Design and Synthesis of Small-Molecule Inhibitors of Two Cancer Targets: mTOR and MDM2/p53

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Declaration

The work described in this thesis was carried out between October 2007 and September 2011 in the Medicinal Chemistry Laboratories, Bedson Building, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK, NE1 7RU and in the Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, Oxford, UK, OX1 3QU.

All of the research described in this thesis is original and does not incorporate any material or ideas previously published or presented by other authors except where due reference is given in the text.

No part of this thesis has been previously submitted for a degree, diploma or any qualification at any other university.
Abstract

Cancer is a disease in which cellular control over growth and differentiation has been lost. Targeted therapies for the treatment of cancer are becoming an increasing area of focus within the pharmaceutical industry and academia, due to the increasing understanding of the biology behind tumourgenesis.

There are 518 protein kinases within the human genome and they play a significant role within cellular signalling. Aberrant signalling of kinases can contribute to the development of cancer, and inhibition of kinase targets can result in either a cytostatic or cytotoxic effort. Kinases have a discrete ATP-binding domain, which presents an ideal target for small-molecule inhibitors. mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase, which forms two complexes, mTORC1 and mTORC2, as part of the PI 3-K/Akt pathway, a growth/survival pathway which has aberrant signalling in a number of cancers.

![Chemical structures of NU6027 and NU6227](image)

NU6027:
- mTOR IC\textsubscript{50} = 2.6 µM
- CDK\textsubscript{2} IC\textsubscript{50} = 2.2 µM

NU6227:
- mTOR IC\textsubscript{50} = 1.5 µM
- CDK\textsubscript{2} IC\textsubscript{50} = 62 µM

The development of ATP-competitive inhibitors of mTOR has been based on a series of 2,6-diaminostubstituted pyrimidines, with modest activity against mTOR, as exemplified by NU6027 and NU6227 originally designed as CDK2 inhibitors. Structure-activity relationships for inhibition of mTOR have been explored. The 4-substituent was either modified to a smaller alkoxy group or completely removed, giving reduced activity. At the 5-position compounds with other substituents were then synthesised. Modifications of the 2- and 6-amino groups were also investigated. The pyrimidine heterocycle was also replaced with two pyridine regioisomers. None of the synthesised compounds showed improved mTOR inhibitory activity over NU6227 and NU6027.

The tumour suppressor p53 is activated as a response to DNA-damage, oncogene activation and cellular stress. Once activated p53 acts as a transcription initiator, inducing the
transcription of a number of genes, including those involved in halting the cell cycle, repairing DNA-damage and initiating apoptosis. A further transcriptional target of p53 is MDM2, a negative regulator of p53. Inhibitors of the MDM2-p53 interaction have been reported including the isoindolinones e.g. NCL-00008406.

Structure-activity relationships (SAR) studies for the identification of replacement of the 4-nitro group showed that a 4-ethynyl substituent had a similar level of potency, along with the 4-bromo, 3-fluoro substituents. SARs around the isoindolinone A-ring identified the 6-tert-butyl substituent as equipotent to the parent. Synthesis of an oxetane derivative such as NCL-00018327 has demonstrated that replacing the cyclopropyl group with a 3,3-oxetane substituent either maintained or improved activity against MDM2.

Synthetic efforts have identified highly potent, low nanomolar, isoindolinone-based inhibitors of the MDM2-p53 protein-protein interaction. Optimal substituents for the benzyl group have been identified, avoiding the use of a nitro group which is toxic within drugs. Synthesis of an oxetane derivative has been shown to be either equi- or more potent than cyclopropyl derivatives, and this modification is predicted to improved the aqueous solubility by reducing the clogP. Synthesis of a tert-butyl analogues using an alternative synthetic route has
developed the SAR around the A-ring and resulted in an improved synthetic scheme. Purification of a range of MDM2 mutant proteins has identified a crystallisable form of MDM2 and the recently solved crystal structure of an isoindolinone bound to MDM2 should guide further improvements to potency and aid the incorporation of groups to improve physical properties.

Cancer is a disease in which control over cellular growth is deregulated. Synthetic efforts have been directed toward the development of two targeted anti-cancer agents; the kinase mTOR and the protein-protein interaction MDM2/p53. 2,6-diaminopyrimidine were identified as most inhibitors of mTOR, but demonstrated a flat SAR and no increase in potency was observed. Isoindolinones have been identified as a valuable scaffold to inhibit the protein-protein interaction and have demonstrated excellent potencies.
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Abbreviations

ap.  Apparent
Ar  Aryl
ATM  Ataxia-telangiectasia mutated kinase
ATP  Adenosine triphosphate
ATR  Ataxia-telangiectasia and Rad3-related kinase
CAN  Cerium ammonium nitrate
CDK  Cyclin-dependent kinase
d  doublet
DCM  Dichloromethane
DDQ  3,4-dichloro-5,6-dicyanoquinone
DIPEA  Diisopropylethylamine (Hünig’s base)
DMAP  4-Dimethylaminopyridine
DME  Dimethoxyethane
DNA  Deoxyribose nucleic acid
DNA-PK  DNA dependent protein kinase
ELISA  enzyme linked immunosorbent assay
Eq  Equivalent(s)
ESI  Electrospray ionisation
HATs  Histone acetyl transferases
HPLC  High-performance liquid chromatography
HRMS  High-resolution mass spectroscopy
Hz  Hertz
IC\textsubscript{50}  concentration required for inhibition of 50 % of target
IR  Infrared spectroscopy
K\textsubscript{d}  dissociation constant
K\textsubscript{i}  inhibition constant
KLISA  kinase linked immunosorbent assay
LCMS  Liquid chromatography mass spectroscopy
MDM2  Murine double minute protein 2
MDM4/X  Murine double minute protein 4
MS  Mass spectroscopy
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Full Form</th>
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<tr>
<td>mTOR</td>
<td>Mammalian target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mTOR complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mTOR complex 2</td>
</tr>
<tr>
<td>mp</td>
<td>Melting point</td>
</tr>
<tr>
<td>N.D</td>
<td>Not determined</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether (b.p 40-60 °C)</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol 3-kinase related kinase</td>
</tr>
<tr>
<td>PMB</td>
<td>para-Methoxybenzyl</td>
</tr>
<tr>
<td>RE</td>
<td>Responsive elements</td>
</tr>
<tr>
<td>R_f</td>
<td>Retardation factor</td>
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<tr>
<td>R_t</td>
<td>Retention time</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>s</td>
<td>singlet</td>
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<tr>
<td>SAR</td>
<td>Structure activity relationships</td>
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<td>sat.</td>
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<tr>
<td>TIPS</td>
<td>Triisopropylsilyl</td>
</tr>
<tr>
<td>TMEDA</td>
<td>Tetramethylethylene diamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Spectroscopy</td>
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1. Cancer: An Introduction

Cancer is a disease in which cellular control over growth and replication has been lost. There are around 200 different types of cancer,\(^1\) each characterised by significant changes within the genome. 298,000 new cases of cancer are diagnosed each year within the UK, with around 54% of cases being breast, lung, large bowel or prostate cancer. The 20 most common cancers by incident in 2007 are shown below (Figure 1).

