PKcs deficiency does not have a profound effect on the proportion of DNA DSBs repaired but decreases the kinetics with which it occurs
- A secondary (slow) DSB repair activity is present in the absence of DNA-PKcs
- Ku may have DNA-PKcs independent functions in DNA DSB repair
- Inhibition of PARP-1 and DNA-PK retards NHEJ and BER and may block further downstream processing of DNA ends leading to an almost total inhibition of repair

CHAPTER 6

RECIPROCAL REGULATION OF PARP-1 AND DNA-PK AT DNA ENDS

6.1 Introduction

Both PARP-1 and DNA-PK have important roles to play in DNA damage recognition and repair and therefore it is reasonable to suggest that they may co-operate to minimise genomic damage caused by DNA strand interruptions.

Both enzymes are present at similar levels of approximately 1×10^6 molecules per cell, both are non inducible gene products, present constitutively within the cell and they share many of the same proposed mechanisms in DNA repair. Specifically, both PARP-1 and DNA-PK are suggested to have anti-recombinational, protection and DNA damage-inducible signalling properties (see sections 1.6.4 and 1.10.4). Moreover, they are activated by the same types of DNA conformation and they share many of the same in vitro substrates, which include p53. In addition, both enzymes are substrates for ICE-like proteases during apoptosis (Shakelford *et al.*, 1999; Kauffman *et al.*, 1992). It is therefore possible that PARP-1 and DNA-PK might interact with one another either functionally or physically and that the two enzymes may regulate one another through posttranslational modification. Furthermore, PARP-1 and DNA-PK may compete or co-operate with one another at sites of DNA damage. The properties of the two enzymes are compared in more detail in Table 6.1.

Property	DNA-PK	PARP-1
Abundance	10 ⁶ molecules per cell	10 ⁶ molecules per cell
Inducible	No	No
Structure	Trimeric : Ku70, Ku80 + catalytic subunit	Monomeric : 3 domains ; DBD, AMD and catalytic
Activated by	DNA DSBs	DNA SSBs and DSBs
Fold activation by DNA ends	10-fold (when Ku is present)	100-500-fold
Mechanism	Acts as two heterotrimers, one either side of the break	Kinetic studies suggest 2 molecules bind 1 either side of the break
DNA repair pathway	NHEJ	BER Implicated in NHEJ
Proposed mechanism(s) of repair	Phosphorylation mediates recruitment/activation/inactivation of key DNA repair machinery	Chromatin relaxation and/or PAR formation mediates recruitment/activation of repair factors.
Proposed mechanism in signalling	P53	P53
DNA damaging agents that enzyme deficient cells are hypersensitive to	IR, Bleomycin, Nitrogen mustards, topo II poisons	IR, bleomycin, monofunctional alkylating agents, Topo I poisons
Sensitisation by inhibitors to	IR, bleomycin, nitrogen mustards, topoI and II poisons,	IR, bleomycin, monofunctional alkylating agents, Topo I poisons
Phenotype of KO mice	Deficient in V(D)J recombination, DSB repair, hypersensitive to IR	Hypersensitive to MNNG and IR
Limitations of current inhibitors	Primarily PI3-K inhibitors	Lack potency, specificity and solubility
Novel Inhibitors	NU7026 IC ₅₀ DNA-PK = 0.44 µM	AG14361 Potent IC ₅₀ PARP-1= 23 nM

Table 6.1 Comparison of the properties of DNA-PK and PARP-1

Evidence that PARP-1 and DNA-PK may interact physically comes from a study by Ruscetti *et al.*, 1998, that utilised a Ku antibody to trap proteins from a HeLa cell nuclear extract. Amongst other proteins, both PARP-1 and DNA-PK were trapped and eluted from the column, thus implicating a physical association for these two enzymes. Further evidence implicating a physical association between the two enzymes comes from the demonstration that PARP-1 and DNA-PK consistently co-immunoprecipitate, suggesting that these enzymes are co-localised within the cell (Morrison *et al.*, 1997; Ruscetti *et al.*, 1998). Furthermore, it has been demonstrated that PARP-1 and the Ku autoantigen, form a complex before binding to matrix attachment sequences (Galante and Kohwi-Shigematsu, 1999).

In addition, Ruscetti *et al.*, 1998, showed that PARP-1 could be phosphorylated by DNA-PK and that PARP-1 can poly(ADP-ribosylate) DNA-PK. Given that PARP-1 is known to alter the activities of various nuclear proteins through ADP-ribosylation, it might be expected that poly(ADP-ribosylation) of DNA-PK would affect its activity. Consistent with this, the study by Ruscetti *et al.*, 1998, showed that poly(ADP-ribosylation) of DNA-PK caused activation of the enzyme.

PARP-1 activity is thought to be regulated through phosphorylation, *in vivo*, (Aoufouchi and Shall, 1997) and PARP-1 serves as a substrate for DNA-PK, *in vitro*, (Ruscetti *et al.*, 1998). These latter studies showed that phosphorylation of PARP-1 has no detectable effect on activity and therefore phosphorylation of PARP-1 may affect some other property of the enzyme. Ariumi *et al.*, 1999, also found that phosphorylation of PARP-1 by DNA-PK had little effect on PARP-1 activity. However, the authors showed that the presence of DNA-PK with PARP-1 markedly inhibited PARP-1 activity both in the presence or absence of ATP. Since phosphorylation is therefore not likely to be responsible for this inhibition, the authors suggest that an alternative mechanism may be that of competition for DNA ends between these two enzymes although they point out that a more direct mechanism may be involved.

In order to investigate whether there is a functional interaction between the two enzymes, studies by Weinfield *et al.*, 1997 and D'Silva *et al.*, 1999, have investigated the relative affinities of the two enzymes for DNA strand break types. Evidence suggests that the two enzymes have preferences for different types of breaks. For example, PARP-1 has a greater preference for blunt ends and single stranded nicks. In addition, PARP-1 is attracted to a wider range of DNA breaks and ends. Both enzymes, however, are activated by DSBs and therefore it is possible that despite competing for DNA ends, they may co-operate at the site of a break to repair DSBs. For example, PARP-1 may bind initially in order to open the chromatin structure and protect the strand ends thus enabling access to DNA-PK.

Further evidence for a functional interaction between PARP-1 and DNA-PK comes from a study by Morrison *et al.*, 1997. SCID mice, defective in DNA-PKcs are unable to carry out V(D)J recombination whilst SCID-PARP-1 knockout mice are able to carry out this process and therefore the absence of PARP-1 appears to rescue V(D)J recombination in DNA-PK defective mice. This implies that PARP-1 normally serves as an anti-recombinational factor in which it binds to DNA ends, preventing illegitimate recombination reactions from occurring. These findings serve to reinforce the notion that PARP-1 might bind DNA ends first, thereby acting as a protection factor prior to DNA-PK-mediated DSB rejoining. The authors also demonstrate that animals defective in both enzymes have a far lower survival rate than those with deficiencies in either one alone. The two enzymes may serve as 'back up' functions for one another. Indeed, PARP-1 may have a function in DNA damage-inducible signalling (see section 1.8.4.2). However, since there are no apparent cell cycle control defects in PARP-1 *-/-* cells, Lindahl *et al.*, 1995 have tentatively proposed that PARP-1 does indeed play a role in a damage inducible signalling pathway but that a backup function may exist in which DNA-PK might be involved.

The final evidence, to date, for a functional interaction between PARP-1 and DNA-PK comes from investigations which have employed inhibitors of the two enzymes alone and in combination. When used in conjunction with one another, the DNA-PK inhibitor, wortmannin and the PARP-1 inhibitor, NU1025, were shown to produce at least additive effects for inhibiting DSB break repair and potentiating IR cytotoxicity (Boulton, *et al.*, 1999). For example, wortmannin gave a dose

enhancement factor (at 10 % survival) of 4.5, whilst NU1025 gave a dose enhancement factor of 1.7. When used in combination, an enhancement factor of 7.8 was observed. These data are supported by the results presented in Chapters 4 and 5 with AG14361 and NU7026.

6.2 Aims

Given that PARP-1 and DNA-PK bind with high selectivity to DNA strand breaks they may regulate one another's activity through post-translational modification and/or they may compete with one another or co-operate for binding at sites of DNA damage. Previous Chapters have investigated the effect of inhibitors of these enzymes on survival (Chapter 4) and DNA repair (Chapter 5) following exposure to IR. However, in order to fully exploit the potential of PARP-1 and DNA-PK inhibitors for cancer therapy, a mechanistic understanding of their interactions is essential. The aim of this final results Chapter was therefore to investigate the interaction of PARP-1 and DNA-PK at DNA ends using both purified enzyme assays and a permeabilised cell assay. Enzyme activity was modulated by the use of NU7026 or AG14361, and by inclusion or exclusion of enzyme substrates. The paired cell lines, proficient or deficient in PARP-1 or DNA-PK, utilized in the previous Chapters, were also used to compare the effects of loss of enzyme as opposed to enzyme inactivation.

AG14361 and NU7026, were used at 0.4 μ M and 10 μ M, respectively, and a 30 bp double stranded blunt-ended oligonucleotide (see appendix) was used to stimulate the activity of both enzymes in all studies described in this Chapter, unless otherwise stated.

6.3 Results

6.3.1 Concentrations of enzymes and DNA ends

There are about one million molecules each of PARP-1 and DNA-PK in the cell (Lindahl *et al.*, 1995; Jackson, 1997). Therefore, approximately equimolar concentrations of PARP-1 and the DNA-PK holoenzyme were used in the experiments described here, (based on their published molecular weights of 116 and ~623 kDa, respectively), unless otherwise specified. In order to minimize direct competition of enzyme molecules for DNA oligonucleotide ends, the ratios of the numbers of DNA ends to enzyme molecules were at greater than 10:1 in all assays. Exact ratios (to the nearest whole number), which are dependent on the assay used, are given in Table 6.2.

Enzyme/Assay	DNA ends: PARP-1	DNA ends: DNA-PK
PARP-1 Assay	12:1	10.:1
DNA-PK Assay	13:1	12:1

Table 6.2 Ratio of individual enzymes to DNA ends in the purified PARP-1 and DNA-PK assays

6.3.2 Regulation of purified PARP-1 activity

The effect of AG14361, NU7026 and purified DNA-PK in the presence or absence of its substrate, ATP, on purified PARP-1 activity was investigated (Figure 6.1A) using a purified PARP-1 assay as described in section 2.1.2..

As expected, PARP-1 activity was almost completely inhibited by the inclusion of AG14361 ($99 \pm 5 \%$, $p = <0.001$), whereas NU7026 alone had no significant effect ($p = 1$). However, when DNA-PK was added to the assay, PARP-1 activity was significantly inhibited by $30 \pm 3 \%$ ($p = <0.001$). The addition of ATP as substrate for DNA-PK reversed this DNA-PK-dependent PARP-1 inhibition such that there was no significant difference in activity between PARP-1 alone and PARP-1 in the presence of DNA-PK and its substrate, ATP ($p = 0.07$). Further inclusion of NU7026 restored the PARP-1 inhibition to $43 \pm 5 \%$ ($p = <0.001$). In summary, whereas active DNA-PK did not modulate PARP-1 activity, the presence of inactive DNA-PK (due to lack of ATP substrate or inhibition by NU7026) caused significant PARP-1 inhibition.

The effects of inactive DNA-PK were investigated further by increasing the molar ratios of DNA-PK to PARP-1 (Figure 6.1B). PARP-1 activity was decreased in a concentration-dependent manner by DNA-PK in the absence of ATP, with a molar ratio of 3:1 resulting in $>90 \%$ inhibition of PARP-1 activity. However, this inhibition was completely reversed by the inclusion of ATP ($p = <0.001$). When compared to PARP-1 alone, although a 3-fold excess of DNA-PK in the presence of its substrate, ATP, did not inhibit PARP-1 activity, it did not stimulate it either thus suggesting that DNA-PK does not stimulate PARP-1 activity.

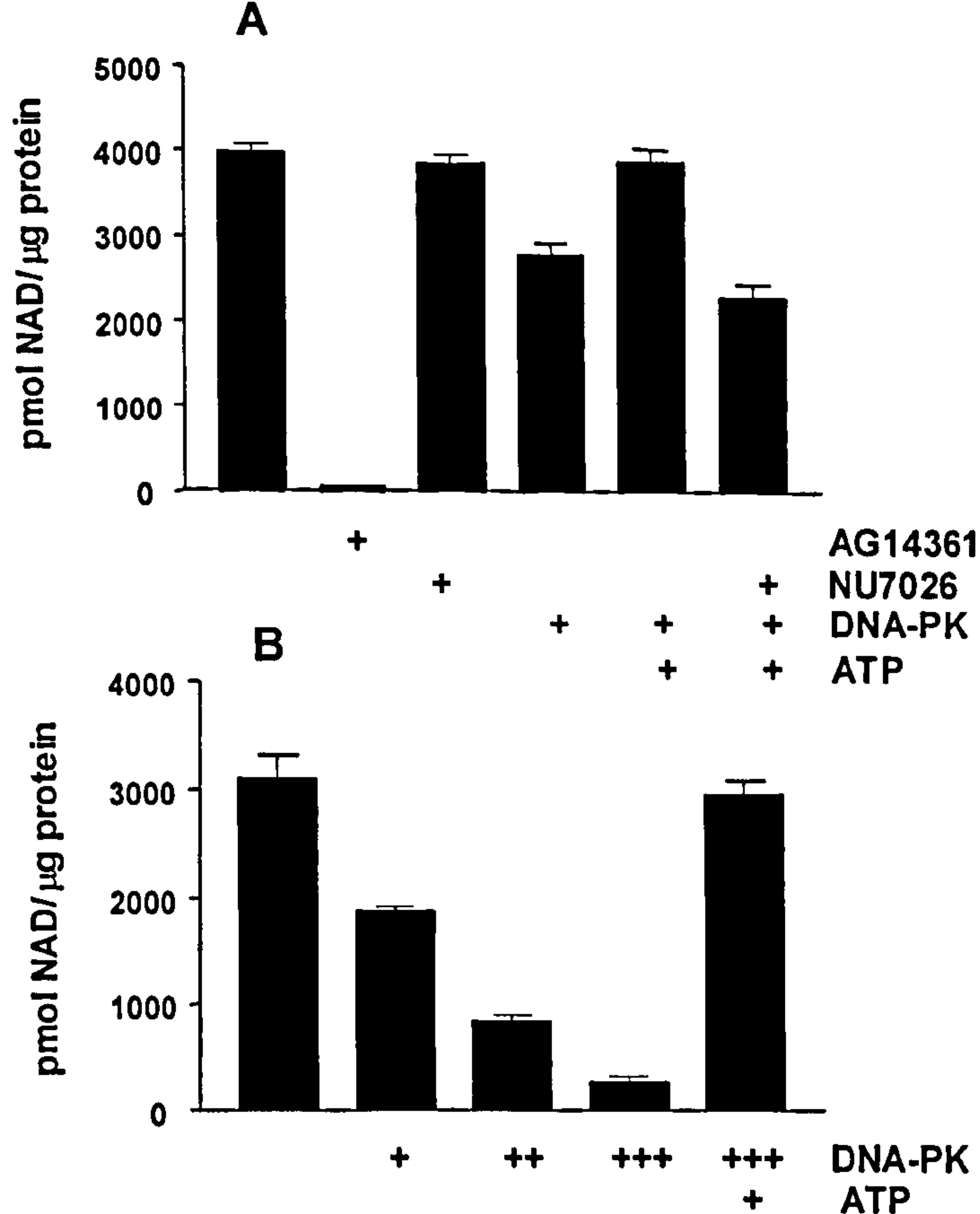


Figure 6.1 (A) The effect of AG14361, NU7026 and DNA-PK in the presence or absence of ATP on purified PARP-1 activity; (B) The effect of increasing the molar ratio of DNA-PK to PARP-1 from 1:1 to 3:1 on PARP-1 activity. Data are the mean of 3 replicate samples, each from 3 independent experiments \pm SEM.

6.3.3 Regulation of purified DNA-PK activity

The effect of AG14361, NU7026 and purified PARP-1 in the presence or absence of its substrate, NAD^+ , on purified DNA-PK activity was investigated (Figure 6.2A) using a purified DNA-PK assay as described in section 2.1.4. DNA-PK activity was completely inhibited by $99 \pm 5 \%$ ($p = <0.001$) when NU7026 was present in the reaction mixture, whereas AG14361 alone had no significant effect ($p = 0.09$). When PARP-1 was included in the assay, DNA-PK activity was inhibited by $82 \pm 3 \%$ ($p = <0.001$) and, similar to the effects of DNA-PK on PARP-1 activity, when its substrate NAD^+ was added, the inhibition of DNA-PK activity was relieved, unless AG14361 was also included, in which case DNA-PK activity was inhibited by $92 \pm 4 \%$ ($p = <0.001$). In summary, whereas active PARP-1 did not modulate DNA-PK activity, the presence of inactive PARP-1 (due to lack of NAD^+ substrate or inhibition by AG14361) caused significant DNA-PK inhibition.

As with the effect of DNA-PK on PARP-1 activity, increasing the molar ratios of PARP-1 to DNA-PK resulted in a concentration-dependent inhibition of DNA-PK, which was reversible by the inclusion of NAD^+ (Figure 6.2B). A molar ratio of 3:1 inhibited DNA-PK activity by >95 %. In addition, when compared to DNA-PK alone, although a 3-fold excess of PARP-1 in the presence of its substrate, NAD^+ , did not inhibit PARP-1 activity, nor did it stimulate it thus indicating that PARP-1 does not stimulate DNA-PK activity in this assay.