![Figure 1 The twenty commonest cancers by incident in 2007 (Taken from reference 1).](image)

Cancer is predominantly a disease of older people, with 75% of cases reported in 2007 being in the over sixties age group, and 76% of cancer deaths being in the over sixty fives age group.\(^1\) Traditionally cancer has been treated by surgery, with radiation therapy emerging at the end of the 19\(^{th}\) century with the discovery of X-rays.\(^2\) However, for metastasised tumours and haematological cancers a more system wide approach is needed. The development of chemotherapeutic agents began with the observation that cytotoxic agents killed rapidly proliferating cells.\(^2\) The challenge lies in selectively killing cancer cells over normal cells and ensuring complete elimination of the tumour cell.\(^2\) Novel anti-cancer agents are constantly being developed, assisted by the increasing understanding of the biology of tumour cells.

The tumourgenesis process can be considered a Darwinian process, in which a build up of mutations confers a growth advantage over normal cells. Mutations within the genome lead either to the activation of oncogenes or the inactivation of tumour suppressors.\(^3\) The combination of the two allows cells to continue reproducing without restraint, despite the
presence of DNA damage. Hanahan and Weinberg identified six ‘hallmarks of cancer’ which can be used to describe the behaviour of nearly all cancer cells, allowing the cell to overcome the regulatory pathways within the cell to prevent cancer. The six hallmarks of cancer are

- Self sufficiency to growth signals
- Insensitivity to antigrowth signals
- Evasion of apoptosis
- Limitless replicative potential
- Angiogenesis
- Tissue invasion and metastases

In a normal cell growth signals are required before progressing through the cell cycle. However, within tumour cells the growth signals, which are transmitted via transmembrane receptors, are not required for growth. The tyrosine receptor kinases are overexpressed in a number of cancers, leading to cancer cells being hyper-sensitive to ambient levels of growth signals. Structural modifications to the receptors may lead to growth signal independent signalling, as well as mutations within the downstream signalling proteins. Growth signalling pathways are deregulated in a high number of cancers, allowing the continuing growth of tumours.

Alongside the self sufficiency to growth signals, cancer cells must also be insensitive to antigrowth signals. Within a normal cell, the cell is maintained in a quiescent state by both soluble growth inhibitors and immobilised inhibitors on the surface of neighbouring cells. For tumourgenesis to occur cancer cells must evade these signals.

These two ‘hallmarks of cancer’ allow cells to grow in an uncontrolled manner. However, for a tumour to develop, cells must also avoid death. A resistance to apoptosis is a hallmark of all tumour cells, commonly (~ 50%) this resistance is acquired via a mutation of p53, a tumour suppressor, which normally cells detects DNA-damage and can initiate programmed cell death.

A further fundamental difference between ‘normal’ cells and tumour cells is a limitless replicative ability. Normal cells cannot replicate endlessly, even in the presence of growth signals, replication can only occur for 60-70 rounds of mitosis. Telomeres are repeating units
of six base pairs at the ends of chromosomes. At each round of replication some of the telomeres are lost, and will eventually be completely degraded. This can result in chromosome fusion, which results in cell death. However, within nearly all tumour cells telomere maintenance is observed, in 85-90% upregulation of telomerase activity, which adds base pairs, giving an endless replicative ability.³

To support continuing growth angiogenesis must occur. To supply sufficient nutrients and oxygen, cells must lie within 100 µm of capillary blood vessels. Within normal cells angiogenesis is a tightly controlled process, but for tumours to progress, the development of these blood vessels must occur at an early to mid point in tumour formation.³

The final ‘hallmark of cancer’ described by Hanahan and Weinberg, is the ability to invade local tissue and metastasise to distant sites. The process of metastasis occurs within most human cancer, with tumour cells seeking a location where growth is not limited by space or a lack of nutrients. Metastasise are thought to cause 90% of cancer deaths.³

A further publication from Hanahan and Weinberg describes further characteristics which enable the development of tumours.⁴ The enabling characteristics are

- Deregulation of cellular energetics
- Genome instability and mutations
- Avoiding immune destruction
- Tumour-promoting inflammation

Deregulation of cellular energetics allows continuous growth and proliferation.⁴ Genome instability and mutations will be discussed in greater detail shortly. Avoiding immune destruction is also crucial for tumour development. Cells and tissue are continuously monitored by the immune system, which can recognise and eliminate emerging tumours. Those which survive must avoid detection by the immune system. Immunocompromised mice are more prone to carcinogen derived tumours than immunocompetent mice.⁴ The fourth enabling characteristic is tumour-promoting inflammation. Inflammation can contribute towards the development of tumours, by supplying bioactive molecules to the tumour such as growth factors, survival factors and proangiogenic factors such as extracellular matrix modifying enzymes which allows angiogenesis and metastases to occur.⁴
Luo et al suggest that the key to developing new treatments lies with identifying critical signalling pathways, and members of signalling pathways within tumour cells whose inhibition results in ‘system failure’. Luo et al have identified a further set of ‘hallmarks’ which whilst not uniquely found in cancer cells, must be tolerated for cancer cells to survive. They term these the ‘stress phenotype’.

The first of the stress phenotypes is high levels of DNA-damage. Tumours have high levels of genetic instability, partially due the loss of telomeres, allowing fusion of unprotected chromosomes, partially due to mutation within the DNA repair and DNA stress response pathway, which allows the cancer cells to survive, but also allows for accumulation of DNA damage.

The fusion of chromosomes can lead to translocation and amplification of genes. This in turn can lead to the second stress phenotype – proteotoxic stress, the stress caused by the misfolded proteins. Translocated genes and anuploidy can alter the stoichiometry of protein complexes within cells, leading to a build up of unfolded proteins which aggregating within cells.

Further ‘stress phenotypes’ include: mitotic stress, in which the shift in chromosome distribution within cancer cells leads to rapid evolution; metabolic stress, where tumour cell get their energy from glycolysis; and oxidative stress, where higher levels of reactive oxygen species are generated by oncogenic signalling, which in turn damages DNA, leading to further transformation.

These stress phenotypes contribute further to the difference between cancer cells and normal cells which can be exploited in developing selective anti-cancer chemotherapeutic agents. Luo et al have characterised cancer cells as having oncogenic and non-oncogene addiction. Oncogene addiction is the reliance of tumour cells on oncogenes to drive the tumourgenesis process, whereas non-oncogenic addiction is the reliance on genes which are not mutated, but are necessary for maintaining the tumour cell. The non-oncogene addiction includes genes which allow the tumour cells to cope with the cellular stress caused by the ‘stress oncogene’.

There are two approaches which can be taken to exploit the difference between cancer and normal cells. The first is ‘stress sensitisation’, where vital proteins within the stress support
pathway are inhibited and the cell is sensitised to cellular stress, which should induce cell death. The second approach is stress overload in which the existing stress is intensified, to overwhelm the stress support pathways, which should also lead to cell death.\textsuperscript{5}
2. mTOR: An Anti-Cancer Target

2.1 Protein Kinases and Protein Kinase Inhibitors

Protein kinases transfer the terminal (γ) phosphate group of adenosine triphosphate (ATP) to the hydroxyl groups of amino acid side-chains within proteins (Figure 2). This process activates proteins, usually by inducing a change in conformation. There are three classes of protein kinase: serine/threonine kinases, tyrosine kinases and dual kinases. The phosphorylation process can be reversed through the activity of phosphatases, resulting in free hydroxyl groups and orthophosphate (P$_i$). Without the activity of phosphatases, phosphorylation would essentially be irreversible as it is a highly favourable process, the hydrolysis of ATP to ADP has $\Delta G$ of -50.2 kCal/mol.$^6$

![Figure 2](image)

There are 518 protein kinases within the human genome.$^7$ All 518 share a highly conserved catalytic domain, alike in both sequence and structure.$^7$ The ATP-binding site lies in a deep cleft between the C- and N-terminal lobes,$^8$ and consists of: a hydrophobic pocket, which binds the purine of ATP; a hinge region, which connect the C- and N- lobes; a P loop, which acts as ceiling to the pocket and the activation loop.$^8$ The activation loop can undergo significant conformational changes as a result of its phosphorylation state. When phosphorylated (the ‘active’ form) the activation loop moves to close up a hydrophobic pocket adjacent to the ATP-binding site which is present in the unphosphorylated configuration (inactive), also known as the ‘DFG out’ configuration.$^8$ The aspartate of the DFG binds two Mg$^{2+}$ ions, which co-ordinate to the three phosphate groups of ATP on binding.$^9$
Protein kinases play a vital role within the cell, with virtually every cellular process governed by a cascade of phosphorylation. Disregulation of kinase is observed in a number of diseases, including cancer, immunological, neurological, metabolic and infectious disease. Since the success of imatinib (I) (Gleevec) protein kinases have become a major focus of anti-cancer drug discovery efforts, with compounds targeting 30 different kinases reaching the level of phase I clinical trials.

The majority of kinase inhibitors target the ATP binding site. The compounds, usually heterocycles, form one to three hydrogen bonds with the hinge region, mimicking those formed by ATP. Inhibitors may also bind to other regions around the adenine binding site including the hydrophobic pockets I and II, the ribose binding region and phosphate binding region. Selectivity can be gained from these regions, but generally not from the adenine binding residues. ATP-competitive inhibitors are also known as type I kinase inhibitors. As this class of inhibitors target the ATP-binding site the structure should be highly conserved and not alter significantly due to mutation, as mutation may not be tolerated for ATP-binding and lead to a lack of function.

**Figure 3** Representation of ATP-competitive binding site (Taken from reference 11)
Type II kinase inhibitors occupy a hydrophobic pocket adjacent to the ATP-binding site. This hydrophobic pocket is not present when the kinase is in its active form, instead it occupies a pocket formed when the activation loop is in the ‘DFG’ out configuration. Inactive conformation inhibitors occupy the hydrophobic pocket created by the inactive configuration via a hydrophobic ‘tail’ but also usually have a ‘head’ group which can interact with the adenine binding site. Inhibitors which bind to the inactive conformation are sometimes known as allosteric inhibitors, however, this term has also been applied to inhibitors such as rapamycin, which bind at a distant site and do not interact with the ATP-binding site.

![Figure 4](image.jpg)

**Figure 4** Representation of the allosteric binding site (Taken from reference 11)

Accessing the ‘DFG’ out conformation may allow greater selectivity for kinase inhibitors as residues exposed within the inactive conformation hydrophobic pocket are less conserved. Inhibitors which bind to the inactive conformation may also have a greater cellular potency, as ATP has a lower affinity for inactive conformation kinases. Imatinib (I) binds to the inactive conformation and locks the kinase in an inactive conformation, thus ensuring that the aspartate (from the ‘DFG’) cannot co-ordinate to the magnesium ion in the catalytic site.

There are two further classes of kinase inhibitors. Class III refers to the previously discussed ‘allosteric’ inhibitors which bind at a distant site, far removed from the ATP-binding site (rather than adjacent like type II inhibitors). These inhibitors usually bind in a unique manner, and use an individual mechanism to inhibit kinase activity. By avoiding the highly conserved ATP-binding site the type III inhibitors are usually highly selective. Rapamycin, a potent and highly selective inhibitor of mTORC1, acts in this manner.
Type IV are covalent kinase inhibitors. These form an irreversible covalent bond with the active site, usually by reacting with a cysteine residue in a Michael-type reaction. Type IV inhibitors usually consist of a group which bind to the ATP-binding site, thus targeting the molecule, which can then react with the nucleophilic residue.

Selectivity of a kinase inhibitor is particularly important, especially during early development of a new class of kinase inhibitor. A selective kinase inhibitor should be less toxic and will make target validation simpler, with closely related kinases most likely to share sensitivity to a particular inhibitor. A single residue within the ATP-binding site can control the sensitivity of a kinase to a particular inhibitor. This residue, known as the gatekeeper residue, controls access to the hydrophobic pocket. Kinases with a larger residue at this site have a greater range of resistance to kinase inhibitors. The gatekeeper residue does not affect binding of ATP as the hydrophobic pocket it controls access to is not exploited by ATP. Mutation of the gate keeper residue is a common mechanism of developing resistance to inhibitors, and as this area in not exploited by ATP, the kinase is still functional. Kinase inhibitors which can tolerate a variety of residues at this point can avoid this loss of efficiency. Zuccotto et al have demonstrated the influence of the gatekeeper residue on access to the hydrophobic pocket by comparing the crystal structure of CDK2 (Phe gatekeeper) and bRAF (Thr gatekeeper). The bRAF crystal structure has a larger hydrophobic pocket accessible to a small molecule, in comparison to the CDK2 inhibitor (Figure 5).

**Figure 5** Comparison of the CDK2 and bRAF kinase domain, with differing gatekeeper residues (Taken from reference 13)
With the emergence of rationally-targeted agents, kinase inhibitors have been increasingly important. Alongside this, sophisticated techniques for designing of new inhibitors have been developed, aided by NMR and protein crystallography. High-throughput screening has helped identify starting points for a number of drug discovery programmes and this has now been joined by fragment-based screening to identify new starting points.