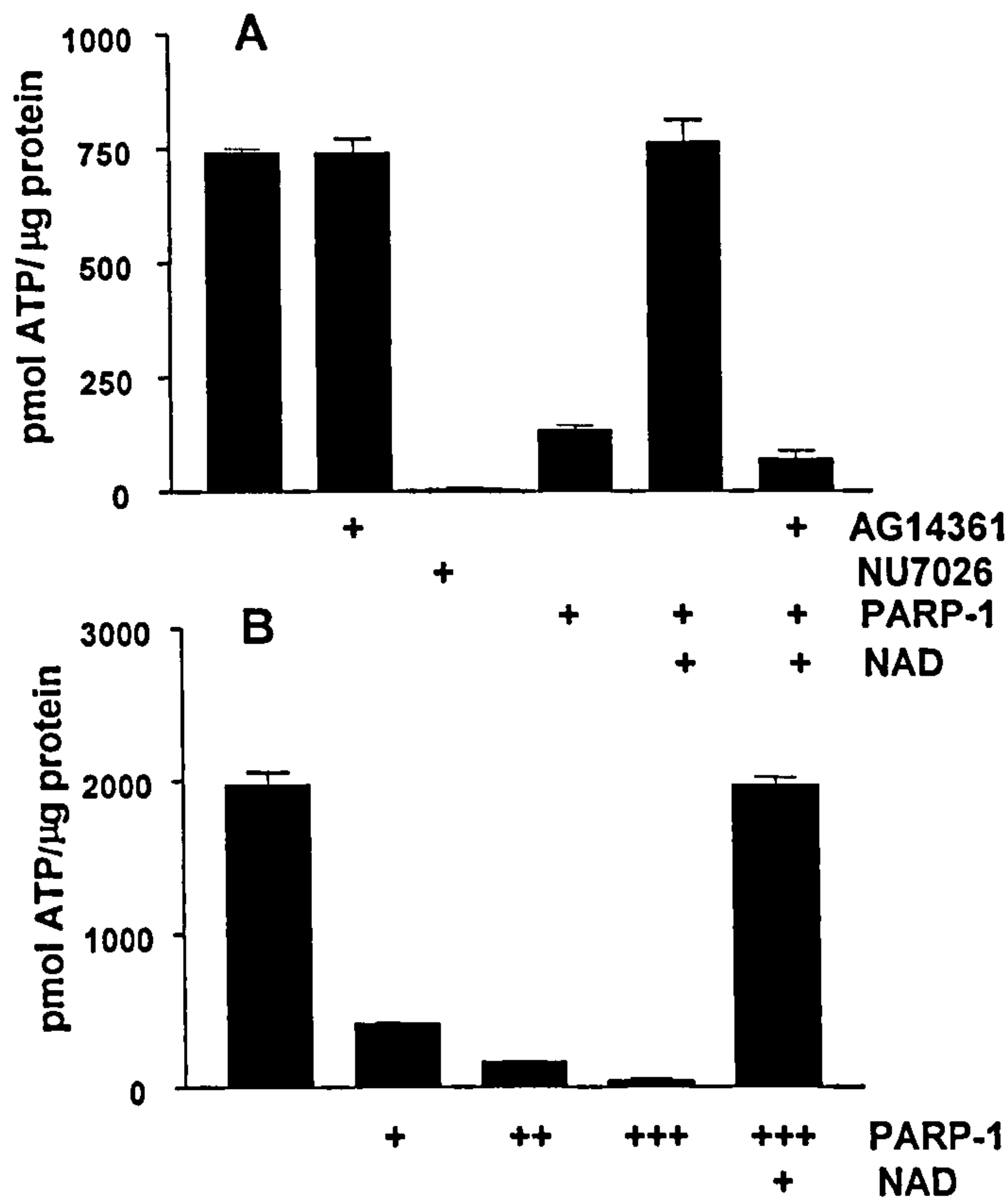


Figure 6.2 (A) The effect of AG14361, NU7026 and PARP-1 in the presence or absence of NAD^+ on purified DNA-PK activity; (B) The effect of increasing the molar ratio of PARP-1 to DNA-PK from 1:1 to 3:1 on DNA-PK activity. Data are the mean of 3 replicate samples, each from 3 independent experiments \pm SEM

6.3.4 PARP-1 and DNA-PK heteromodification

In the purified enzyme assays used here, histones were used as substrates for poly(ADP-ribosylation) by PARP-1, and a p53 peptide as a substrate for phosphorylation by DNA-PK. In order to investigate whether PARP-1 and DNA-PK could act as acceptors for covalent modification, purified PARP-1 and DNA-PK assays were carried out in the absence of histones or p53 peptide, respectively. No PARP-1 activity was detectable in the absence of histones, but the addition of DNA-PK caused a concentration-dependent increase in activity, which was stimulated further by the inclusion of ATP (Figure 6.3A). Similarly, no DNA-PK activity was detectable in the absence of the p53 peptide, but the addition of PARP-1 caused a concentration-dependent increase in activity, which was stimulated further by the inclusion of NAD^+ (Figure 6.3B).

PARP-1 activity was restored to similar levels in the presence of DNA-PK and ATP as achieved by the inclusion of histones (Figure 6.1 (a)). In contrast, the highest activity obtained for DNA-PK, in the presence of PARP-1 and NAD^+ , was ~10 fold lower compared to the inclusion of p53 peptide (Figure 6.2 (a)).

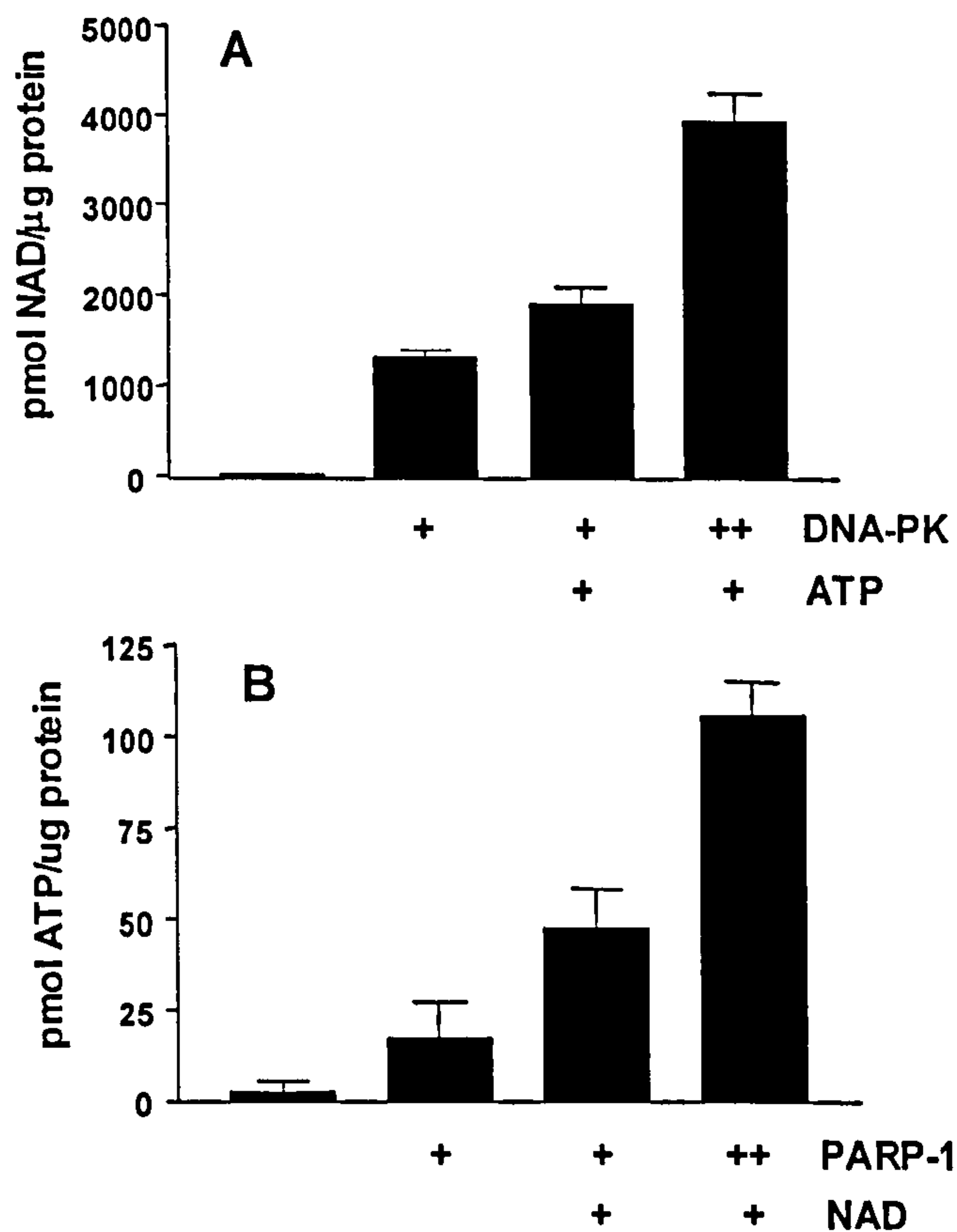


Figure 6.3 (A) Effect of increasing concentrations of DNA-PK (\pm ATP) on PARP-1 activity in the absence of histone substrate; (B) Effect of increasing concentrations of PARP-1 (\pm NAD⁺) on DNA-PK activity in the absence of p53 peptide substrate. Data are the mean of 3 replicate samples from 3 independent experiments \pm SEM.

6.3.5 Effect of the inhibitors on PARP activity in permeabilised cells

Cell lines proficient or deficient in either DNA-PK (V3YAC and V3) or PARP-1 (PARP-1^{+/+} and PARP-1^{-/-}) were rendered nucleotide-permeable by digitonin treatment and PARP-1 activity was measured using a permeabilised PARP-1 cell assay, as described in section 2.11. PARP-1 activity was slightly, but significantly different in the V3YAC and V3 cells (129 ± 7 pmol compared to 149 ± 2 pmol NAD/ 10^6 cells, respectively, $p = 0.05$), (Figure 6.4A and 6.4B). The inclusion of 50 μ M ATP in the V3YAC cells significantly stimulated PARP-1 activity to 150 ± 2 pmol NAD/ 10^6 cells, $p = 0.02$, up to the level of the V3 cells. PARP-1 activity remained unchanged in the V3 cells with the addition of ATP ($p = 0.7$). As expected, AG14361 completely inhibited PARP-1 activity (97 ± 5 %) in all PARP-1 proficient

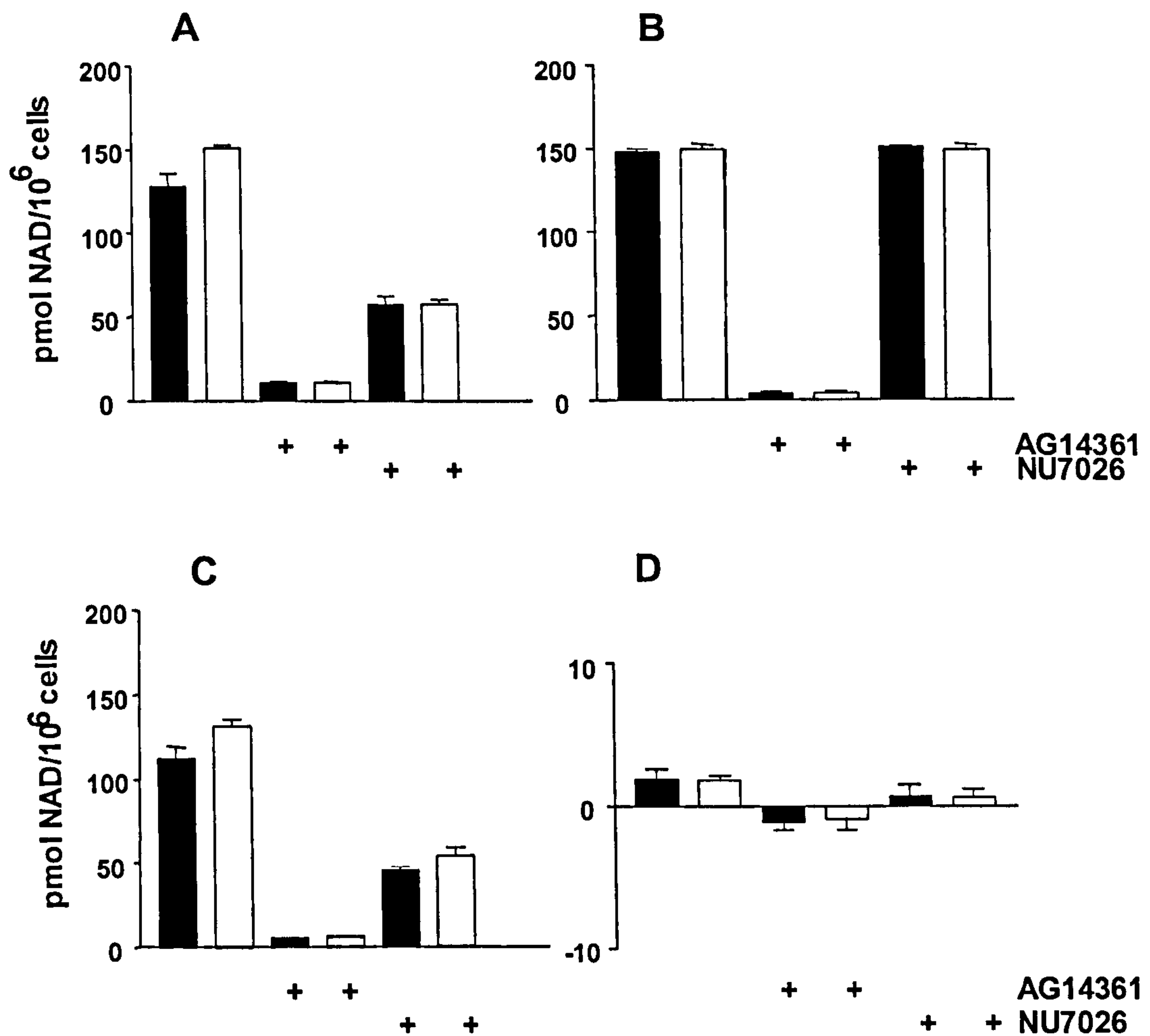


Figure 6.4 The effect of AG14361 and NU7026 on PARP-1 activity in permeabilised cells.

Samples were treated either in the presence of ATP (white bars) or absence of ATP (black bars). Results are the mean of 3 replicate samples each from 3 independent experiments \pm SEM. (A) V3YAC cells; (B) V3 cells; (C) PARP-1^{+/+} cells; (D) PARP-1^{-/-} cells.

cell lines. In contrast, NU7026 reduced PARP-1 activity by $61 \pm 4 \%$ ($p = <0.001$) in the V3YAC cells, by $59 \pm 5 \%$ in the PARP-1 +/+ cells and by $71 \pm 3 \%$ in the PARP-1 -/- cells, whilst having no significant effect in the V3 cells ($p = 1$).

As described in Chapter 3 section 3.3.3, the PARP-1^{-/-} cells retained a small, but significant residual activity (4 ± 0.3 compared to 132 ± 3.8 pmol in the PARP-1^{+/+} cells) (Figure 6.4C and 6.4D) and this minority activity is probably attributable to PARP-2, which together with PARP-1 has also been implicated in BER (Schreiber *et al.*, 2002). AG14361 completely inhibited this residual activity ($p = <0.01$). NU7026 inhibited PARP activity in the PARP-1^{-/-} cells to a similar extent to its inhibition of PARP activity in PARP-1 +/+, V3YAC and V3 cells ($p = <0.001$), indicating that DNA-PK inhibition also modulates this second PARP activity.

Earlier studies were carried out in the V3YAC and V3 cells using a self complementing palindromic sequence of 10 nucleotides which forms a double stranded blunt ended hairpin that stimulates PARP-1 activity (Grube, 1991) (see appendix). Whilst very similar results were obtained in the V3YAC cells, with a comparable level of PARP-1 activity (136 ± 8 pmol compared to 129 ± 7 pmol, Figure 3.5) and $99 \pm 3 \%$ and $45 \pm 5 \%$ inhibition of activity when AG14361 and NU7026 were added to the reaction mix, respectively, this oligonucleotide failed to stimulate DNA-PK activity when added to a purified DNA-PK assay (data not shown). This effect of NU7026 in the presence of this type of oligonucleotide is therefore interesting and the implications of this observation will be considered in the discussion.

6.3.6 Immuno dot-blot method for the detection of ADP-ribose polymer formation

The activity measured by [³²P] incorporation from radiolabelled NAD⁺ into TCA precipitable counts in the permeabilised cell assay may not be solely attributable to the activity of PARP-1. Poly(ADP-ribosylation) reactions are also carried out by other enzymes which include the mono(ADP-ribosyl) transferases and NAD glycohydrolases, as well as other members of the PARP family. Furthermore, although label from the ADP-ribose moiety of NAD⁺ is incorporated into acid

insoluble material, this still does not constitute as proof of ADP-ribose polymer formation.

An alternative ‘immuno’ dot-blot strategy was therefore used as a means to confirm the permeabilised cell assay results. Briefly, the technique involves the permeabilisation of cells and activation of PARP-1 by oligonucleotide in the same manner as the radiolabelled permeabilised cell assay. To measure PARP-1 activity, however, samples are blotted onto a membrane and a specific antibody to ADP-ribose polymer which shows no cross reactivity with DNA, RNA, monomers of ADP-ribose or NAD⁺ (Menard *et al.*, 1987) is used to detect the levels of ADP-ribose polymer synthesized during the reaction and bound to the membrane, as described in section 2.1.3. The intensity of the ‘dot’ is a reflection of the amount of polymer bound to the membrane.