2.2 Phosphatidylinositol 3-kinase Lipids (PI 3-K)

Phosphatidylinositol lipids are comprised of an inositol ring with a phosphatidic acid group attached via the 1'-OH group. The inositol group can be phosphorylated by ATP at all positions around the ring apart from the 2' and 6' position by lipid kinases, with the position and combination of phosphorylation effecting function. Phosphatidylinositol 3-kinases (PI 3-K) phosphorylate the membrane bound inositol ring at the 3-position. There are multiple isoforms of PI 3-K, which can be divided into 3 distinct classes, which share a homologous core catalytic domain.

Class I consists of a heterodimer made up of ~110 kDa catalytic subunit known as p110 and a regulatory or adaptor subunit. The preferred substrate of class I is phosphatidylinositol (4, 5) biphosphate (PtdIns (4,5) P_2) giving phosphatidylinositol (3,4,5) triphosphate (PtdIns (3,4,5) P_3). The activity of class I PI 3-K is reversed by the lipid phosphatise Pten (phosphatase and tensin homologue deleted on chromosome 10).

Class I can be further divided into class I_A and class I_B. Class I_A consists of one of three isoforms of p110, either α, β or δ and an adaptor protein, one of the isoforms of p85, α, β or γ. p110 isoforms α and β are ubiquitously spread throughout cells, however p110δ is far less common, mostly found within leukocytes. The adaptor protein p85 has two Src-homology 2 (SH2) domains which bind to phosphorylated tyrosine kinase receptors, allowing the PI 3-K to relocate from the cytoplasm to the membrane where the phosphatidylinositol lipids are
located. Stimulation of almost every receptor induces activation of class IA. The strengths and extent of these signals differs between signals, with insulin and PDGF signalling strongly, whilst EGFR has much weaker signalling. Class IA is also activated by Ras though the mechanism of this process is unclear.

PtdIns (3,4,5) P_3 product of class I activity acts as a secondary messenger recruiting a number of proteins to the membrane. These proteins are recruited via a domain known as the pleckstrin homology (PH), a structurally conserved area of around 100 amino acids which bind inositol lipids with high affinity.

Class IB has only one isoform of p110, the γ isoform and an adaptor protein of p101 kDa, which has no sequence homology to any other known protein. Class IB is activated by heterotrimeric G-protein coupled receptors.
Class II is larger than either classes I and III with a molecular mass of larger than 170 kDa, due to an extra C2 domain, which can bind to phospholipids. In vitro class II prefer PtdIns as substrate. Mammals have 3 class II isoforms - α, β and γ, with α and β found ubiquitously and γ primarily found in the liver. Class II is found at the cell membrane unlike class I which is found in the cytoplasm under resting conditions. The effect of class II signalling is unclear.

Class III is a homology of yeast vesicular protein sorting protein Vsp34p. In vitro class III only acts on PtdIns and is not stimulated by cell signalling. Class III is found in complex with p150, a Ser/Thr protein kinase – which targets class III to the membrane. In yeast Vsp34p has a role within protein trafficking to the vacuole and within mammals the role is thought to be similar, moving proteins to the lysosome.

2.3 Phosphatidylinositol 3-kinase-like Kinases

The phosphatidylinositol 3-kinase-like kinases (PIKK) are a family of serine/threonine protein kinases. As the name suggests, they share significant sequence similarity (20-25%) to a family of lipid kinases, the phosphatidylinositol 3-kinases, rather than other serine/threonine protein kinases. PIKK family members are large, with molecular masses ranging from 280 kDa to 470 kDa.

There are six members of the PIKK family: mammalian target of rapamycin (mTOR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia-telangiectasia mutated (ATM), ataxia- and Rad 3-related (ATR), suppressor of morphogenesis in genitalia (SMG-1) and transformation/transcription domain-associated protein (TRRAP).

The PIKK family have a range of diverse functions. mTOR controls cellular growth in a nutrient and amino acid sensitive manner. DNA-PK plays a role within non-homologous end-joining as a response to DNA double strand breaks. ATM and ATR act within the signalling pathway as a response to genome damage. ATM acts chiefly within the signalling of double strand breaks and ATR with other types of DNA-damage, such as that caused by UV light. SMG-1 is involved in the monitoring of RNA known as ‘nonsense mediated mRNA decay’ (NMD) which recognises and eliminates mRNA species which code for non-functional or harmful residues. TRRAP acts as a transcriptional co-activator, however, TRRAP does not behave as a kinase, instead functioning as a ‘scaffold’ recruiting histone acetyl transferases.
The kinase domain of PIKKs (apart from TRRAP) contains the residues vital for ATP binding seen in other ‘traditional’ kinases, the DXXXXN and the DFG motif. This lack of the kinase motif results in TRRAP being catalytically inactive. Of the catalytically active PIKKs four out of five demonstrate a preference for the phosphorylation of Ser/Thr residues followed by a glutamine residue, known as S/T Q directed kinases. However, the fifth, mTOR shows no such preference for this particular motif, and as yet no mTOR motif for phosphorylation has been identified. It has been proposed that the difference in phosphorylation site preference lies in the role of the kinase, all catalytically active PIKK enzymes which preferentially phosphorylate the S/T Q motif function within genome monitoring and repair, whereas mTOR does not, perhaps ensuring that mTOR does not interfere with genome stability.

The catalytic domain of the PIKKs is flanked on either side by domains known as FAT (FRAP-ATM-TRRAP) and FAT-C, which are always found in combination. The FAT domain lies on the N-terminal side of the catalytic domain and is made up of around 500 residues, however the function of the FAT domain is unclear. The FAT-C domain lies towards C-terminus of PIKK enzymes, it is a highly conserved region of around 30 amino acids. It has been shown to be critical for catalytic activity and mutagenesis experiments have demonstrated the importance of the conserved hydrophobic residues. It has been proposed that this region plays an important role within protein-protein interactions.

The PIKK family also share a further structure motif known as HEAT (Huntington, Elongation factor 3, Alpha-regulatory subunit of protein phosphatise 2A and Tor1) repeats. The PIKK enzymes can have as many as 40-54 HEAT repeats, with each HEAT repeat made up of a pair of interacting anti-parallel helices linked by a flexible intraunit loop. Each helix is made up of 10-20 residues, with the intraloop made up of 5-8 residues. Between 63-83% of the non-kinase domain of ATM, ATR and mTOR has identified as forming HEAT repeats and can form a superhelical structure. The HEAT repeats have been identified in all PIKK family members and are thought to function within forming macromolecules.