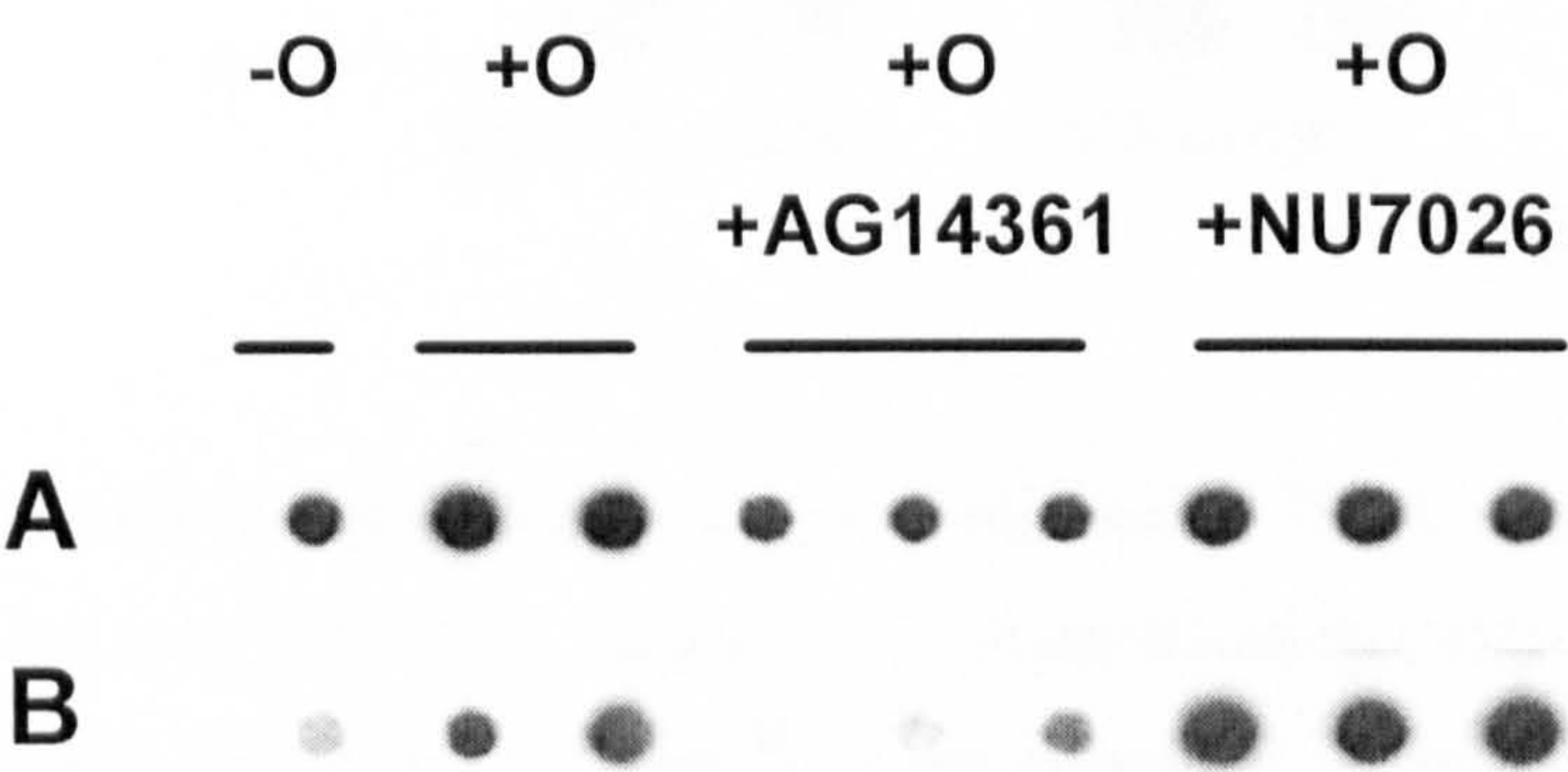


Figure 6.5 Dot blot measuring the effect of AG14361 and NU7026 on PARP-1 activity : A, V3YAC cells; B, V3 cells; -O, absence of oligonucleotide; +O, presence of oligonucleotide

Consistent with the radiolabelled assay data (see Figure 6.4A and 6.4B), oligonucleotide stimulated PARP-1 activity can be seen in both the V3YAC and V3 cells. Furthermore, AG14361 fully inhibited this stimulation in both cell lines. Whereas NU7026 had no effect on PARP-1 activity in the V3 cells, a small reduction in activity can be seen in the V3YAC cells. However, as no densitometry was carried out, this reduction in activity cannot be quantitated although it is clear that the difference is not as large as the 61 % inhibition observed in the radiolabelled assay (Figure 6.4A).

6.3.7 Development of a DNA-PK permeabilised cell assay

Attempts were made to adapt the PARP permeabilised cell assay into a permeabilised cell assay specific for DNA-PK activity (section 2.17). To be considered specific for DNA-PK, the activity measured needed to be both DNA strand break dependent and absent in the DNA-PK deficient cells

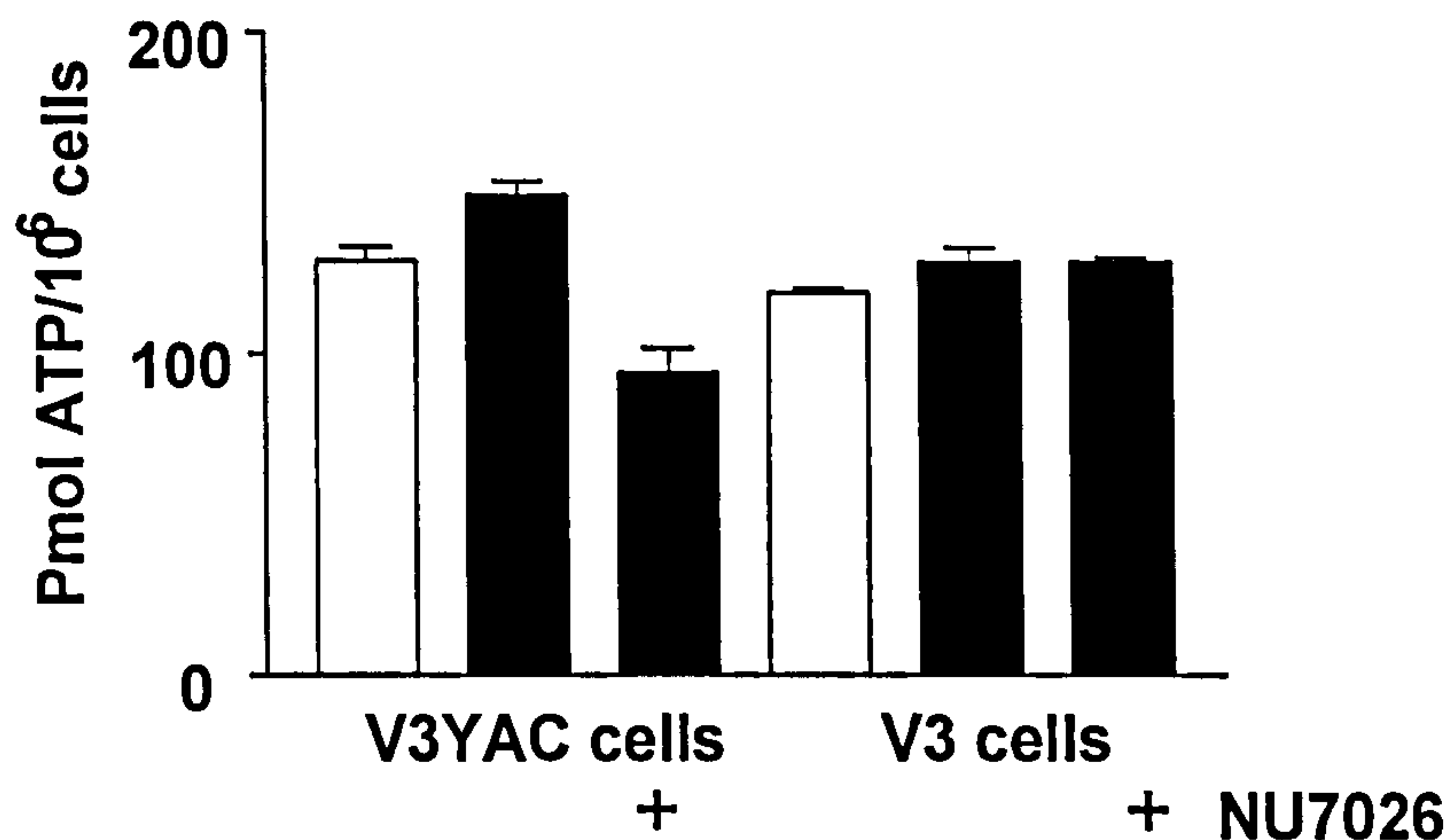


Figure 6.6 DNA-PK activity in permeabilised V3YAC and V3 cells and the effect of 10 μ M NU7026. Absence of oligonucleotide (white bars); presence of oligonucleotide (black bars). Data are the mean of 3 replicate samples from 3 independent experiments \pm SEM.

A small but significant amount of oligonucleotide activatable kinase activity can be seen in the V3YAC cells, however, this only accounts for 14 ± 5 % of the total kinase activity measured, $p = 0.01$ (Figure 6.6). No significant oligonucleotide stimulated activity can be seen in the DNA-PK deficient V3 cells ($p = 0.07$), indicating that the activity measured in the V3YAC cells is DNA-PK specific. NU7026 inhibited total kinase activity in the V3YAC cells by 37 ± 3 % ($p = 0.006$) whilst having no significant effect in the V3 cells ($p = 1$). High levels of non-specific kinase activity were present both in the presence of NU7026 in the V3YAC cells, and in the V3 cells in the absence of NU7026.

6.4 Discussion

The activity of purified PARP-1 and DNA-PK were modulated by the use of specific inhibitors and by deprivation of enzyme substrates. Importantly, in the context of the data presented in this Chapter, the studies described in Chapter 3 demonstrated that NU7026 is not a direct inhibitor of PARP-1 and AG14361 is not a direct inhibitor of DNA-PK. The purified enzyme studies described in this Chapter have confirmed this observation and demonstrated that PARP-1 has no effect on DNA-PK activity towards a peptide substrate and DNA-PK has no effect on PARP-1 for ADP ribosylation of histones, provided their substrates (ATP and NAD^+ , respectively) are present. However, in the absence of substrate, inactive DNA-PK inhibited PARP-1, and inactive PARP-1 inhibited DNA-PK, both in a concentration-dependent manner. Similarly, Ariumi *et al.*, 1999, have shown that co-existence of DNA-PK markedly inhibited PARP-1 activity in the absence of ATP substrate, as assessed by its auto-ADP-ribosylating activity. However, in contrast to the results presented here, this suppression of activity also occurred in the presence of ATP. This group also demonstrated that the presence of PARP-1 had no effect on the auto-phosphorylation activity of DNA-PK in either the presence or absence of NAD^+ .

Since the suppression of one enzyme activity by co-incubation with the other enzyme occurs in the absence of substrate this reduction in activity is not likely to be due to modification of each other (phosphorylation of PARP-1 or ADP-ribosylation of DNA-PK). One possible explanation for the mechanism of inhibition of PARP-1 by DNA-PK and vice versa would be a competition for DNA ends between the enzymes, although a more direct mechanism could also be involved. In the studies described in this Chapter, however, an excess of ends were used, and therefore competition between the enzymes should have been minimized.

An alternative mechanism for the suppression of the activity of one enzyme by the other could be through blocking/sequestering of DNA ends that serve as important activators for both enzymes. Both PARP-1 and DNA-PK undergo automodification (phosphorylation of DNA-PKs and poly(ADP-ribosylation) of PARP-1) when activated by DNA strand breaks, and this reaction is important to allow dissociation of PARP-1 or DNA-PK from DNA ends (Merkle *et al.*, 2002;

Smulson *et al.*, 1998)). Therefore, it is conceivable that in the absence of substrate, one enzyme will remain bound at the site of DNA damage and prevent the access of the other one, thus reducing their activity. In support of this, Satoh and Lindahl, 1992, demonstrated that in the absence of NAD^+ , PARP-1 persisted on the breaks and DNA repair of a nicked plasmid DNA was abrogated. Addition of NAD^+ , such that automodification and release of PARP-1 from breaks occurred, allowed the repair process to proceed.

Accordingly, the data presented in this Chapter have shown that the addition of substrate (NAD^+ for PARP-1 and ATP for DNA-PK) to the reaction mixture negated the enzyme inhibitory effect (Figures 6.1(a) and 6.2(a)). This is therefore likely to be because the presence of substrate enables the dissociation of the enzyme(s) from the DNA ends due to automodification, thus allowing access of the other enzyme. In contrast, however, as described above, Ariumi *et al.*, 1999, showed that the suppression of PARP-1 activity by DNA-PK was unchanged when ATP was present. This could reflect that the mechanisms of PARP-1 inhibition by the lack of ATP or presence of ATP are distinct. For example, in the absence of ATP, a 'blocking' mechanism may be responsible. However, the suppression of PARP-1 activity in the presence of ATP, might reflect phosphorylation of PARP-1 and a subsequent inactivation of its enzyme activity. Indeed, PARP-1 has been shown to exist as a phosphoprotein (Bauer *et al.*, 1994) and heteromodification and modulation of enzyme activity (poly(ADP-ribosylation) of DNA-PK by PARP-1, and phosphorylation of PARP-1 by DNA-PK) has also been demonstrated (Ariumi *et al.*, 1999; Ruscetti *et al.*, 1998).

Under normal, physiological conditions, both enzyme substrates are likely to be present at sufficient levels to maintain full enzyme activity. The data presented in the previous Chapter suggests that, physiologically, in the presence of substrates these enzymes will co-operate in DNA repair. There are instances, however, where a depletion of substrate(s) could occur. For example, following extreme damage to the DNA, PARP-1 activation could seriously reduce the cellular NAD^+ concentration, which in turn could deplete the ATP reserves of the cell. Similarly, perturbations in nucleotide synthesis (for example, with antipurine antimetabolites) or when mitochondrial respiration is compromised (for example, by loss of membrane

potential) could drastically reduce cellular ATP. However, these scenarios are likely to be so cytotoxic that a negative effect on DNA repair whilst it might occur, would be largely irrelevant in terms of cell survival.

The effect of addition of NU7026 to the PARP-1 assay in the presence of DNA-PK (Figure 6.1) and the addition of AG14361 to the DNA-PK assay in the presence of PARP-1 (Figure 6.2) was to inhibit PARP-1 and DNA-PK activity, respectively. As the inhibitor(s) both act by inhibiting the catalytic activity of their respective enzymes and only target their respective enzymes, it is unlikely that this effect is due to an inhibition of heteromodification. This effect could be explained by inhibition of automodification and dissociation of one enzyme preventing access of the other enzyme to DNA in a similar manner to a lack of substrate. In support of this hypothesis, wortmannin has been demonstrated to block DNA-PK at DNA ends, and prevents their processing by DNA polymerization, degradation, or ligation (Calsou *et al.*, 1999). Similarly, 3AB hindered the dissociation of PARP-1 from nicked plasmid DNA thus preventing its repair (Sato and Lindahl 1992).

The observation that inactive PARP-1 inhibited DNA-PK activity by 82 % but that inactive DNA-PK only inhibited PARP-1 by 30 % suggests that PARP-1 has a greater affinity than DNA-PK for the 30 bp blunt ended oligonucleotide, used in the studies described in this Chapter, if the model of blocked ends as a cause of inhibition is valid. These data are consistent with the lower $K_{d\text{ app}}$ value of PARP-1 (116 pmol) for blunt ends compared to DNA-PK (1312 pmol) (D'silva *et al* 1999). This suggests that when DNA-PK and PARP-1 are in direct competition for binding to a break it would be bound preferentially by PARP-1. This could be to protect the ends from illegitimate recombination prior to DNA-PK binding and executing repair of the break. Surprisingly, despite its purported predominant role in BER, which produces only DNA SSBs, these authors also found that PARP-1 had a higher affinity for blunt ends compared to nicks and 3' single base overhangs ($K_{d\text{ app}} = 467$ pM and 332 pM, respectively). These data are also consistent with the findings of Benjamin and Gill, 1980, who showed that PARP-1 had a higher affinity for blunt ends over 3' overhangs, 5' overhangs and SSBs.

Under competitive binding conditions, PARP-1 bound ends would be predicted to predominate. However, the numbers of DNA ends in the assays used here were in at least 10-fold excess compared to the numbers of molecules of each enzyme, and therefore direct competition of the two enzymes for binding to a single DNA end should have been minimised. Even under these conditions, the presence of one inactive enzyme had a significant effect on the activity of the other. A possible explanation for these observations is that PARP-1 and Ku form a molecular complex in the absence of DNA (Galande and Kohwi-Shigematsu, 1999). The location of Ku at DNA ends serves to recruit and activate DNA-PKcs. Thus it may be envisaged that Ku, by forming a DNA-independent complex with PARP-1, may co-locate DNA-PKcs and PARP-1 to the same DNA ends, even in the presence of excess free ends. Under these conditions, interactive functioning of the enzymes would be inevitable.

From the data presented in this thesis it is apparent that in the event of co-localisation of the two enzymes, each enzyme needs to be catalytically active (i.e. in the presence of substrate and absence of inhibitor) to permit the catalytic activity of the other. Whether this functionality is dependent on auto- or heteromodification was not determined. However, it has been proposed that co-localisation of PARP-1 and DNA-PK on the same DNA ends increases the likelihood that they modify one another in response to DNA damage. PARP-1 possesses 6 sites which match the DNA-PK target sequence (Anderson and Lees-Miller, 1992; Bannister *et al.*, 1993) and 4 of these sites reside in the catalytic domain of PARP-1. Conversely, DNA-PKcs contains a peptide motif which binds free ADP-ribose polymers with high specificity (Pleschke *et al.*, 2000).