Within the FAT domain on mTOR lies an area known as the FKBP12·rapamycin binding (FRB) domain. The FRB lies adjacent to the catalytic domain, and binds to the mTOR inhibitor rapamycin-FKBP12 complex (to be discussed in greater detail in chapter 2.5).
110 residue region\textsuperscript{23} forms four helices, a pair of HEAT repeats, one of which is unique to mTOR.\textsuperscript{20}

\textbf{Figure 8} Structure of the PIKK with domains highlights

\textbf{2.4 Mammalian Target of Rapamycin}

\textbf{2.4.1 mTOR Complex 1 and 2}

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) forms two complexes, both of which function within the PI 3-K/Akt growth/survival pathway. mTOR
complex 1, also known as mTORC1, is the rapamycin sensitive complex and co-ordinates growth signals and the availability of nutrients and cellular energy, to induce protein synthesis. It is made up of mTOR protein, raptor, mLST8, deptor and PRAS40. Both deptor and PRAS40 (proline-rich Akt substrate of 40 kDa) inhibit the catalytic activity of mTORC1, which is relieved on cellular signalling and will be discussed in more detail in Chapter 2.4.2. mLST8 (lethal with sec13 protein 8) helps to activate mTORC1 by stabilising the mTOR/raptor interaction, and although it associates with the kinase domain of mTORC1, it does not interact with substrates. Raptor is a 150 kDa protein, with a highly conserved N-terminal domain, it has three HEAT domains and seven WD40 domains, which are important for protein-protein interactions. Raptor acts as a scaffold, recruiting and interacting with mTORC1 substrates. Disruption of either mTOR or raptor genes results in embryonic lethality, with death at around 5.5-6.5 days.

mTORC1 signalling is implicated in a number of cancers, due to its role within protein synthesis and its function within the PI 3-K pathway, which commonly has aberrant signalling within cancer. This includes mutant or over expressed PIK3CA, which is the gene coding for the PI 3-K p110 α subunit, mutation or amplification of Akt or loss of the tumour suppressor Pten.

Yip et al have determined the 3D structure of mTORC1 by cyroelectron microscopy. mTORC1 was observed to form a dimer of two mTORC1 units with a cavity within the complex, the function of which is unclear. Antibody labelling identified location of PRAS40 and mLST8 binding. Incubating with rapamycin, a macrolide inhibitor of mTORC1 (see chapter 2.5) significantly reduced the levels of mTORC1, suggesting that rapamycin acts by destabilising the complex. This effect was not observed on treatment with the ATP-competitive inhibitor Torin1.
mTORC2 is the rapamycin and nutrient insensitive mTOR complex.\textsuperscript{27} mTORC2 is made up of mTOR protein, rictor, mLST8, protor, deptor and mSIN1 (also known as mitogen-activated-protein-kinase-associated protein 1).\textsuperscript{24, 31} Rictor is the rapamycin insensitive companion of mTOR and protor the protein observed with rictor.\textsuperscript{27} Interaction of mTOR with either raptor or rictor is mutually exclusive, complexes either contain raptor or rictor.\textsuperscript{32} Rictor knockouts result in embryonic lethality slightly later at \textasciitilde 10.5 days. Interestingly, mSIN1 knockout also results in embryonic lethality after 10.5 days, indicating that mSIN1 is only necessary for mTORC2 function but not mTORC1.\textsuperscript{28} Each complex has a distinct cellular function, though both lie within the PI 3-K/Akt pathway and there are several interactions between both mTOR substrates and activators at various points within the pathway.
Guertin et al have observed that loss of the tumour suppressor Pten is associated with prostate cancer, and that mTORC2 activity is required in development of the tumour. Pten-deficient cell-line PC-3C requires rictor to form tumours with nude mice and transforms prostate epithelial cells in vivo. In adenocarcinoma driven by loss of Pten, loss of rictor impairs development of tumours, but has little effect on normal prostate tissue.

**Figure 11**

### 2.4.2 The Function of mTORC1 and 2 Within the PI 3-K/Akt Pathway

As a response to the binding of growth signals to tyrosine receptor kinases PI 3-Kα is activated. PI 3-Kα is comprised of a p110α and p85 subunits and phosphorylates membrane bound PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃. The phosphorylation of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃ can be reversed by the activity of the phosphatase and tumour suppressor Pten (phosphatase and tensin homolog deleted on chromosome ten). PtdIns(3,4,5)P₃ is a secondary messenger and recruits a number of proteins via their pleckstrin homology domain, a conserved domain of approximately 100 residues, which binds PtdIns(3,4,5)P₃ with high affinity. Recruited proteins included Akt and PDK1. mTORC2 is also recruited via the pleckstrin homology domain of mSIN1. Mutation or upregulation of the PI3KCA gene coding for p110α or loss of Pten either through mutation or deletion can result in an aberrant signalling of the PI 3-K/Akt pathway. PI3KCA mutation is common and is observed within a number of cancers including ovarian, colorectal, breast, gastric, brain and cervical whilst Pten inactivation is observed in glioblastomas, endometrial, and prostate cancers, amongst
Figure 12 A simplified PI 3-K/Akt pathway

The exact mode of mTORC2 activation is unclear. mTORC2 can be stimulated on exposure to serum, in particular serum containing insulin and insulin-like growth factor 1, suggesting that mTORC2 is activated by the PI 3-K pathway. Both mTORC2 and rictor are phosphorylated multiple times. Rictor phosphorylation at Thr1135 has been demonstrated to be sensitive to both rapamycin treatment, growth factors and levels of amino acids, which indicates that Thr1135 is phosphorylated downstream of mTORC1. An in vitro kinase assay indicated that rictor Thr1135 was phosphorylated by S6K, which is one of the target proteins of mTORC1. The TSC1/2 complex, which has previously been thought to act purely as an upstream suppressor of mTORC1 activity (discussed below) has been proposed to act as a positive regulatory of mTORC2 activity. Inactivated TSC1/2 reduces mTORC2 activity, but inactivated Rheb, which is a target of TSC1/2 activity has no effect. However the full mechanism of mTORC2 activation has yet to be deduced.

In addition to mTORC2 playing a part within actin polymerisation and cell spreading via PKCα and Rho, its crucial role within the PI 3-K pathway is phosphorylation of Akt at Ser473. This site lies within the C-terminus and is vital for full activation of Akt.
also phosphorylated by PDK1 at Thr308, but phosphorylation at Ser473 is sufficient to ensure that some kinase targets of Akt can be phosphorylated, including the TSC1/2 complex. Full phosphorylation of Akt is required before other targets such as FOXO1/3 can be phosphorylated. Akt controls the cell-cycle by regulating the FOXO protein, cyclin D1, p27 and GSK3. Akt also regulates MDM2, caspase-9, IKKα and Bad, controlling apoptosis. Akt phosphorylation of MDM2 induces translocation of MDM2 into the nucleus where it can inhibit the anti-apoptotic activity of p53. mTORC2 has also been proposed to phosphorylate SK61, a target protein of mTORC1, which belongs to the same family as Akt.