Several groups have attempted to assess whether these enzymes modify one another and if so, what effect this has on enzyme activity. Both Ruscetti, *et al.*, 1998 and Ariumi *et al.*, 1999, independently showed that DNA-PK could phosphorylate PARP-1 and that PARP-1 can poly(ADP-ribosylate) DNA-PK using purified enzymes and autoradiography. In addition they both demonstrated that this heteromodification only occurred when dsDNA was present. Ariumi *et al.*, 1999, further demonstrated phosphorylation of PARP-1 by DNA-PK in nuclear extracts obtained from the DNA-PK proficient M059K human glioma cell line whilst this

phosphorylation was absent in the DNA-PK deficient M059J cells.
(immunoprecipitation of enzyme assay products).

In the studies presented by Ruscetti *et al.*, 1998, phosphorylation of PARP-1 had no apparent effect on PARP-1 activity. Conversely, they showed that poly(ADP-ribosylation) activated DNA-PK since activation of DNA-PK by PARP-1 was dependent on the presence of NAD^+ and did not occur in the presence of the PARP-1 inhibitor, 1,5-dihydro-isoquinoline. One point of caution, however, is that their data could be alternatively interpreted. Thus, rather than causing the 'activation' of DNA-PK, the addition of NAD^+ to the reaction mix could actually be reflecting the abrogation of 'inhibition' and this interpretation would be more consistent with the data presented here (Figure 6.2A), and the 'competition for ends' hypothesis. Moreover, the authors did not measure DNA-PK activity alone in the absence of PARP-1 for comparison and thus it is not possible to differentiate between these different scenarios.

Consistent with the data presented here, Ariumi *et al.*, 1999, demonstrated that whilst purified DNA-PKcs and Ku70/80 can be poly(ADP-ribosylated) by PARP-1 in vitro, the presence of PARP-1 had no effects on the autophosphorylation activity of DNA-PK. Similarly, DNA-PK can phosphorylate PARP-1 but in support of previous findings by (Dvir *et al.*, 1992) this has no effect on its DNA binding or catalytic activity.

The studies described in this Chapter have shown that in the absence of histone cofactors, PARP-1 activity was not measurable, but activity was restored by the inclusion of DNA-PK, and stimulated further by the addition of ATP. Similarly, DNA-PK activity was not measurable in the absence of the p53 peptide, unless PARP-1 was included, and stimulated further by the addition of NAD^+ . It is interesting that neither enzyme was able to automodify itself in the absence of histones or peptide substrate. Without carrying out SDS-PAGE, followed by autoradiography on the reaction mixtures, it is not possible to distinguish whether the phosphorylation or poly(ADP-ribosylation) reactions represent automodification, heteromodification or a mixture of both.

The studies presented here, in the presence of histones or peptide have shown that the addition of one enzyme in the presence of its substrate does not stimulate the activity of the other above that of the enzyme alone. However, in the absence of histone or peptide, the restoration of one enzyme activity by the presence of the other is likely to reflect availability of acceptors/targets rather than a direct stimulation of PARP-1 by DNA-PK and vice versa. Consistent with this, the stimulatory effect of DNA-PK (+ATP) on PARP-1 activity was similar to the levels achieved by the inclusion of histones. In contrast, the highest DNA-PK activity observed following stimulation by PARP-1 and NAD⁺ was 10-fold lower compared to the inclusion of the p53 peptide. In the case of PARP-1, histones (1.32 pmol) were replaced by a roughly equivalent amount of DNA-PK, (0.81 pmol), whereas in the case of DNA-PK, peptide (2 nmol) was replaced by PARP-1 protein (0.77 pmol), resulting in a much lower concentration (> 2000-fold) of available substrate for phosphorylation.

In conclusion, inactive DNA-PK can inhibit PARP-1 and vice versa, and each enzyme can act as a target for heteromodification by the other; i.e. DNA-PKcs, or Ku, is a target for poly(ADP-ribosylation) and PARP-1 is a target for phosphorylation. Modification of one another could affect, negatively or positively, some other property of the enzymes apart from their catalytic activity, such as, interactions with other components of DNA repair complex. For example, it would be interesting to determine whether the interaction(s) between PARP-1 and pol β and/or XRCC1 (Caldecott *et al.*, 1996) are affected by DNA-PK mediated phosphorylation. Similarly, it will be interesting to analyse the effect of PARP-1 mediated poly(ADP-ribosylation) on the interactions between DNA-PK, ligase IV and XRCC4.

A direct confirmation of the experiments with purified enzymes was provided by the PARP-1 assays in permeabilised cells proficient or deficient in DNA-PK or PARP-1, where the enzymes are present in normal cellular concentrations, and the introduced oligonucleotides may associate with other chromatin proteins. Briefly, in permeabilised cells, NU7026 or reduced ATP concentration inhibited PARP-1 activity in the DNA-PK proficient cells. Earlier studies with a self complementing palindromic sequence of 10 nucleotides which forms a double stranded blunt ended hairpin showed a 45 % reduction in PARP activity in permeabilised V3YAC cells

when NU7026 was added. However, recent studies by Leuther *et al.*, 1999, have shown that a 14mer oligonucleotide is the smallest DNA capable of forming a stable Ku-DNA complex and studies by Yoo and Dynan, 1999, have shown that efficient kinase activation requires DNA longer than 12 bp. Consistent with these findings, this 10 mer oligonucleotide was unable to stimulate DNA-PK activity in a purified DNA-PK assay (data not shown). As DNA-PK would be unlikely to be able to assemble on this oligonucleotide, a potential mechanism for the effect of NU7026 could be that DNA-PK was able to bind to endogenous breaks. The process of permeabilisation has been shown to cause DNA damage and hence induce intracellular stress responses (Haldrosson *et al.*, 1978).

Whilst the methodology of the dot blot is very similar to a standard permeabilised cell assay, it has the advantages of not using radioactivity and requires lower cell numbers (Table. 2.4). Consistent with the radiolabelled assay data (Figure 6.4A and 6.4B), oligonucleotide stimulated PAR formation can be seen in both the V3YAC and V3 cells and this was fully inhibited by AG14361 in both cell lines. Whereas NU7026 had no effect on PARP-1 activity in the V3 cells, a small reduction in activity can be seen in the V3YAC cells. However, as no densitometry was carried out, this reduction in activity cannot be quantitated although it would appear that the difference is not as large as the 61% inhibition observed in the radiolabelled assay. This could be due to the number of cells loaded per well being too high such that the oligonucleotide treated sample is already at the saturation level of the assay in terms of signal intensity thus resulting in a loss of differentiation between oligonucleotide alone and oligonucleotide in the presence of NU7026. The cell number therefore needs to be optimized. The data from this experiment are only preliminary and clearly this assay requires optimisation. Nevertheless, the data obtained are in general agreement with those presented in the rest of this Chapter (Figure 6.4A and B).

A disadvantage of this technique is that whilst only de novo synthesis of polymer is measured in the radiolabel assay, in this technique, all polymer, including any pre-existing polymer, will be bound by the antibody. This phenomenon could be responsible for the small amount of polymer detected in the presence of AG14361 and in the absence of oligonucleotide. During the course of the work described in this

thesis, this assay has been optimized within the Northern Institute for Cancer Research (NICR) for use in a clinical trial which began in May 2003, using potent PARP-1 inhibitors in combination with the anti-cancer agent temozolomide.

Attempts were made to use the permeabilised cells for an oligonucleotide-activatable DNA-PK-specific assay but there proved to be very high levels of non-specific kinase activity both in the presence of NU7026 in the V3YAC cells, and in the V3 cells in the absence of NU7026. This is in contrast to the PARP permeabilised cell assay, where < 5% PARP activity was present in the absence of oligonucleotide, and none in the presence of AG14361. The strongest indication that this assay can measure at least to an extent, DNA-PK activity is the fact that the activity was stimulated by oligonucleotide in the V3YAC cells but not in the V3 cells. These results are consistent with those of Ariumi *et al.*, 1999, who attempted to measure DNA-PK phosphorylation in nuclear extracts from M059 J and K cells in the presence or absence of sonicated calf thymus DNA. The group showed that although there was a high level of phosphorylation in the absence of DNA, a large induction of protein phosphorylation was induced in the presence of DNA. Moreover, this only occurred in the DNA-PK proficient M059K cells and not in the M059J cells.

Although NU7026 fully inhibited the 'kinase' activity in the V3YAC cells, it brought activity to below that seen in the absence of activating oligonucleotide. There are two possible reasons for this. Firstly, despite being tested against other members of the PI3-K family and showing no activity against any of them at the concentration used in these assays, NU7026 could be inhibiting other kinases in the cell of which there is estimated to be greater than 500 (estimated that 2% of all proteins are kinases). However, NU7026 had no effect in the V3 cells which would also possess these kinases. Alternatively, therefore, NU7026 could be inhibiting DNA-PK activity that has been stimulated by endogenous breaks or those which have been induced during the permeabilisation process.

The data presented in this Chapter provide evidence for interaction and reciprocal regulation of DNA-PK and PARP-1 activity at DNA ends. PARP-1 and DNA-PK share similar biochemical properties (Table 6.1) which include the ability to bind DNA strand breaks, automodify in response to DNA binding and dissociate from DNA following automodification. Whereas DNA-PK is known to play an important role in the DSB repair pathway NHEJ, prior evidence for the participation of PARP-1 in this pathway has not been established. The results presented in this Chapter demonstrate that PARP-1 is able to bind blunt ended DSBs with high affinity.

It is worth noting that since both PARP-1 and DNA-PK are present at approximately 1 million copies per human cell, both are likely to be present in excess over the levels of DNA strand breaks generated under normal physiological circumstances, even following 75 Gy IR which at approximately 40 DSBs per Gy would induce 3000 DSBs per cell. All studies described in this Chapter were carried out when activating ends were in excess. The data therefore do not provide direct insights into the interplay between DNA-PK and PARP-1 *in vivo*.

6.5 Summary

In summary, the data presented in this Chapter have shown that:-

- PARP-1 and DNA-PK activities were not affected by the presence of the other enzyme, as long as it was active (substrate present or absence of inhibitor).
- Inactive DNA-PK inhibited PARP-1, and inactive PARP-1 inhibited DNA-PK, both in a concentration-dependent manner.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

The primary aim of the work described in this thesis was to investigate the interactive effects of PARP-1 and DNA-PK in the cellular responses to ionising radiation-induced DNA damage. In order to achieve the aim, data presented in this thesis utilised cell lines proficient or deficient (by mutation or gene knockout) in PARP-1 or DNA-PK in combination with novel specific inhibitors of the two enzymes. These cell lines were characterised in Chapter 3. The effect on a number of biological endpoints known to be modulated by PARP-1/ DNA-PK function following DNA damage were investigated. These included clonogenic survival (Chapter 4) and DSB formation and repair (Chapter 5). In addition, the ability of the two enzymes to compete for strand breaks and utilise one another as a substrate using both purified enzymes and a permeabilised cell assay was explored (Chapter 6).

At the time of writing this thesis, AG14361 and NU7026 were the current benchmark inhibitors of PARP-1 and DNA-PK, respectively. AG14361 is the first inhibitor of PARP-1 with the required pharmacological properties of potency, specificity, solubility and in vivo activity to address the therapeutic potential of PARP-1 inhibition for the treatment of human cancer. On the basis of extremely promising pre-clinical data (Calabrese *et al.*, In press) further analogue development resulted in the identification of a compound which combines the potency and efficacy of AG14361 with improved pharmacological properties. This PARP-1 inhibitor is currently undergoing Phase 1 clinical trials under the auspices of Cancer Research UK. Wortmannin is primarily a strong and selective inhibitor for PI3-kinase whilst the DNA-PK inhibitor used in the studies described in this thesis is, in contrast, a potent and selective inhibitor of DNA-PK. Thus, NU7026 has limited activity against PI3-Kinase and members of the PI3-kinase like family of enzymes, including ATM and ATR, which share structural similarities in the kinase domains. More recently, the Northern Institute for Cancer Research (NICR) in collaboration with KuDOS Pharmaceuticals (Cambridge) has identified new candidate compounds of sufficient

potency to be clinical candidates. These compounds are now currently being characterised in preclinical antitumour efficacy studies.

The work described in this thesis, exploring the properties of these inhibitors in cell culture, has been instrumental in providing the information required to facilitate the design of experiments for in vivo evaluation using tumour xenografts in nude mice. Mechanisms by which these novel inhibitors function were studied by evaluating the relationship between the inhibition of DNA repair and potentiation of cytotoxicity.

The main cytotoxic lesion induced by IR is the DNA DSB, which occur at a low frequency of approximately 40 per Gray per cell. Critically, their induction has been correlated with IR-induced cell death (Macmillan and Steel, 1993). Details of DSB repair mechanisms are not well understood in mammalian cell systems. However, radiosensitive mutants have been isolated which are defective in the repair of DSBs (reviewed in sections 1.8.4 and 1.12.2). Genetic analysis of these radiation sensitive cell lines has generated a large body of evidence suggesting crucial roles for DNA-PKcs and Ku70/80 in NHEJ but it is not clear whether they function directly or whether they primarily function indirectly by sensing damage and activating other factors involved in the processing of DNA ends.

Although the precise biological role of PARP-1 has not been elucidated, there is a wealth of evidence to suggest that inhibition of PARP-1 impedes DNA repair (Sato and Lindahl 1992) and enhances the cytotoxicity of DNA damaging agents such as IR (Ben-Hur et al 1984) and alkylating agents (Durkacz 1980). PARP-1 is a member of the BER complex comprised of XRCC1, DNA ligase III and polymerase β . The generation of PARP-1 deficient mice by homologous recombination has clearly demonstrated the involvement of PARP-1 in the maintenance of genomic integrity due to its role in BER. In contrast, the literature is lacking an analysis of PARP-1 function in DSB repair although molecular evidence indicates that PARP-1 interacts with DSBs as well as SSBs (reviewed in section 5.4.3).

The studies described in Chapter 4 demonstrated that under conditions that resulted in inhibition of DNA single and double strand break repair (hallmarks of PARP-1 and DNA-PK deficiency, respectively), NU7026 and AG14361 sensitised cells to IR as measured by colony forming assay. NU7026 did not potentiate IR in the DNA-PKcs deficient cell line, confirming its specificity for DNA-PK. Similarly, AG14361 did not potentiate IR in the PARP-1 $-/-$ cells, confirming that radiosensitisation in the PARP-1 proficient cells was due to the inhibition of PARP-1. In the absence of IR, the inhibitor(s) did not affect cell survival, indicating that they are non toxic per se. As with the inhibition of DNA DSB repair, when used in combination, the effects on survival were at least additive. Further data presented in Chapter 4 suggest that inhibition of PLDR in radioresistant non-proliferating cells by novel potent and specific inhibitors of PARP-1 and DNA-PK may be an important means by which to achieve clinical radiosensitisation.

The data presented in Chapter 5 evaluated the repair of DSBs and SSBs following treatment with IR in paired cell lines proficient or deficient in PARP-1 or DNA-PK in order to elucidate the mechanism underlying the increased radiosensitivity of PARP-1 and DNA-PK deficient cell lines. In addition, the effect of AG14361 and NU7026 on the repair of DSBs and SSBs was investigated in order to allow comparison with cytotoxicity data presented in Chapter 4. A lack of or inhibition of either DNA-PK or PARP-1 resulted in a deficiency in DSB rejoining. Notably, these data implicate PARP-1 in the processing and repair of DNA DSBs. In contrast, SSB repair was not reduced in the absence of or inhibition of DNA-PK indicating that whilst DNA-PK is involved in DNA DSB repair, it is not involved in SSB repair. When the inhibitors were used in combination, a profound and long term inhibition of DNA DSB repair was observed.

The results presented in Chapter 4 and 5, demonstrate a correlation between the ability to recover from IR-induced DNA damage and DSB repair capacity. This was particularly striking when comparing the ability to recover from PLD in non-cycling cells with their ability to repair DSBs. For example, the xrs-6 cells which carried out no DSB repair were also completely deficient in PLDR. Repair processes following IR may therefore be a key determinant of cell survival. Despite having a defect in the initial fast component of DSB repair, when repair was followed over 24

h in DNA-PKcs deficient cells, all breaks were eventually repaired. However, these cells are hypersensitive to IR and therefore slower kinetics of repair would lead to longer-term persistence of DSBs that could cause cytotoxicity. The almost complete and long-term abrogation of DNA DSB repair observed with the combined use of inhibitors following 24 h post-IR incubation could easily explain their radiosensitising effects in both proliferating and growth arrested cells. Since most cells in human tumours cycle slowly or are non-cycling, the investigation of the DNA repair processes in these cells may be important for cancer therapy.

Overall, a correlation between enzyme status, DNA repair and survival was observed and very similar results were obtained when use of inhibitors was compared to the use of knockout or mutant cell lines. The studies presented in this thesis have shown that inhibition of DNA-PK or PARP-1 activity increased sensitivity of cells to IR possibly due to an inhibition of repair. The evidence presented also indicates that the combination of a PARP-1 inhibitor with a DNA-PK inhibitor could prove a powerful strategy for radiosensitisation of tumours.

As described in Chapter 4, increased DNA-PK activity (specifically caused by elevation of DNA-PKcs levels) has been widely demonstrated both in cell culture and in samples from patients with relapsed chronic lymphocytic leukaemia and correlates with the resistance of tumour cells to IR and bifunctional alkylating agents. Such tumour specific alterations are likely to be important in terms of therapeutic exploitation and where DNA-PK is overexpressed, the use of a selective inhibitor would be predicted to allow preferential treatment of these tumours, which would otherwise become radio- or chemo-resistant. Disregulation of cellular functions is a hallmark of cancer and DNA repair pathways may be lost, making the cell dependent on complementary repair pathways. In such a case, the cells would be hypersensitive to inhibitors of the remaining pathway. For example, a cell which has lost the capacity for homologous recombination would be dependent on NHEJ to repair DNA DSBs. Combination of a DNA-PK inhibitor with an agent causing DNA DSBs would be predicted to be selectively toxic. Human tumours are frequently defective in ATM or BRCA1 and BRCA2, implicated in HR of DSBs. The additional inhibition of DNA-PK mediated NHEJ may be predicted to highly radiosensitise these tumours.