Phosphorylation and activation of Akt can either control cell survival or in aberrant signalling potentially drive tumourgenesis by increasing cell growth, proliferation and migration, as well as the shift to glycolytic metabolism. Akt phosphorylates TSC2 of the TSC1/2 heterodimeric complex at Ser939 and Thr1462. TSC1 (also known as hamartin) is a 130 kDa protein and TSC2 (also known as tuberin) a 200 kDa protein, which without Akt signalling form a stable heterodimer. When forming a stable heterodimer the TSC complex acts as a GTPase activating protein (GAP). TSC1/2 activates Rheb (Ras homolog enriched in brain) to convert bound GTP to the inactive GDP bound form. On phosphorylation by Akt the heterodimer dissociates, inactivating the GAP activity of TSC1/2. The Ras/MAPK pathway can also phosphorylate TSC1/2 complex through the kinase activity of Rsk.

![Figure 13](image-url)
As protein synthesis is both an energy-rich and nutrient-rich pathway protein synthesis must be halted in times of nutrient and/or energy starvation. The energy-sensing AMP-dependent protein kinase (AMPK), which is activated by phosphorylation at Thr172 and Thr1227 by LKB1, phosphorylates TSC2 at Ser1345 and Ser1227, which activates the TSC1/2 complex.\textsuperscript{25,35} AMPK is activated as a response to a change in AMP:ATP ratio.\textsuperscript{25} LKB1 also phosphorylates raptor at Ser792 and Ser722, promoting 14-3-3 protein binding and inactivating mTORC1.\textsuperscript{25} The tumour suppressor p53 also acts via AMPK. On sensing DNA-damage p53 upregulates the transcription of AMPK, preventing continued cell growth in the face on DNA-damage.\textsuperscript{35} AMPK can also phosphorylate p53 at Ser15,\textsuperscript{24} stabilising p53 allowing increased levels of transcription of p53 cellular targets and halting the cell cycle whilst the cell is in energy-starvation conditions.

Hypoxia is also proposed to influence the PI 3-K/Akt pathway through the TSC1/2 complex. On phosphorylation, TSC2 is proposed to binds 14-3-3 proteins. As a response to hypoxia HIF (hypoxia inducible factor) is stabilised, inducing the expression of a number of genes, including those associated with angiogenesis, and Redd1 and 2. Redd1 can complete with TSC2 for binding with 14-3-3 proteins, relieving the inhibition of the TSC1/2 complex.\textsuperscript{25} TSC1/2 can then act as a GAP, resulting in mTORC1 remaining inactive.

Destabilisation of the TSC1/2 complex results in loss of GAP-activity ensuing that Rheb existing as its active GTP-bound form. Rheb interacts with mTORC1, through a poorly understood mechanism, activating mTOR.\textsuperscript{23} As Rheb is selective for mTORC1, it is thought that the protein may interact with raptor, rather than the mTOR protein itself.\textsuperscript{28} Bai et al proposes that Rheb activation of mTORC1 is due to its interaction with FKBP38.\textsuperscript{39} FKBP38 is related to FKBP12, a protein which, when bound to rapamycin, can inhibit mTORC1 (see Chapter 2.5). FKBP38 binds to the FRB domain of mTOR (but only mTOR in mTORC1) and inhibits the kinase activity of mTORC1. Bai et al propose that GTP-bound Rheb interacts with FKBP38, which disrupts FKBP38 binding with mTORC1, relieving its inhibitory activity.\textsuperscript{39}

Cellular nutrient levels have also been demonstrated to influence mTORC1 signalling. The strongest link between mTOR and nutrients is the Rag family of small GTPases.\textsuperscript{35} The Rag family forms a heterodimer composed of either Rag A or B and Rag C or D. In the inactive state either Rag A or B is bound to GDP and RAG C or D is bound to GTP. However, in the
presence of amino acids the nucleotide binding switches through an unknown mechanism, to
the active form. The RAG heterodimer can then interact with raptor and induces the
translocation of mTORC1 to the surface of endosomes and lysosomes, where it can interact
with Rheb. Interestingly, it has been shown that withdrawal of Leu has similar effect to
starvation of a range of amino acids. The amino acid levels may be detected by a mechanism
reliant on leucine levels.

PRAS40 is an inhibitory member of the mTORC1 complex that has been proposed to bind to
the kinase domain of mTOR, preventing kinase activity. On Akt activation PRAS40 is
phosphorylated by Akt at Thr246, targeting PRAS40 to 14-3-3 binding, which relieves the
inhibitory activity of PRAS40 on mTORC1 by sequestering PRAS40. However, this may
not be the full picture for PRAS40, as it also has a TOF sequence (discussed in more detail
below), which suggests that it could be a substrate of mTORC1.

After activation mTORC1 can phosphorylate a number of target proteins involved in protein
synthesis. mTORC1 targets are thought to have a TOF (target of TOR) motif, which consists
of five amino acids – a phenylalanine, followed by two hydrophobic amino acid, then two
acidic residues. This TOF motif is thought to be important for target protein to interact with
raptor.

The two best described targets of mTORC1 kinase activity are the p70 S6 kinase 1 (S6K1)
and eukaryotic initiation factor 4E-binding protein 1 (4E BP1). Phosphorylation of 4E BP1
causes it to dissociate from eukaryotic initiation factor 4E (eIF4E). Without phosphorylation
4E BP1 is bound to eIF4E, inhibiting the translational initiation activity eIF4E. eIF4E binds
and recognises the cap, which contains a 7-methyl guanosine moiety, at the 5’ end of
mRNA and mediates transcription of the mRNA. 4E BP1 is phosphorylated at numerous
sites including Thr37, Thr46, Ser65, Thr70, Ser83, Ser101 and Ser112. Of these four are
linked to mTOR kinase activity: Thr37, Thr46, Ser65 and Thr70, with Ser65 and Thr70 lying
close to the eIF4E binding site.

After repression of eIF4E is relieved it can form the initiation complex with proteins
including eIF4G, allowing translation of mRNA with highly structured 5’-UTRs
(untranslated regions). The complex also contains eIF4F (which binds to the mRNA 5’
cap), eIF4A (an ATP-dependent helicase which unwinds mRNA secondary structure) and eIF4B which enhances eIF4A activity.\textsuperscript{33, 35, 41}

![Diagram of mTORC1 activation]

**Figure 14**

Phosphorylation of S6K1 at Thr389 in the linker region of S6K1 and Ser371 activates S6K1 as a kinase.\textsuperscript{25-26} S6K1 phosphorylates S6, the function of which is unclear. S6 was thought to increase transcription of mRNA with a TOP (tract of pyrimidine) mRNA, which is common within RNA coding for ribosomes and other regulators within the protein translation process.\textsuperscript{26} However, later evidence did not support this theory.\textsuperscript{38} Ribosome biogenesis is controlled through the activity of S6K1, which induces the translational activity of RNA polymerase I, resulted in transcription of ribosome proteins.\textsuperscript{35} S6K phosphorylates a member of the initiation complex (mentioned above) eIF4B, as well as PDCD4 (phosphorylation-dependent degradation of programmed cell death 4) which blocks association of eIF4A to the complex.\textsuperscript{33} S6K can also phosphorylate a number of other targets, including mTORC2. S6K1 is also involved in a negative feedback loop, once activated it can phosphorylate and degrade IRS-1 at Ser302, suppressing its activity, including the activation of PI 3-K.\textsuperscript{25, 28}

mTORC1 is also linked to autophagy, the process in which cells degrade damaged, redundant or dangerous proteins, providing substrates during low nutrient availability.\textsuperscript{35} mTORC1 regulates autophagy, inhibiting it at nutrient-rich conditions.\textsuperscript{25} In yeast, Atg1 plays a vital role in the inducement of autophagy. When forming a complex with Atg13 and Atg17, Atg1 functions as a kinase inducing autophagy. TORC1 phosphorylates Atg13, destabilising the complex and inhibiting Atg1.\textsuperscript{42} In humans, the homolog of Alg1 is Ulk1 and 2, and Ulk1 is
known to function in autophagy. mTORC1 interacts with Ulk1/Atg13/FIP200 (homolog of Atg17) complex and phosphorylate Ulk1 and Atg13.\textsuperscript{42}

The PI 3-K/Akt pathway has wide-reaching consequences for cellular growth and survival. Aberrant signalling within this pathway has been associated with a wide-range of cancers. As mTOR (both within mTORC1 and 2) is rarely mutated it presents an attractive medicinal chemistry target within cancer. The macrolide rapamycin was the first inhibitor of mTORC1, but also contributed to the discovery of mTOR (hence the name target of rapamycin) and is discussed in chapter 2.5, however, the development of ATP-competitive inhibitors of mTOR, which inhibit both complexes, has becoming an area of wide-ranging focus.

2.5 Rapamycin and ‘Rapalogs’

Rapamycin (2) is a macrolide produced by the bacteria \textit{Streptomyces hygroscopius}.\textsuperscript{34} Discovered during the 1970’s in a soil sample from Easter Island (Rapa Nui)\textsuperscript{34} rapamycin was originally developed by Ayerst as an anti-fungal against \textit{Candida albicans}, \textit{Crytoticoccus neoforms} and \textit{Aspergillus fumigates},\textsuperscript{43} however, development was stopped on the discovery of potent immunosuppressive activity.\textsuperscript{34} The deducing of the mechanism of action of rapamycin led to an increase in interest leading to the approval of rapamycin in 1997 as an anti-rejection agent.\textsuperscript{34} Rapamycin is commonly used in combination with cyclosporine to prevent renal graft rejection.\textsuperscript{43} However, studies at the NCI also observed anti-tumour activity within both \textit{in vivo} and \textit{in vitro} studies.

![Rapamycin Structure](image)

\textbf{Figure 15}
Rapamycin activity is derived from its activity as a potent (low and sub-nanomolar) and highly selective allosteric inhibitor of mTORC1. Rapamycin interacts with the immunophilin FK506-binding protein (FKBP12). The rapamycin-FKBP12 complex then interacts with mTORC1 via the FKBP12 rapamycin binding (FRB) domain, inhibiting the catalytic activity of mTORC1. The FRB domain is a 11 kDa region is adjacent to the PIKK domain, and the formation of the tertiary complex forms a ‘sandwich’ like structure. The mechanism by which rapamycin inhibits mTORC1 catalytic activity is unclear, the rapamycin-FKBP12 complex has been proposed to act as a physical obstruction between the mTOR catalytic domain and its substrates. The formation of this complex also appears to destabilise the interaction between mTOR and raptor, despite raptor binding further than 1000 residues away from the FRB domain. The resulting loss of the mTOR raptor complex may contribute to the loss of mTOR catalytic activity as the mTOR/raptor complex is required for catalytic activity.

![Figure 16 Structure of mTOR FRB, rapamycin and FKBP12 tertiary complex (Taken from reference 34)](image)

Rapamycin is a highly selective molecule, inhibiting only one of the two mTOR complexes. It has been proposed that rictor or a further component of the mTORC2 complex can prevent binding of rapamycin-FKBP12, either by physically blocking the FRB domain or by allosterically altering the FRB binding site on rictor binding. However, long term treatment with rapamycin decreases levels of mTORC2, which in approximately 20% of cell lines is sufficient to reduce Akt phosphorylation. It has been proposed that this is due to the inactivation of mTORC1 by rapamycin, which the cell then compensates for by forming more mTORC1, leaving insufficient mTOR protein available to form mTORC2. Guerin et al also
proposed that rapamycin-FKBP12 can bind to recently synthesised mTOR protein, preventing the formation of mTORC2.\textsuperscript{33}

Rapamycin is a white solid that is soluble in organic solvent; however it is insoluble in aqueous solutions.\textsuperscript{43} Rapamycin can only be given \textit{via} oral administration; it has rapid absorption with peak concentration reached at 2 h. However, bioavailability of rapamycin is low (only around 15\%) and can vary greatly between patients.\textsuperscript{45} Intestinal CYP450 3A enzymes and P-glycoproteins can influence absorption of rapamycin.\textsuperscript{45}

To improve the pharmacokinetic properties of rapamycin a series of semi-synthetic derivatives have been developed. All are very structurally similar to rapamycin, with modifications only at the C40 position, an area not involved in either binding to FKBP12 and mTOR.\textsuperscript{45} Four ‘rapalogs’ have so far been developed – Temsirolimus (3) (or CCI-779 from Wyeth),\textsuperscript{34, 44} Everolimus (4) (RAD001 from Novartis),\textsuperscript{34, 44} Deforolimus (5) (or Ridaforolimus) (AP23573 from Ariad)\textsuperscript{34, 44} and Zotarolimus (6) (ABT-578 from Abbott).\textsuperscript{46} All ‘rapalogs’ act \textit{via} the same mode of action as rapamycin.\textsuperscript{45}
Figure 17

Temsirolimus (3) was the earliest analogue of rapamycin. It is a water-soluble ester analogue of rapamycin and acts as a prodrug form of rapamycin. It is rapidly hydrolysed, with intravenous administration rapamycin can be detected as quickly as 15 min with peak concentration between 0.5 h and 2 h. Temsirolimus was licensed by the FDA in 2007 for use in the treatment of renal cell carcinoma and can be used as either an oral or intravenous administration.