In order to full exploit the potential of PARP-1 and DNA-PK inhibitors for cancer therapy, a mechanistic understanding of their interactions is essential. The studies in Chapter 6 aimed to investigate the interaction of PARP-1 and DNA-PK at DNA ends using both purified enzyme assays and permeabilised cell assays. In the presence of limiting substrate and/ or inhibitor DNA-PK or PARP-1 inhibit the activity of each other indicating competition for DNA ends. However, in the studies described, DNA ends were in excess over the enzymes, thereby minimising competition. In addition, cell based studies suggest that in the presence of adequate substrate, these enzymes co-operate. The data presented in this thesis therefore suggests reciprocal regulation and co-operation of DNA-PK and PARP-1 at sites of DSBs.

The interaction between PARP-1 and DNA-PK at the site of a break requires further investigation as the results described in Chapter 6 were still at a preliminary stage at completion of this project. A CASE studentship project in collaboration with KuDOS pharmaceuticals is currently underway to further test the hypothesis that PARP-1 and DNA-PK compete for DNA double strand breaks and that different affinities for the type of end affects this competition. The relative affinities of purified DNA-PK and PARP-1 for different types of DNA end will be investigated by measuring their activation by synthetic oligonucleotides with a variety of ends, including blunt ends and 3', 5' overhangs. The effect of altering substrate concentrations, oligonucleotide concentration and enzyme ratios will also be investigated. The downstream effects of enzyme activation on DNA repair will be determined by measuring the rejoining of oligonucleotides with different ends in cell extracts from cell lines proficient and deficient in PARP-1 and DNA-PK. The role of PARP-1 and DNA-PK on the fidelity of repair will also be investigated using plasmid rejoining assays.

Although both PARP-1 and DNA-PK are thought to play a number of different roles within the cell, the most researched area and that evaluated in this thesis, is their function in DNA repair. PARP-1 activation has also been recognised as a novel factor mediating ischaemia/reperfusion injury and inflammatory tissue damage, and a dependence of NF- κ B activation following DNA damage on PARP-1 protein or activity has been proposed as a mechanism. Thus, PARP-1 inhibitors could

be important in the treatment of a broad spectrum of diseases, including cancer, strokes and heart attacks.

In conclusion, the results presented in this thesis demonstrate the therapeutic potential of PARP-1 and DNA-PK inhibitors as radiosensitisers, confirm that the inhibitors mediate their effects specifically by inhibition of their target enzymes and indicate that inhibition of DSB repair is the most plausible mechanism for their radiosensitising effects. Furthermore, the studies employing inhibitor combinations suggest reciprocal regulation and interaction of DNA-PK and PARP-1 and demonstrate that the use of combined inhibitors provides extremely potent radiosensitising effects with implications for future anticancer clinical strategies.

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Interaction of PARP-1 and DNA-PK at DNA strand ends : Implications for Cancer Therapy

Radiosensitization and DNA Repair Inhibition by the Combined Use of Novel Inhibitors of DNA-dependent Protein Kinase and Poly(ADP-Ribose) Polymerase-1¹

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ABSTRACT

The DNA repair enzymes, DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase-1 (PARP-1), are key determinants of radio- and chemo-resistance. We have developed and evaluated novel specific inhibitors of DNA-PK (NU7026) and PARP-1 (AG14361) for use in anticancer therapy. PARP-1- and DNA-PK-deficient cell lines were 4-fold more sensitive to ionizing radiation (IR) alone, and showed reduced potentially lethal damage recovery (PLDR) in G₀ cells, compared with their proficient counterparts. NU7026 (10 μ M) potentiated IR cytotoxicity [potentiation factor at 90% cell kill (PF₉₀) = 1.51 \pm 0.04] in exponentially growing DNA-PK proficient but not deficient cells. Similarly, AG14361 (0.4 μ M) potentiated IR in PARP-1^{+/+} (PF₉₀ = 1.37 \pm 0.03) but not PARP-1^{-/-} cells. When NU7026 and AG14361 were used in combination, their potentiating effects were additive (e.g., PF₉₀ = 2.81 \pm 0.19 in PARP-1^{+/+} cells). Both inhibitors alone reduced PLDR ~3-fold in the proficient cell lines. Furthermore, the inhibitor combination completely abolished PLDR. IR-induced DNA double strand break (DNA DSB) repair was inhibited by both NU7026 and AG14361, and use of the inhibitor combination prevented 90% of DNA DSB rejoining, even 24-h postirradiation. Thus, there was a correlation between the ability of the inhibitors to prevent IR-induced DNA DSB repair and their ability to potentiate cytotoxicity. Thus, individually, or in combination, the DNA-PK and PARP-1 inhibitors act as potent radiosensitizers and show potential as tools for anticancer therapeutic intervention.

INTRODUCTION

Enzyme-mediated repair of DNA DSBs³ is a major mechanism of resistance to radiotherapy, and inhibition of DNA DSB repair is thus a strategy for radiopotentialization. This study focuses on the use of novel inhibitors of the DNA-PK and PARP-1, both of which are DNA strand break-activated enzymes and key components of DNA damage recognition, repair, and signaling pathways.

Two DNA DSB repair pathways in eukaryotes are NHEJ and HRR (1). Important components in these repair pathways are the PI 3-K-related protein kinase family of enzymes. These DNA damage-activated serine/threonine protein kinases include DNA-PK, ATM, and ATR (2). The DNA-PK holoenzyme comprises a heterodimer of M_r ~70,000 and ~80,000 polypeptides, known as Ku, which binds to DNA strand breaks, recruiting and activating the M_r

470,000 catalytic subunit, termed DNA-PKcs (3). Numerous studies have shown that cells lacking either functional DNA-PKcs or Ku80 through mutation or gene knockout are hypersensitive to IR and cross-linking agents (3–6). DNA-PK, together with the XRCC4/DNA ligase IV complex and the recently identified cofactor Artemis, is specifically required for NHEJ (7, 8), with ~80% of DNA DSBs repaired by this pathway (9).

The first identified inhibitor of the PI 3-K-related protein kinase enzyme family was the fungal metabolite Wortmannin. Although primarily used as a PI 3-K inhibitor, it was also shown to potentiate IR-induced cytotoxicity and inhibit DNA DSB repair at concentrations that inhibit cellular DNA-PK (10–13). The recently synthesized molecule NU7026, 2-(morpholin-4-yl)-benzo[h]chomen-4-one, a novel and specific inhibitor of DNA-PK, has been evaluated in this study.

PARP-1 is a M_r 116,000 enzyme that is an important component of the BER complex required for DNA SSB repair (14–16). Loss of PARP-1 function, using molecular genetic techniques or inhibition, causes radiosensitization and potentiates the cytotoxicity of monofunctional alkylating agents (17–19). Thus, PARP-1 inhibitors are predicted to improve the efficacy of radio and certain types of chemotherapy. It is generally assumed that the inhibition of BER is the cause of the enhanced cytotoxicity. However, we have recently reported that a PARP-1 inhibitor (NU1025) also inhibits DNA DSB repair in cells exposed to IR, and this may contribute to the cytotoxic mechanism (12).

Convincing evidence for *in vivo* radiopotentialization with a PARP-1 inhibitor was obtained using PD128763 (20). Using a murine tumor model, Leopold and Seebolt-Leopold demonstrated that PD128763 + IR caused a 10–15-day increase in growth delay compared with irradiation alone. The clinical potential of this class of agents has led to the development of diverse classes of potent PARP-1 inhibitors (21–23). The tricyclic benzamidazole 1-(4-dimethylaminomethylphenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one) AG14361 has been used in this study (23).

Radiosensitization by deficiency or inhibition of either DNA-PK or PARP-1 is not limited to proliferating cells. Nonproliferating cells exposed to IR are known to undergo PLDR leading to substantial increases in survival compared with proliferating cells or cells induced to start a proliferative cycle shortly after irradiation (24, 25). Several early reports, using either cell lines mutationally inactivated in DNA-PK or PARP-1 inhibitors, showed a reduction in PLDR in nonproliferating (quiescent) cells (26–30). Local tumor conditions, such as cell and vascular density, as well as availability of oxygen and nutrients, frequently result in a high fraction of nonproliferating cells. PLDR after irradiation is considered to be an important determinant of radioresistance and has been demonstrated in tumor cell lines and in an experimental tumor model (31–33). Thus, the potential of small molecule inhibitors of DNA-PK and PARP-1 to prevent PLDR is an important consideration in the assessment of their therapeutic application.

Here, we describe the biological effects of both AG14361 and

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³ The abbreviations used are: DSB, double strand break; ATM, ataxia telangiectasia-mutated kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LD₉₀, dose of ionizing radiation that causes 90% cell kill; ATR, ataxia telangiectasia and Rad3-related kinase; BER, base excision repair; DNA-PK, DNA-dependent protein kinase; HRR, homologous recombination repair; IR, ionizing radiation; MEF, mouse embryonic fibroblast; NHEJ, nonhomologous end joining; PARP-1, poly(ADP-ribose) polymerase-1; PF₉₀, potentiation factor at 90% cell kill; PI 3-K, phosphatidylinositol 3-kinase; PLDR, potentially lethal damage recovery; SSB, single-strand break; YAC, yeast artificial chromosome.

NU7026 in cell culture models. We have investigated the ability of these inhibitors, used alone or in combination, to radiosensitize both proliferating and quiescent paired cell lines proficient or deficient in either DNA-PK or PARP-1. In addition, their effects on DNA DSB repair were investigated.

MATERIALS AND METHODS

Drugs. NU7026 was synthesized in the Department of Chemistry, University of Newcastle upon Tyne. AG14361 was synthesized by Pfizer GRD, CA (23). NU7026 and AG14361 were dissolved in anhydrous DMSO at stock concentrations of 5 and 10 mM, respectively, and stored at -20°C . Drugs (alone or in combination) were added to cell cultures so that the final DMSO concentrations were kept constant at 1% (v/v).

Cell Lines and Culture. Primary PARP-1^{+/+} and PARP-1^{-/-} MEFs were a gift from Professor Gilbert de Murcia, École Supérieure de Biotechnologie de Strasbourg, France. Spontaneously immortalized cell lines were derived from the primary MEFs to enable clonogenic survival assays to be performed. It was noted that the immortalized PARP-1^{+/+} MEFs expressed very high basal levels of p53 protein compared with the PARP-1^{-/-} MEFs, and therefore, the p53 gene was sequenced. It was found that the PARP-1^{-/-} cells had a wt p53 sequence, but the PARP-1^{+/+} had an Asp to Glu substitution at codon 278 in p53, within a conserved region of the DNA binding domain. This rendered the p53 unable to act as a transcriptional transactivator.⁴ The significance of the difference in the functional status of p53, with respect to the results obtained, is explored in the "Discussion." The Chinese hamster cell lines V3 (mutated in DNA-PKcs) and V3YAC [V3 transfected with a YAC carrying the complementing human DNA-PKcs gene] were kindly provided by Dr. Penny Jeggo, University of Sussex (34).

All cell lines were cultured as monolayers in DMEM medium [supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin]. Glutamine was added at a final concentration of 2 mM. The V3YAC cell line was maintained under antibiotic selection with gentamicin (Life Technologies, Inc., Paisley, United Kingdom) at a final concentration of 500 $\mu\text{g}/\text{ml}$ to ensure retention of the YAC.

Purified Enzyme Assays. Mammalian DNA-PK (500 ng/ μl) was isolated from HeLa cell nuclear extract after chromatography using Q-Sepharose, S-Sepharose, and Heparin agarose. DNA-PK (250 ng) activity was measured at 30°C , in a final volume of 40 μl , in buffer containing 25 mM HEPES (pH 7.4), 12.5 mM MgCl_2 , 50 mM KCl, 1 mM DTT, 10% v/v Glycerol, 0.1% w/v NP-40, and 1 mg of the substrate GST-p53N66 (the NH_2 -terminal 66 amino acid residues of human wild-type p53 fused to glutathione *S*-transferase) in polypropylene 96-well plates. To the assay mix, varying concentrations of inhibitor (in DMSO at a final concentration of 1% v/v) were added. After 10 min of incubation, ATP was added to give a final concentration of 50 μM , along with a 30-mer double-stranded DNA oligonucleotide (final concentration of 0.5 ng/ml), to initiate the reaction. After 1 h with shaking, 150 μl of PBS were added to the reaction, and 5 μl were then transferred to a 96-well opaque white plate containing 45 μl of PBS per well, where the GSTp53N66 substrate was allowed to bind to the wells for 1 h. To detect the phosphorylation event on the serine 15 residue of p53 elicited by DNA-PK, a p53 phosphoserine-15 antibody (Cell Signaling Technology, Beverly, MA) was used in a basic ELISA procedure. An antirabbit horseradish peroxidase-conjugated secondary antibody (Pierce) was then used in the ELISA before the addition of chemiluminescence reagent (Renaissance, New England Nuclear) to detect the signal as measured by chemiluminescent counting via a TopCount NXT (Packard).

The protocols used to detect ATM kinase and ATR kinase activities were performed essentially according to the methodologies described previously (35, 36). ATM and ATR were immunoprecipitated using rabbit polyclonal antisera raised to the COOH-terminal 400 amino acids of ATM and antisera raised to amino acids 400–480 of ATR, respectively. The PI 3-kinase assay was performed essentially as described previously (37), using baculoviral derived recombinant p110 α and p85 α (a kind gift from Prof. Mike Waterfield, Ludwig Institute, London, United Kingdom).

The IC_{50} s for the compounds in all of the enzymes assays were derived from

sigmoidal plots using the graphic package Prism, in which the enzyme activity in the varying concentration of compounds was plotted against the concentration of compound.

Cytotoxicity Assays. Clonogenic assays were performed as described previously (12). Briefly, exponentially growing cells (\pm AG14361 and/or NU7026) were exposed to IR. Drug(s) were added 60 min before irradiation followed by a 16-h postincubation at 37°C before harvesting and reseeding for colony formation in the absence of drugs. Data were normalized to untreated controls (1% v/v DMSO). PF_{90} values were calculated from the ratio of the individual LD_{90} values, *i.e.*, the LD_{90} divided by LD_{90} in the presence of inhibitor(s).

PLDR. The effects of AG14361 and NU7026, alone or in combination, were investigated in growth arrested cells after approximately equitoxic doses of IR. Cells were grown to confluence to induce G_1 arrest (G_0 status) before irradiation and irradiated in conditioned medium obtained from plateau phase cells. Cell cycle distribution to confirm G_1 arrest in plateau phase cells was assessed by flow cytometric analysis (data not shown). Drug(s) were added in conditioned medium 60 min before irradiation. After exposure to IR, cells were trypsinized and reseeded for colony formation in the absence of drugs, either immediately after exposure to IR or after a 24-h postincubation (\pm AG14361 and/or NU7026) at 37°C .

Western Blot Analysis of Protein Expression. Cell lysates were prepared from exponentially growing cells and loaded onto denaturing polyacrylamide gels using standard protocols. DNA-PKcs was resolved on 3–8% (w/v) Tris acetate gradient gels and all other proteins on 4–20% (w/v) Tris glycine gradient gels (Invitrogen Ltd., Paisley, United Kingdom). After electrophoresis, proteins were electrotransferred onto nitrocellulose (Bio-Rad, Herts, United Kingdom) and probed for PARP-1 (H-250 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) and DNA-PKcs (Ab-4 cocktail mouse monoclonal; Stratech Scientific, Cambs, United Kingdom). As a loading control, an antiactin antibody (mouse clone AC-40; Sigma, Dorset, United Kingdom) was used. This was followed by binding of peroxidase-conjugated goat antimouse/rabbit antibody and detection of proteins by enhanced chemiluminescence (DAKO, Ely, United Kingdom).

Cell-based Enzyme Activity Assays. For comparison of enzyme activities in the cell lines, the following techniques were used: DNA-PK was assayed in nuclear extracts. Briefly, DNA was removed from the extracts using high salt elution (fast flow DEAE Sepharose; Amersham Pharmacia, Bucks, United Kingdom). Fifty microgram aliquots were assayed for DNA-PK activity using the SIGNATECT DNA-PK assay system (Promega, Hants, United Kingdom). PARP-1 was assayed by measuring incorporation of radiolabel from [^{32}P] NAD^+ into acid precipitable counts in a permeabilized cell system (38). Cells were permeabilized using digitonin, and a 30-bp blunt-ended oligonucleotide was used in the assay to maximally activate PARP-1.

DNA Strand-Break Assays. DNA DSB levels were measured by neutral filter elution (39). The radiolabelling, drug treatment, postincubation conditions, and sample preparation used in these experiments were exactly as described by Boulton *et al.* (11). In all experiments, cells were exposed to 75 Gy IR. Cell cultures were preincubated \pm AG14361 and/or NU7026 for 60 min before exposure to IR, and the drugs remained in the culture medium during the postincubation periods. Regression analysis of each elution profile was performed to calculate the relative retention, *i.e.*, the fraction of sample DNA retained on the filter when 50% of the internal standard has eluted (40). Values for cells treated with IR \pm inhibitor(s) were expressed as a percentage of the values of unirradiated controls.

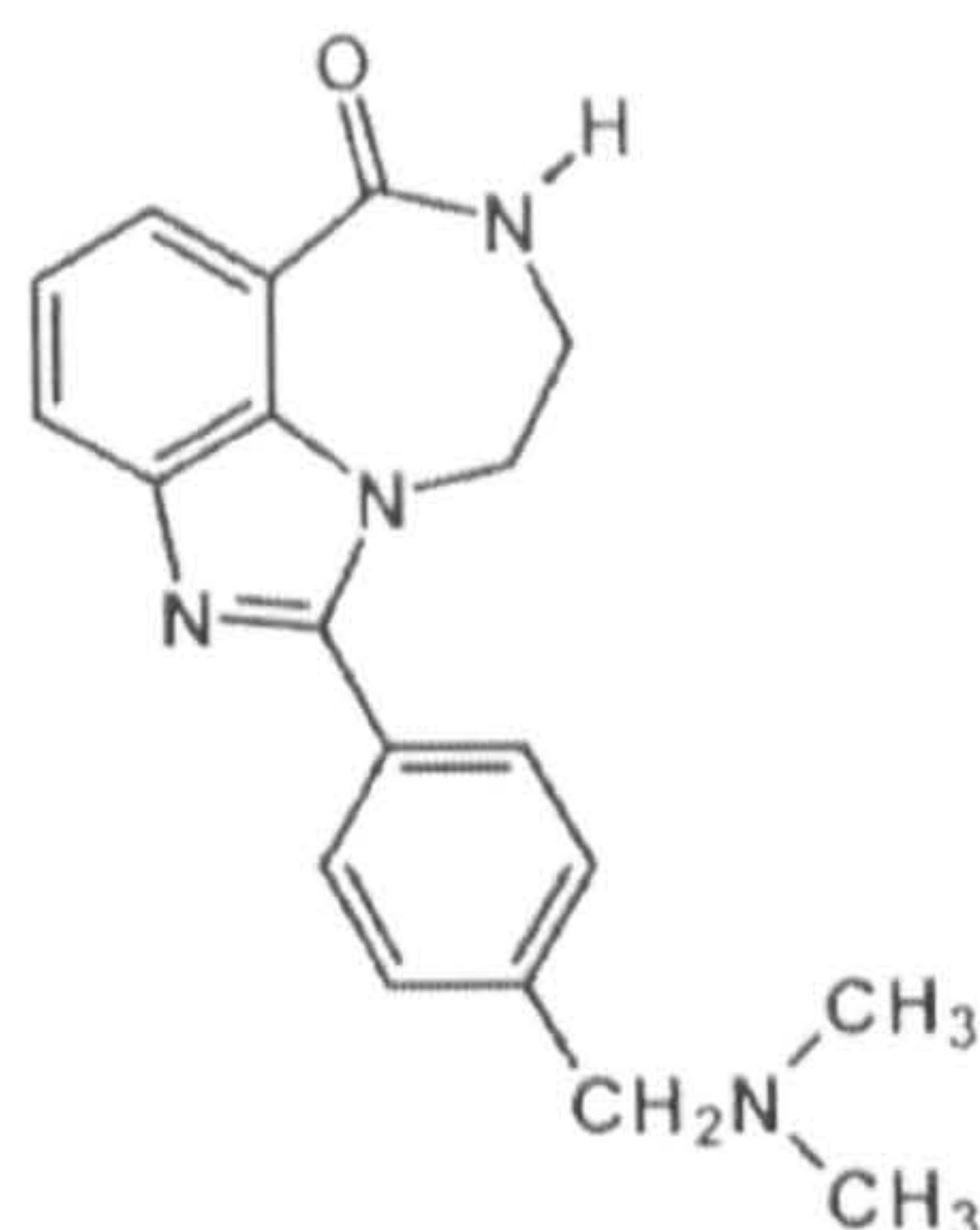
RESULTS

Inhibitor Structures and Evaluation of Enzyme Inhibition. The structure of AG14361 (1-(4-dimethylaminomethyl-phenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one) and NU7026 (2-(morpholin-4-yl)-benzo[h]chomen-4-one) is shown in Fig. 1. AG14361 is an extremely potent competitive inhibitor of PARP-1 with a K_i value < 5 nM (16). In the permeabilized cell assay used here, the IC_{50} for AG14361 in the V3YAC cells was 68.4 ± 3.5 nM and 56.3 ± 4.2 nM in the PARP-1^{+/+} cells.

Wortmannin is a noncompetitive, irreversible inhibitor of DNA-PK (10), whereas NU7026 is competitive with ATP. Shown in Table 1 is

⁴ P. A. Jowsey, unpublished results.

AG14361



NU7026

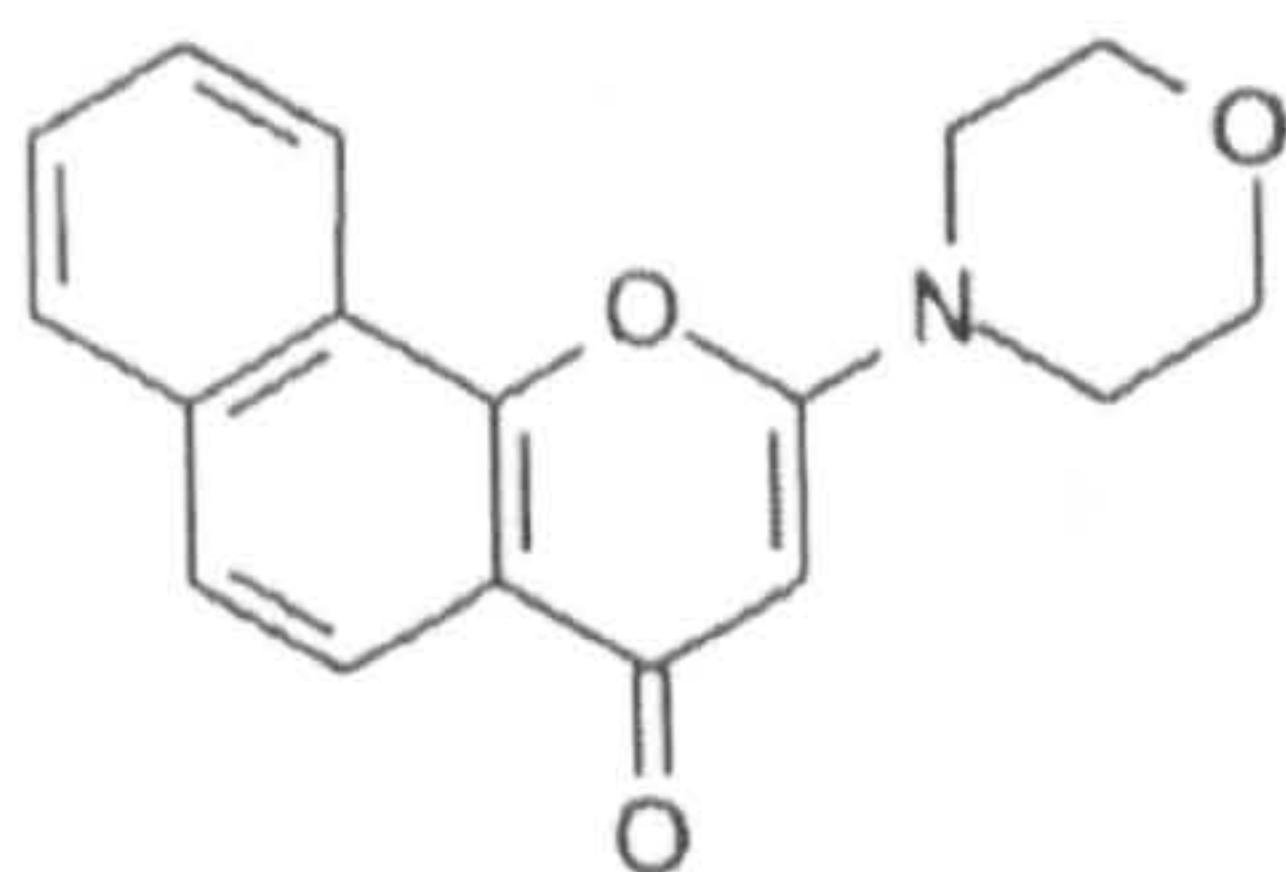


Fig. 1. Chemical structures of AG14361 [1-(4-dimethylaminomethyl-phenyl)-8,9-dihydro-7H-2,7,9a-benzo[cd]azulen-6-one] and NU7026 [2-(morpholin-4-yl)-benzo[h]chomen-4-one].

Table 1 Comparison of inhibitor potencies and specificities against the PIKK family enzymes^a

Enzyme	NU7026 IC ₅₀ (μM)	Wortmannin IC ₅₀ (μM)
DNA-PK	0.23 ± 0.01	0.26 ± 0.02
PI 3-K	13.0 ± 3.00	0.003 ± 0.00
ATM	>100	0.30 ± 0.05
ATR	>100	4.4 ± 0.18

^a Each IC₅₀ value is computed from the mean of more than or equal to three independent experiments ± SE.

a comparison of the relative inhibitory potencies of NU7026 and Wortmannin against PI 3-K, DNA-PK, ATM, and ATR. Although Wortmannin is primarily a PI 3-K inhibitor, being 90-fold more active against PI 3-K than DNA-PK or ATM, NU7026 was more selective for DNA-PK with a 60-fold greater potency against this enzyme than PI 3-K and inactive against both ATM and ATR. Thus, in contrast to Wortmannin, NU7026 demonstrates excellent specificity for DNA-PK.

AG14361 (0.4 μM) completely inhibited purified PARP-1 enzyme activity while having no effect on DNA-PK activity, and similarly, NU7026 (10 μM) completely inhibited purified DNA-PK activity, while having no effect on PARP-1 activity (results not shown). These inhibitor concentrations were used in all subsequent cell culture experiments.

Characterization of Cell Lines. Western blot analyses were carried out to assess PARP-1 and DNA-PK protein levels in the paired cell lines. As expected (Fig. 2A), the PARP-1^{+/+} cell line exhibited a band at *M_r* 116,000 (PARP-1), and this was completely absent in the PARP-1^{-/-} cell line. However, both these cell lines showed bands for DNA-PKcs and Ku80 (results not shown). The V3 cell line lacked the *M_r* 470,000 DNA-PKcs band, but this was restored in the V3YAC cell line (Fig. 2A). Both these cell lines showed the *M_r* 116,000 PARP-1 band (results not shown). DNA-PK and PARP-1 assays were carried out to confirm that the lack of detectable protein in the V3 and PARP-1^{-/-} cells correlated with an absence of the corresponding enzyme activities, to ensure that DNA-PK activity was present in both the PARP-1^{+/+} and PARP-1^{-/-} cell lines and to ensure that PARP-1 activity was present in both the V3 and V3YAC cell lines (Fig. 2, B and C). DNA-PK activity was slightly higher in the V3YAC cell line (750 pmol ATP/mg protein) compared with the PARP-1^{+/+} and PARP-1^{-/-} cell lines (500 pmol ATP/mg protein for both cell lines;

Fig. 2B). This may reflect overexpression of the human DNA-PKcs by the YAC. As expected, DNA-PK activity was undetectable in the V3 cells. PARP-1 activity was similar in all of the cell lines, apart from the PARP-1^{-/-} cells, which retained 3.9 ± 0.5% residual activity compared with the PARP-1^{+/+} cells (Fig. 2C). This residual activity is likely to be attributable to PARP-2, which, together with PARP-1, has also been implicated in BER (16).

Radiosensitization by AG14361 and NU7026. The effects of AG14361 and NU7026 alone and on IR-induced cytotoxicity were investigated in exponentially growing cell lines. Both PARP-1- and DNA-PK-deficient cells were ~4-fold more sensitive to IR than their proficient counterparts (note the different IR dose ranges in Fig. 3, A versus B and Fig. 3, C versus D).

Neither AG14361 (≤40 μM) nor NU7026 (≤50 μM), alone or in combination, reduced clonogenic survival (results not shown). The inhibitors were effective when added immediately before exposure to IR but for convenience were routinely added 1 h before irradiation. Both AG14361 and NU7026 potentiated IR in a concentration-depen-

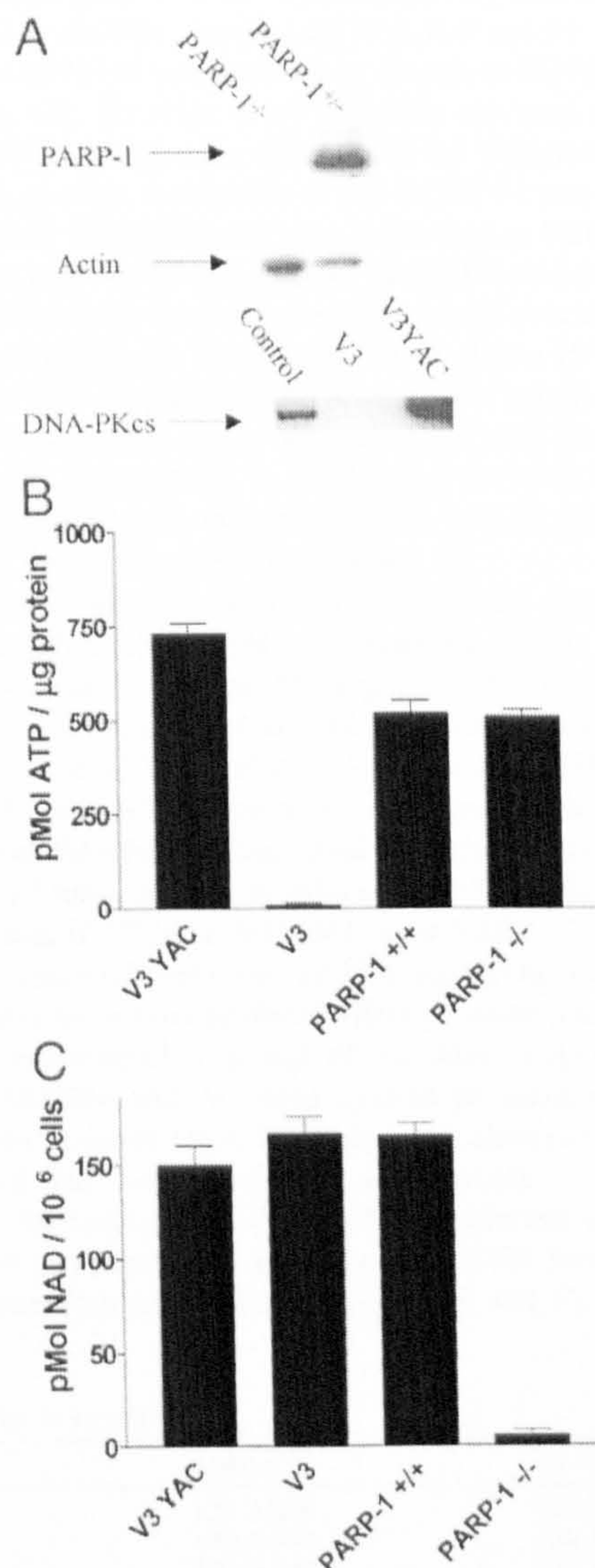


Fig. 2. Characterization of DNA-PK and PARP-1 levels and activities in the cell lines studied. A, Western blot analysis of PARP-1 and DNA-PK. Blots were probed using antibodies against PARP-1 and DNA-PKcs; B, DNA-PK activity; C, PARP-1 activity. Results are the mean of three replicate samples each from three independent experiments ± SE.

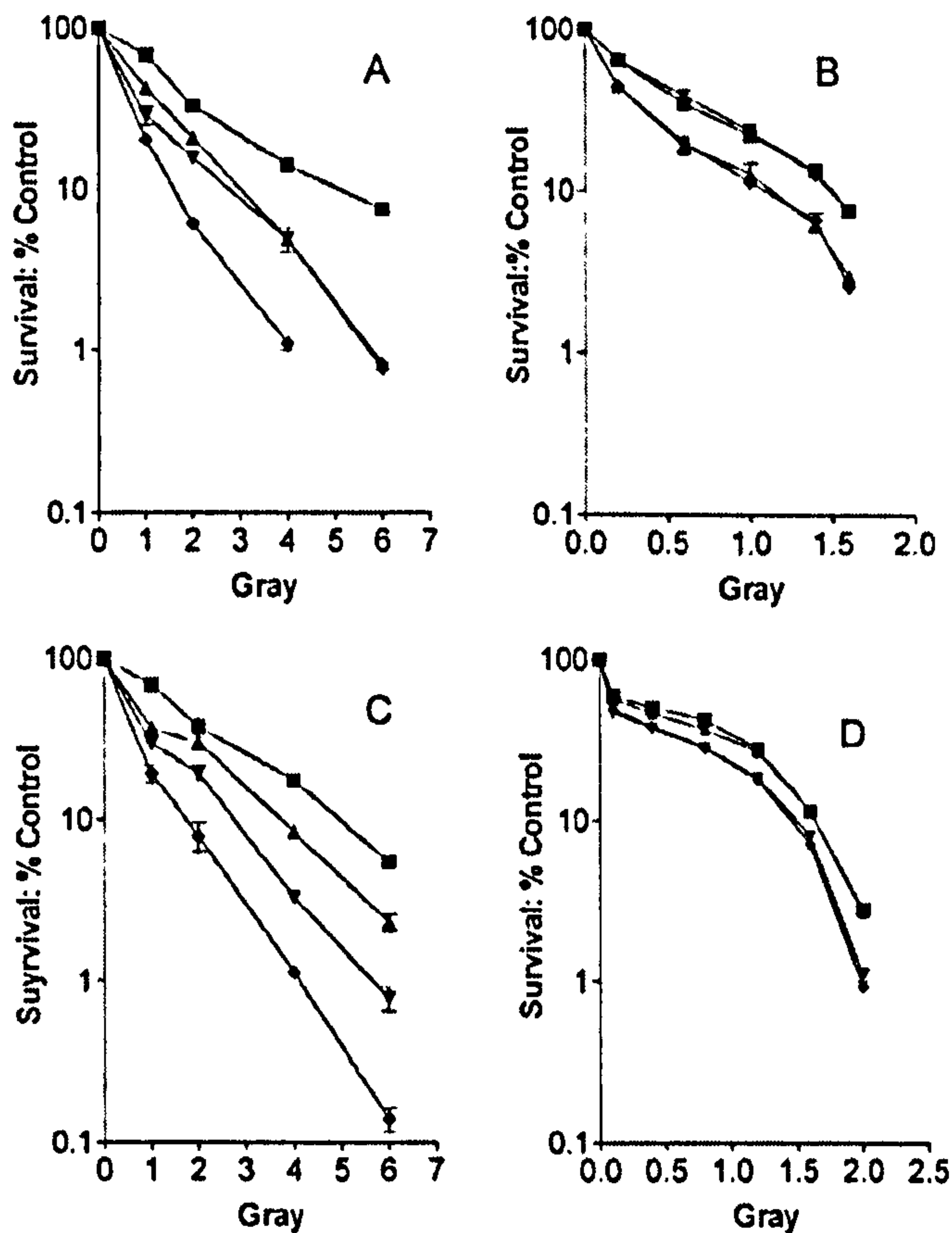


Fig. 3. Effects of increasing doses of IR in the presence or absence of AG14361 and NU7026 on the survival of exponentially growing: A, V3YAC cells; B, V3 cells; C, PARP-1^{+/+} cells; D, PARP-1^{-/-} cells; ■, IR alone; ▲, IR + AG14361; ▼, IR + NU7026; ◆, IR + AG14361 + NU7026. Cells were preincubated with drug(s) for 1 h before exposure to IR, then incubated for 16-h post-treatment before reseeding for colony formation. Data are the mean of at least three independent experiments \pm SE.

dent manner, and maximum potentiation was achieved at 10 μ M NU7026 and 0.4 μ M AG14361 (results not shown). Therefore, these concentrations were used in all subsequent experiments.

The inhibitors used alone, or in combination, potentiated the cytotoxicity of IR in the enzyme proficient exponentially growing V3YAC and PARP-1^{+/+} cells (Fig. 3, A and C). PF₉₀ values were calculated to quantitate the potentiating effects of the inhibitors alone, and in combination, on IR-induced cytotoxicity, and the results are summarized in Table 2. Approximately additive effects were obtained when the inhibitors were used together (Fig. 3, A and C), e.g., the PF₉₀ values for AG14361 and NU7026 in the V3YAC cell line were 1.4 ± 0 and 1.51 ± 0.04 , respectively; when used in combination, the PF₉₀ value was 2.78 ± 0.04 (Table 2). Significant radiosensitization by AG14361 was observed in all of the cell lines with PARP-1 activity (Fig. 3, A–C; PF₉₀ values 1.3–1.4) but not in the PARP-1^{-/-} cells (Fig. 3D). Similarly, NU7026 caused significant radiosensitiza-

tion in the DNA-PK proficient cells (Fig. 3, A, C, and D; PF₉₀ values 1.1–1.7) but not in the DNA-PK-deficient cell (Fig. 3B). These data support the hypothesis that NU7026 and AG14361 mediate potentiation specifically by inhibition of their target enzymes, DNA-PK and PARP-1, respectively.

Prevention of PLDR by AG14361 and NU7026. The effects of the inhibitors on PLDR were investigated. Cells were growth arrested by growing them to confluence. G₀ status was confirmed by flow cytometric analysis (results not shown). Cells were exposed to approximately equitoxic doses of IR (see details in Fig. 4 legend), such that the survival of cells which were immediately replated was $\leq 5\%$. After a 24-h delay in replating, which allows quiescent (G₀) cells to repair PLD, the surviving fraction of V3YAC and PARP-1^{+/+} cells was increased 7–8-fold (Fig. 4, A and C). This recovery was reduced by $\sim 70\%$ by inclusion of AG14361 during the recovery period, whereas NU7026 alone completely abolished PLDR, and the combination of NU7026 and AG14361 reduced survival even further (Fig. 4, A and C). PLDR was reduced (2–3-fold) in both DNA-PK-deficient (V3) and PARP-deficient (PARP-1^{-/-}) cells, compared with their proficient counterparts, and this modest recovery was fully inhibited by AG14361 or NU7026, respectively (Fig. 4, B and D). As expected, no inhibition of PLDR was obtained by the use of NU7026 in the V3 cell line (Fig. 4B). However, when AG14361 was used in the PARP-1^{-/-} cell line (Fig. 4D), there was a small, but significant, inhibition of PLDR. A possible explanation is that AG14361 may additionally inhibit PARP-2, which has also been implicated in BER (16).

Inhibition of DNA DSB Repair by AG14361 and NU7026. The kinetics of DNA DSB repair after exposure to IR over a 60-min time period were investigated. In the DNA-PK-proficient (V3YAC) cells, DNA DSBs were rejoined rapidly with $\geq 80\%$ rejoined by 60 min (Fig. 5A). Very similar results were obtained with the PARP-1^{+/+} cells (Fig. 5B). By contrast, in the V3 and PARP-1^{-/-} cell lines, higher DNA DSB levels remained 60-min post-IR incubation with only $\sim 50\%$ DNA DSB rejoined (5, A and B).

To determine whether the inhibitors similarly modulated DNA DSB repair, DNA DSB levels were assessed in cells \pm AG14361 and/or NU7026 at 60-min post-IR. (Fig. 6, A–D). In the V3YAC and PARP-1^{+/+} cell lines, AG14361 and NU7026 inhibited DNA DSB repair, e.g., in the V3YAC cell line, AG14361 and NU7026 inhibited repair by 40 and 56%, respectively, compared with the drug-free control. When the inhibitors were used in combination in the enzyme-proficient cell lines, repair was reduced by $\geq 90\%$ (Fig. 6, A and C). In comparison, NU7026 or AG14361 in the PARP-1^{-/-} or V3 cells, respectively, only reduced repair by 70%, suggesting that inhibition of both enzymes has a more profound effect on repair than inhibition of one enzyme combined with lack of the other enzyme. Finally, as expected, NU7026 and AG14361 exerted no additional inhibitory effect on the reduced DNA DSB rejoining observed in the V3 or PARP-1 cell lines, respectively (Fig. 6, B and D).

Finally, the longer term effects of the inhibitors on DNA DSB repair were investigated by assessing DNA DSB levels in V3YAC and V3 cells 24-h postirradiation (Fig. 6, E and F). By this time,

Table 2 Comparison of the PF₉₀ values^a derived from IR survival curves

Cell line/treatment	V3YAC	V3	PARP-1 ^{+/+}	PARP-1 ^{-/-}
IR + AG14361	1.4 ± 0.00	1.3 ± 0.01	1.37 ± 0.00	1.03 ± 0.001^b
IR + NU7026	1.51 ± 0.04	1.0 ± 0.00^b	1.69 ± 0.03	1.12 ± 0.01
IR + AG14361 + NU7026	2.78 ± 0.04	1.3 ± 0.03	2.81 ± 0.19	1.13 ± 0.00

^a All mean PF₉₀ values (\pm SE) were calculated from the ratio of the individual LD₉₀ values, i.e., LD₉₀ divided by LD₉₀ in the presence of inhibitor(s). The mean LD₉₀ values for IR + inhibitor(s) were all significantly different from the mean LD₉₀ values for IR alone in the same cell line ($P \leq 0.05$, $n = \geq 3$, two-tailed Student *t* test), excluding the LD₉₀ values for IR + inhibitor in the cell line lacking its corresponding target enzyme (see below).

^b The bold PF₉₀ values, which are not significantly different from unity, demonstrate the lack of potentiation by the inhibitor in the cell line which lacks its corresponding target enzyme.

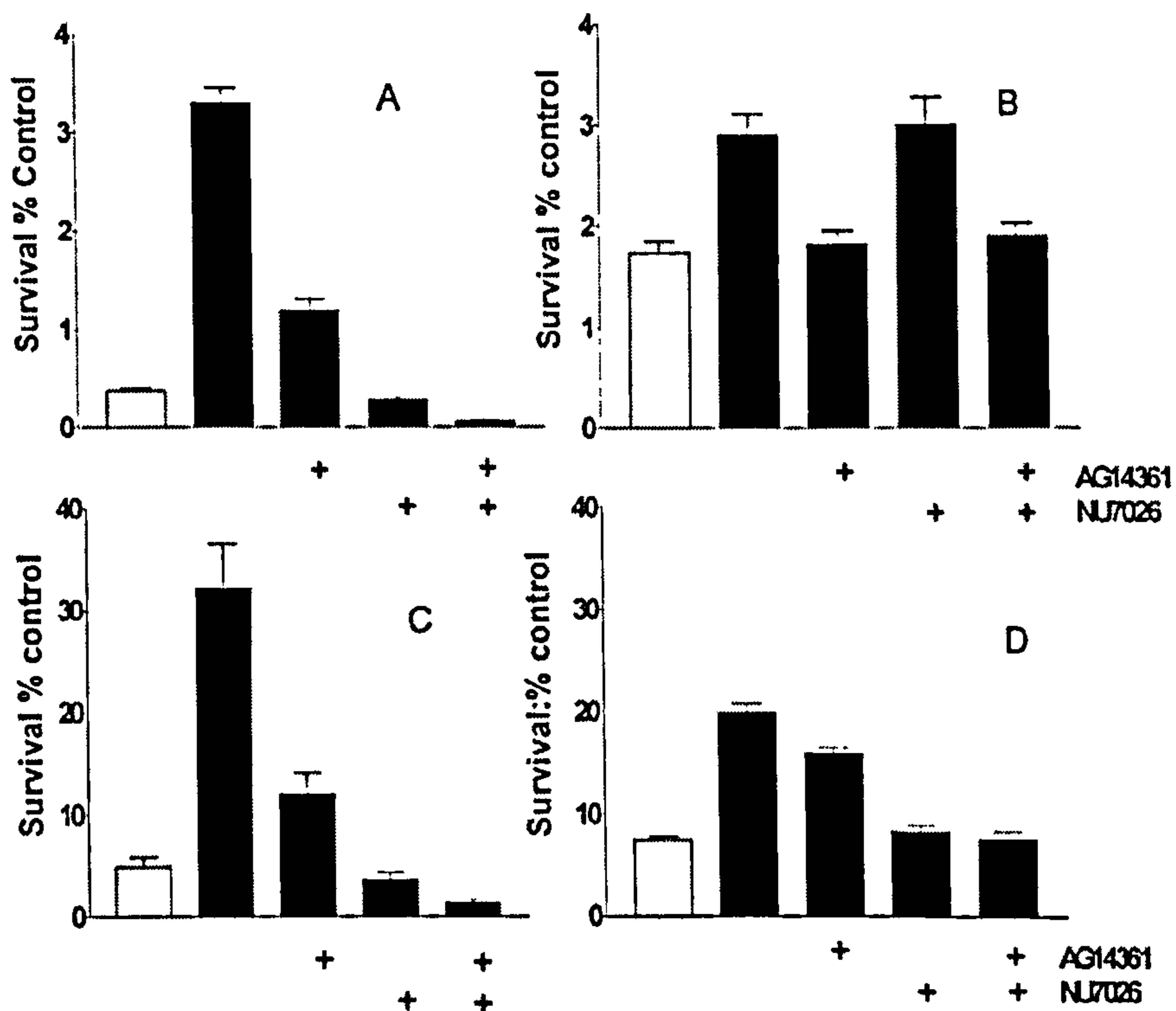


Fig. 4. Effects of AG14361 and NU7026 on recovery from IR-induced potentially lethal damage in growth-arrested cells. Cells were treated with approximately equitoxic doses of IR; 6 Gy for V3YAC, 1.4 Gy for V3, 5 Gy for PARP-1^{+/+}, and 1.2 Gy for PARP-1^{-/-}. The cells were either replated immediately for colony formation (white bars) or after a 24-h postincubation to allow PLDR (black bars). A, V3YAC; B, V3; C, PARP-1^{+/+}; D, PARP-1^{-/-}.

96 ± 3% of DNA DSBs were rejoined in the control V3YAC cells, compared with 7 ± 2% and 60 ± 7% when treated with AG14361 and NU7026, respectively (Fig. 6E). When AG14361 and NU7026 were used in combination, only 10 ± 2% of the breaks had rejoined. The V3 cell line was only slightly less proficient than the V3YACs at rejoining DNA DSBs by 24 h (77 ± 3%), and this was further reduced by the inclusion of AG14361, whereas NU7026 exerted no additional effect.

DISCUSSION

Here, we describe the effects on cellular responses to IR produced by novel specific inhibitors of the repair enzymes PARP-1 and DNA-PK. We demonstrate that these compounds radiosensitize both pro-

liferating and quiescent cells to IR and inhibit DNA DSB repair. Furthermore, when AG14361 and NU7026 are used in combination, their effects on all these biological endpoints are at least additive. In the survival experiments, cells were incubated for 16 h (approximately one cell cycle) after IR in the presence of the inhibitors before replating for colony formation. Additional experiments are required to determine the minimum exposure time to inhibitors necessary to obtain maximum potentiation. Use of the inhibitors in the paired cell lines, proficient or deficient for DNA-PK and PARP-1, has provided confirmation that the inhibitors mediate their effects on IR-induced cytotoxicity and DNA DSB repair specifically via inhibition of their target enzymes. Additionally, the radiosensitization and reduced DNA DSB repair observed in the deficient cell lines were mimicked by the use of the inhibitors in the proficient cell lines. The effect of enzyme inhibition or deficiency on cell survival closely paralleled the effects on DNA DSB repair, and hence, DNA DSB repair inhibition is a plausible mechanism for the radiosensitization observed.

Ideally, paired cell lines should be isogenic, differing only in the gene of interest. However, this is rarely the case, and it was established during the course of this work that, in contrast to the PARP-1^{-/-} MEFs, which had retained wild-type p53 after spontaneous immortalization, the PARP-1^{+/+} MEFs had acquired a p53 mutation in the DNA binding domain and did not induce mdm2 in response to IR.⁴ In consideration of the potential clinical use of DNA-PK and PARP-1 inhibitors, it is pertinent to point out that both inhibitors were able to cause radiosensitization in this cell line, despite the lack of a functional p53. This ability to radiopotentialize cells, regardless of their p53 status, increases the range of tumors for which the use of the inhibitors may be effective.

The observation that both NU7026 and AG14361 not only radiosensitize proliferating cells but also prevent PLDR in quiescent cells is very important. PLDR is a significant factor in determining tumor responses to radiotherapy (reviewed in Ref. 41). Evidence for PLDR in the nonproliferating compartment of tumors is very sparse, but

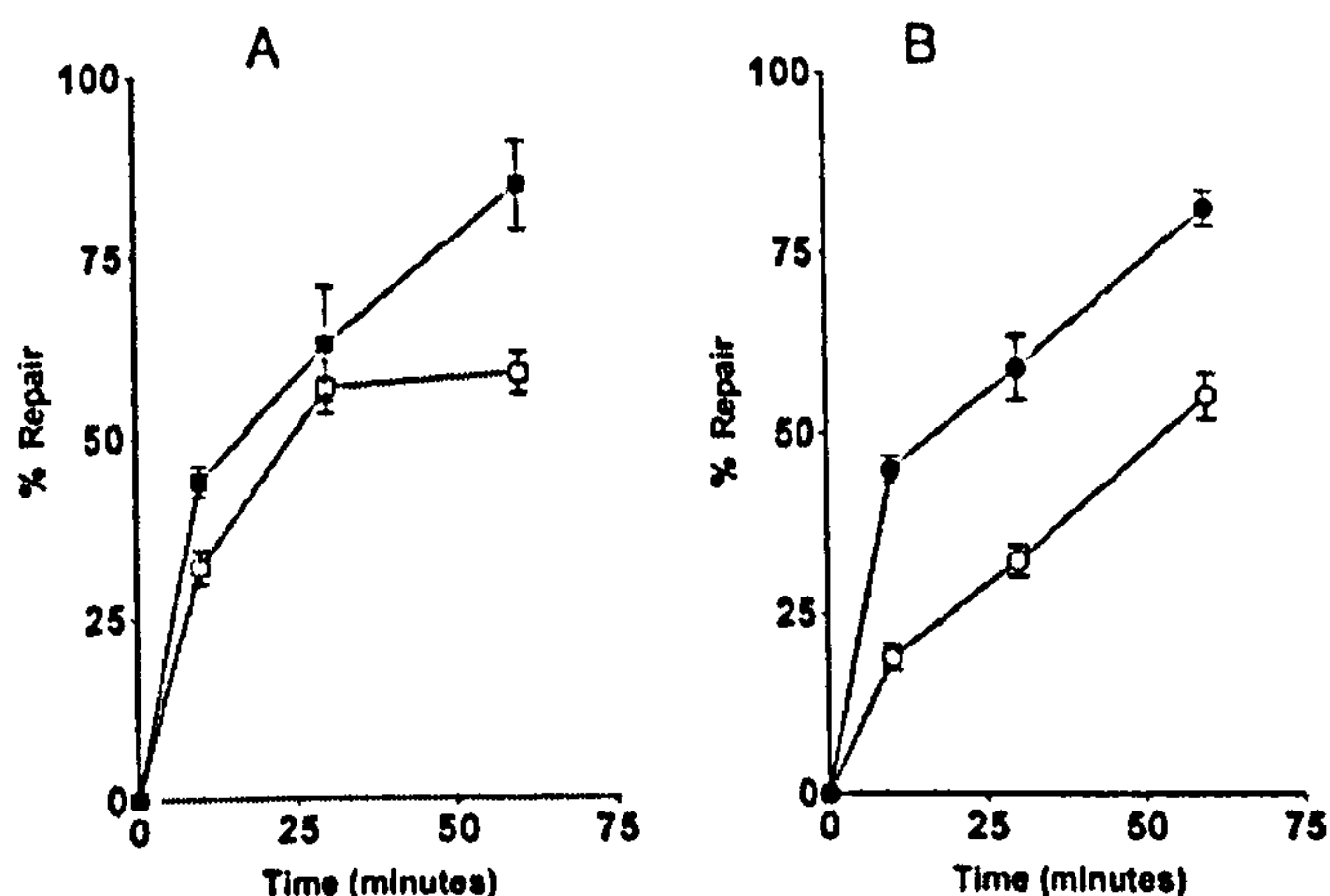


Fig. 5. Kinetics of DNA DSB repair after a fixed dose of IR. A, ■ V3YAC cells; □ V3 cells; B, ● PARP-1^{+/+} cells; ○ PARP-1^{-/-} cells. Cells were exposed to 75 GY IR and postincubated for increasing amounts of time before being harvested for neutral elution.

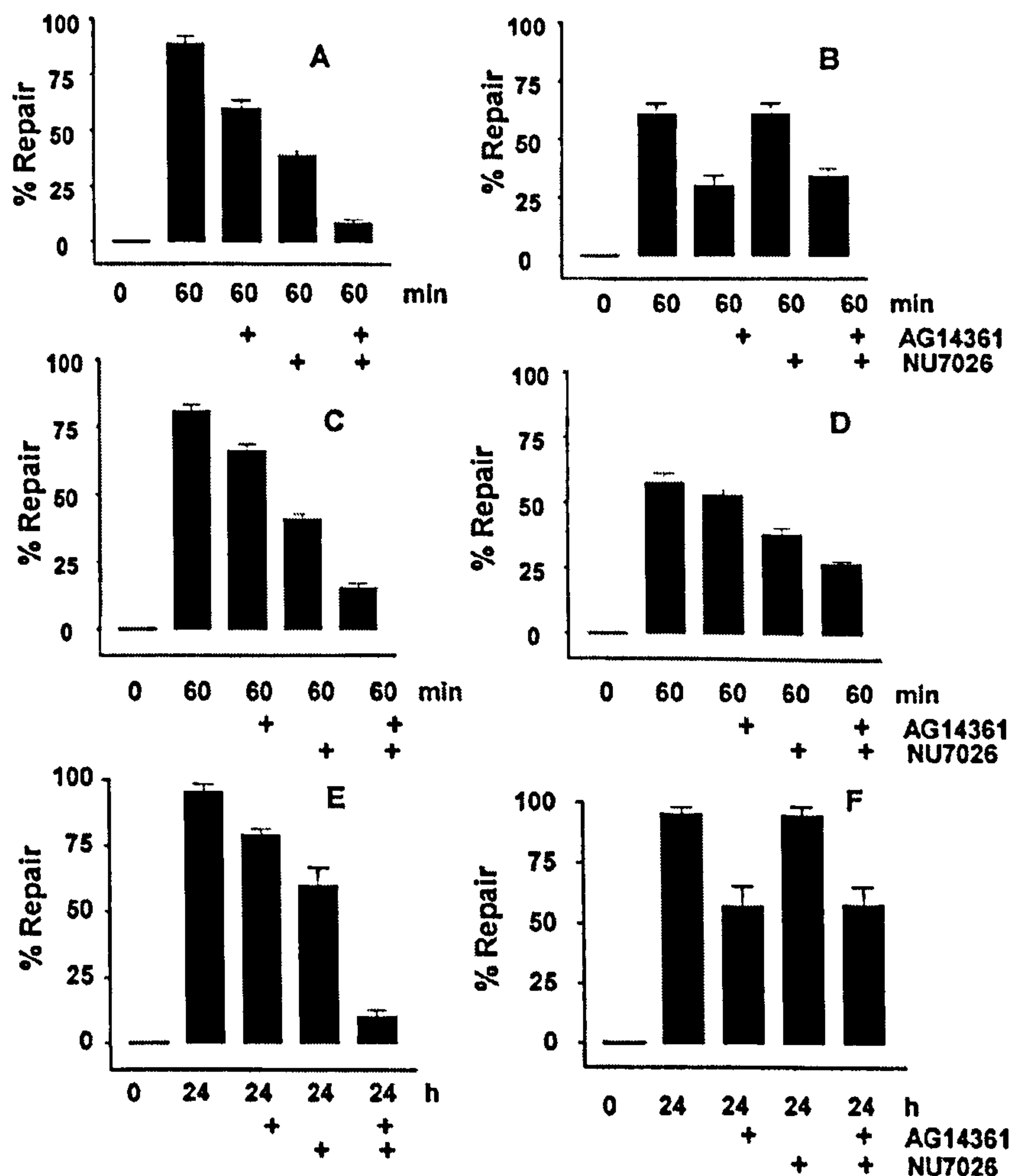


Fig. 6. Effects of AG14361 and NU7026 on DNA DSB repair. *A*, V3YAC cells; *B*, V3 cells; *C*, PARP-1^{+/+} cells; *D*, PARP-1^{-/-} cells. Cells were preincubated with inhibitor(s) for 60 min, exposed to 75 GY IR, and postincubated for 60 min before harvesting for neutral elution (*A-D*) or exposed to 75 GY and postincubated for 24 h. *E*, V3YAC cells; *F*, V3 cells.

experimental and clinical observations suggest that the rapid repopulation after IR-induced killing of the proliferating compartment of tumors results from recruitment of surviving G₀ cells into the proliferative cycle.

Both inhibitors used alone substantially prevented PLDR. More dramatically, when used in combination, AG14361 and NU7026 not only completely abolished PLDR but reduced survival significantly below that obtained in cells exposed to IR alone and replated immediately (*i.e.*, no 24-h recovery period), *e.g.*, when quiescent V3YAC cells were irradiated, survival was reduced to 0.38% if the cells were replated immediately. Survival was increased to 3.3% by a 24-h delay in replating, but the presence of AG14361 and NU7026 during the 24-h delay reduced survival to 0.064%, even lower than the cells which were replated immediately post-IR. This extremely potent radiosensitization (~50-fold) of nonproliferating cells by the combined use of the NU7026 and AG14361 is one of the major aims of radiobiologists and radiotherapists alike. Current interest in clinical radiosensitization has focused on hypoxic radiosensitizers and chemical radiosensitizers, such as the halogenated pyrimidine analogues (42-44), whereas the potential of PLDR inhibitors has largely been ignored. These data point to inhibition of PLDR as a tool for clinical radiosensitization that merits further investigation.

PARP-1 inhibition clearly results in higher levels of IR-induced DNA DSBs, but these data do not prove a direct role for PARP-1 in NHEJ. IR-induced clustered damage involves, *e.g.*, near neighbor base oxidation and hydrolysis of the phosphodiester backbone on

opposite DNA strands (45). BER at these sites will generate DNA SSB intermediates. Absence or inhibition of PARP-1 by preventing BER-mediated strand rejoining by ligase IV (46, 47) may result in the longer lived DNA SSBs converting to DNA DSBs, rather than by directly inhibiting NHEJ.

A number of lines of evidence indicate that although DNA-PKs highly stimulates the NHEJ pathway, in its absence, a slower repair pathway still operates (9, 48). The slower component of repair is probably mediated by DNA-PKs-independent NHEJ but may also involve HRR, which requires a complex of DNA repair proteins, including RAD51, BRCA1, BRCA2, XRCC2, and ATM (1). Both the slow component of NHEJ and HRR are considered to act independently of DNA-PKs and PARP-1. It was therefore a striking observation that even 24-h postirradiation, the use of the inhibitors resulted in a reduction in the percentage of DNA DSBs rejoined. In particular, a profound effect on DNA DSB repair was observed in the V3YAC cells with the combined use of the inhibitors at 24 h, where only ~10% of DNA DSBs had rejoined, compared with >95% of the control (Fig. 6A). DNA DSBs are considered to be the most cytotoxic lesion that cells encounter, and this almost complete and long-term abrogation of DNA DSB repair by the combined use of the inhibitors would easily explain their potent radiosensitizing effects in both proliferating and growth arrested cells. These data also indicate that the combined inhibition of PARP-1 and DNA-PK has downstream effects on the slow component of DNA DSB repair. One possible mechanism, which is suggested by the data, is that the inhibited

enzymes have more deleterious consequences for DNA DSB repair (and hence survival) than the lack of enzymes. DNA-PK and PARP-1 bind avidly to DNA DSBs, and automodification by phosphorylation and poly(ADP-ribosylation), respectively, is essential for dissociation of these enzymes from the DNA (49, 50). Furthermore, Wortmannin has been demonstrated to block DNA-PK at DNA ends and prevents their processing by either DNA polymerization, degradation, or ligation (51). Similarly, NU7026 and AG14361, by inhibiting the automodification reactions of their target enzymes, are predicted to tether the enzymes irreversibly to the DNA ends. These protein-bound DNA termini could hinder assembly of the enzyme complexes required for the successful execution of NHEJ and HRR.

A number of publications demonstrate interaction and/or cooperation between DNA-PK and PARP-1. Both enzymes have been shown to have high affinities for binding to DNA DSBs (52). Modulation of enzyme activity by poly(ADP-ribosylation) of DNA-PK by PARP-1 and phosphorylation of PARP-1 by DNA-PK has also been demonstrated, suggesting reciprocal regulation of enzyme activity (53, 54). Finally, an intimate association between PARP-1 and Ku has been demonstrated in coimmunoprecipitation studies (55). These observations point to an, as yet, poorly understood functional association of the two enzymes in response to DNA damage.

Increased DNA-PK activity has been widely demonstrated both *in vitro* and *in vivo* and correlates with the resistance of tumor cells to IR and bifunctional alkylating agents (56–59). Conversely, acquired resistance to chemotherapeutic agents has been shown to correlate with increased DNA-PK activity (60–62). Therefore, increased DNA-PK activity has been proposed as a novel cellular and tumor resistance mechanism. Such tumor-specific alterations are likely to be important in terms of therapeutic exploitation. Where DNA-PK is overexpressed, the use of a selective inhibitor is predicted to allow treatment of these tumors which would otherwise be radio and chemoresistant. The effects of NU7026 in model systems with increased DNA-PK activity merits further investigation. Whether there will be a therapeutic gain associated with the use of DNA-PK and PARP-1 inhibitors remains to be established, and an *in vivo* evaluation using human tumor xenografts in nude mice is planned.

In four recent reports, antisense oligonucleotides, small interfering RNAs, and a COOH-terminal peptide which targets Ku80 and prevents DNA-PKs binding to Ku have been used to selectively deplete or inhibit DNA-PK function in human cell lines (63–66). Loss of DNA-PK activity correlated with radiosensitization, increased mutation, and inhibition of DNA damage repair. Our results, the first to use a selective small molecule inhibitor of DNA-PK, are consistent with these data which highlight the current high interest in this enzyme as a target for radio and chemotherapeutic modulation. A PARP-1 inhibitor is currently entering Phase I clinical trials under the auspices of Cancer Research United Kingdom, and the evidence presented here indicates that the combination of this inhibitor with a DNA-PK inhibitor could prove a powerful chemotherapeutic strategy.

In conclusion, the data presented here suggest that pharmacological inhibition of DNA-PK and PARP-1, both alone and in conjunction, represents a promising strategy for tumor radiosensitization.

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APPENDIX

General Reagents

1 x TE: 10 mM Tris base, 1mM EDTA, adjusted to pH....with concentrated HCl. Filter sterilised and stored at room temperature.

Tris-HCl (1M): 12.1 g of Tris base dissolved in 800 ml of distilled water. Adjusted to the desired pH value by adding concentrated HCl and made up to 1 litre with distilled water. Stored at room temperature.

Carnoy's Methanol:Acetic acid 3:1

Western blotting Reagents

SDS lysis Buffer: 100 mM Tris-Hcl pH 6.8, 20 % v/v Glycerol, 4 % w/v SDS. Stored in 10 ml aliquots at -20 °C

Sample Buffer (4x): 0.0005 % w/v bromophenol blue solution (stored as 0.001 % in water), 62.5 mM DTT (1 M stock solution in water stored at -20 °C).

TBS 20 mM Tris, 140 mM NaCl adjusted to pH 7.6

TBS-Tween: 1 x TBS solution containing 0.1 % Tween-20

Tris Glycine;

10 x Running Buffer 250 mM Tris, 1.9 M Glycine, 0.1 % SDS. 1x buffer adjusted to pH 8.3

1 x Transfer 25 mM Tris, 190 mM Glycine, 20 % v/v Methanol

Tris Acetate

20 x Running Buffer 1M Tricine, 1M Tris Base, 2 % SDS. 1 x adjusted to pH 8.25

20 x Transfer: 500 mM Bicine, 500 mM Bistris, 20 mM EDTA. Adjusted to pH 7.2.

DNA Strand Break Assay Reagents

Lysis Buffer: 69 mM SDS, 25 mM EDTA, adjusted to pH 10 using 1 M NaOH. Stored at room temperature

Alkaline Elution Buffer: 2 mM EDTA-acid form, 5 M tetrapropylammonium hydroxide adjusted to pH 12.2 with tetrapropylammonium hydroxide. Stored at room temperature.

Neutral Elution Buffer: 0.05 M Tris, 0.05 M glycine, 0.025 M Na₂EDTA, 2 % w/v SDS. Adjusted to pH 9.6 and stored at room temperature.

Enzyme assays : General Reagents

Dithiothretol (DTT) : 100 mM in aqueous solution stored in 500 µl aliquots at – 20°C

Digitonin : 150 µg/ml in aqueous solution, stored at 4 °C

Oligonucleotide(s)

For PARP-1 activation, a 200 µg/ml stock solution (10 mM Tris-HCl, pH 7.8) of a palindromic sequence (CGGAATTCCG) was prepared from dry pellets synthesised by Dr J. Lunec (Cancer Research Unit, University of Newcastle, UK) on an Applied Biosystems 392 DNA/RNA synthesiser (PE Applied Biosystems, Warrington, Cheshire, UK). The pellets were dissolved in water and the UV absorbance at 260 nm measured in order to determine the concentration. Given

that 1 O.D unit = 50µg, stock solutions of 200 µg/ml. The stock solution was stored in 20 µl aliquots at –20°C.

For PARP-1 and DNA-PK activation, a 30bp double stranded oligonucleotide (5'-ACTTGATTAGTTACGTAACGTTATGATTGA-3') was purchased from Invitrogen (Paisley, UK). This was annealed to a reverse complementary oligonucleotide (5'-TCAACTATAACGTTACGTAACATAATCAAGT-3') ; Each oligonucleotide was resuspended in TE buffer at a final concentration of approximately 100 µM and vortexed for 15 seconds before mixing and heating to 65°C. The solution was then slowly cooled at a rate of approximately 1°C/min to allow them to accurately anneal. The final concentration was quantified by dilution in water and the UV absorbance read at 260 nm as described above. Stock solutions of 100 µg/ml and 200 µg/ml were stored in 50 µl aliquots at –20°C.

Permeabilised PARP Assay Reagents

Hypotonic Buffer : 9 mM HEPES, 4.5 % Dextran, 4.5 mM MgCl₂, adjusted to pH 7.8 with NaOH/HCl. The buffer was filter sterilised and stored at 4°C. DTT was added to a final concentration of 5 mM using 100 mM stock solution prior to use.

Isotonic buffer : 40 mM Hepes, 130 mM KCl, 4 % Dextran, 2 mM EGTA, 2.3 mM MgCl₂, 225 mM sucrose, adjusted to pH7.8 with NaOH/HCl. The buffer was filter sterilised and stored at 4°C. DTT was added to a final concentration of 2.5 mM using 100 mM stock solution prior to use.

Purified PARP Assay Reagents

NAD⁺ buffer 110 mM Tris, 13.5 mM MgCl₂, adjusted to pH 8.0 with HCl. The buffer was filter sterilised and stored at 4°C

PARP Buffer 30 mM Tris, 40 mM MgCl₂, adjusted to pH 8.0 with HCl. The buffer was filter sterilised and stored at 4°C. Just before use, stock 100 mM DTT was added to give a concentration of 400 µM in buffer.

Histone solution 1 mg/ml histone Type IIS dissolved in water was stored in aliquots at -20°C

Immunodotblot Reagents

Permeabilisation Buffer

10 mM Tris-HCl pH 7.8, 1 mM EDTA, 4 mM MgCl₂, 30 mM 2-mercaptoethanol, adjusted to pH 7.8. The buffer was filter sterilised and stored at 4 °C. (Supplemented with digitonin on the day of assay to a final concentration of 0.015 % using a 150 µg/ml stock).

Reaction Buffer 100 mM Tris-HCl pH7.8, 1 mM NAD, 120 mM MgCl₂. The buffer was filter sterilised and stored at 4°C. (NAD⁺ added fresh on day of assay)

Termination Buffer PARP-1 inhibitor, AG14361 10 mM (100 % DMSO v/v) stock solution stored at -20°C 10 mM (diluted 1/8000 in PBS to give final concentration 1.25 µM on day of assay)

PBS-Milk-Tween PBS, 0.05 % (v/v) Tween 20, 5% Milk powder (w/v)

Antibody 10H mouse monoclonal recognising p(ADPr) kindly provided by Alexander Burkle. Stored in stocks of 1.5 mg/ml at 4°C.

Purified DNA-PK Assay Reagents

Peptide p53 peptide substrate (EPPLSQEAFADLKK) G.C.M
Smith, KuDOS Pharmaceuticals, Cambridge, UK

ATP Stock solution in sterile water of 500 μ M. Aliquots of 500 μ l were stored at -20°C.

DNA-PK Buffer 50 mM KCl, 25 mM Hepes, pH7.6, 12.5 mM MgCl₂, 20 % Glycerol, 0.1 % NP40. The buffer was filter sterilised and stored at 4°C. Just before use, DTT was added to a final concentration of 1 mM in buffer.

Combined Assay Buffer 48 mM Tris HCl, 24 mM MgCl₂, 0.2 mM DTT

Purified Enzyme Assay Reagents for PIKK family enzymes

Reaction Buffer 25 mM Hepes, pH 7.4, 12.5 mM MgCl₂, 50 mM KCl, 1mM DTT, 10% v/v Glycerol, 0. 1% w/v NP-40