Everolimus (4) is a hydroxyl ethyl ether analogue of rapamycin. It has immunosuppressive activity, can be used in a synergistic manner with cyclosporine and was approved in Europe for its immunosuppressant activity. In vitro everolimus has immunosuppressive activity three times lower than of rapamycin, however in vivo activity is comparable, due to the improved pharmacokinetics. Everolimus also has anti-cancer activity and was licensed in the US for treatment of kidney cancer in 2009.
Deforolimus (5) is a phosphinate analogue of rapamycin.\textsuperscript{34} Deforolimus is the newest addition to the series of rapamycin analogues.\textsuperscript{43} It is stable in organic solutions and aqueous solution at a range of pH. Deforolimus is also stable in both plasma and blood, indicating that 5 does not act as a prodrug \textit{in vivo}.\textsuperscript{43} Deforolimus had activity against a range of cell lines \textit{in vitro}\textsuperscript{43} and is currently in phase III clinical trials.\textsuperscript{48}

Zotarolimus (6) is a rapamycin analogue designed to be used as a drug to elute from coronary stents to prevent restenosis.\textsuperscript{46} It replaces the C40 hydroxyl group with a tetrazole moiety.\textsuperscript{44}

Rapamycin and its analogues are well tolerated and share similar side-effects.\textsuperscript{45} However, identification of the correct subset of patients who will benefit from rapamycin or rapalog treatment is vital as within trials less than 10\% of patients responded.\textsuperscript{34} Rapamycin treatment can trigger an S6K1 dependant negative feedback loop which leads to increased signalling from IRS1/2, leading to increased activation of Akt, as well as MEK-ERK signalling.\textsuperscript{23,33} This reduces the usefulness of rapamycin and rapalogs as a treatment for cancer. Rapamycin and rapalogs have been found to be useful in mantle cell lymphoma, renal cell carcinomas and endometrial cancer, a small number of cancers, given that aberrant signalling within the PI 3-K/Akt pathway is very common.\textsuperscript{49} However, it is thought that an ATP-competitive inhibitor of mTOR, which should in theory target equally both complexes of mTOR, will avoid this issue by targeting the pathway at two points, leading to a more pronounced effect on cellular growth and survival. An ATP-competitive inhibitor will also be useful as mTORC2 has been implicated in some cancers.\textsuperscript{33}

\textbf{2.6 mTOR Inhibitors Targeting the Kinase Domain}

The PI 3-K/Akt pathway is a key cellular pathway regulating cellular proliferation and growth, survival and protein synthesis.\textsuperscript{29} The significance of this pathway within cancer lies within frequent mutation, such as mutation or amplification of the \textit{PIK3CA} gene, which is mutated in around 15\% of tumours, making it the most commonly mutated kinase within the human genome.\textsuperscript{29} In view of the frequency with which the PI 3-K/AKT pathway has aberrant signalling in cancers, it is thought that targeting this pathway could have a wide range of therapeutic uses. There is a wide range of evidence validating kinases within this signalling cascade as therapeutic targets.\textsuperscript{29} Applying Luo et al’s theory of oncogene and non-oncogene addiction,\textsuperscript{5} a number of tumours are ‘addicted’ to the PI 3-K/Akt pathway. Targeting this
pathway on which the tumour is ‘addicted’ should selectively target tumour cells over normal cells. Crucially, mTOR itself, which is a key component of this pathway, is rarely mutated,$^{34}$ making mTOR a non-oncogene addiction. This can prove beneficial for the purpose of drug discovery as the tumour relies on this kinase for continual growth and replication but genetically mTOR is stable and unlikely to mutate, leading to small-molecule inhibitors becoming ineffective. For these reasons, mTOR has become an increasingly popular target within both academic and pharmaceutical drug discovery.

An early inhibitor of both complexes of mTOR was the natural product Wortmannin (7), which inhibits mTOR with an IC$_{50}$ of 40 nM.$^{50}$ Wortmannin is a pan PI 3-K and PIKK inhibitor, which binds in the ATP-binding site and then covalently modifies the protein, through a reaction with a Lys residue (Lys833 in PI 3-K$\gamma$)$^{51}$ and the C-21 position of Wortmannin in a Michael-type reaction.$^{52}$ Wortmannin is unstable in solution, but has some uses as probe compound.$^{51}$

![Scheme 1](image)

Scheme 1 Mechanism of covalent attack by the PI 3-K on Wortmannin

The first example of an ATP-competitive inhibitor of mTOR is the pan PI 3-K and PIKK ATP-competitive inhibitor LY294002 (8). The compound, developed by Lilly, inhibits mTOR with an IC$_{50}$ of 5 µM.$^{53}$
The compound PI-103 was synthesised as part of a programme to develop isoform selective inhibitors of PI 3-K but was also found to have off-target activity including inhibition of mTOR.\textsuperscript{54-55} PI-103 inhibits mTORC1 89\% at 0.50 µM, but has greater activity against PI 3-K with an IC\textsubscript{50} of 2, 3 and 3 nM against α, β and γ isoforms respectively.\textsuperscript{56} PI-103 also inhibits the PIKK family member DNA-PK, with an IC\textsubscript{50} of 14 nM.\textsuperscript{56} PI-103 demonstrated a rapid effect, with phosphorylation of Akt at both Ser473 (mTORC2 site) and Thr308 (PDK1 site) inhibited within 15 min of treatment.\textsuperscript{56} PI-103 also showed activity with human tumour xenografts with upregulation of the PI 3-K pathway.\textsuperscript{56} However, PI-103 has a poor metabolic profile, with greater than 70\% metabolism after 30 min incubation with human microsomes which combined with the ‘dirty’ inhibition profile of PI-103 would be undesirable as a drug.

\[ \text{PI-103} \]

Stauffer et al have demonstrated that the imidazo[4,5-c] scaffold is a privileged scaffold for the development of ATP-competitive kinase inhibitors.\textsuperscript{57} From this series two dual PI 3-K/mTOR inhibitors have been developed, NVP-BEZ235 (10) and NVP-BBD130 (11), are both potent and stable, orally administered agents.\textsuperscript{58} NVP-BEZ235 has IC\textsubscript{50}s of 4, 75, 7, 5 nM against PI 3-K isoforms α, β, δ and γ respectively. 10 also inhibits mTOR with an IC\textsubscript{50} of 20.7 nM within a KLISA assay and \textit{in vitro} has an IC\textsubscript{50} of 6.5 nM measuring reduction of phosphorylation of S6 at Ser235.\textsuperscript{59} NVP-BBD130 has IC\textsubscript{50}s of 72, 2340, 201 and 382 nM against PI 3-K isoforms α, β, δ and γ respectively, and an IC\textsubscript{50} against mTOR of 7.7 nM determined by measuring phospho S6.\textsuperscript{58} By measuring autophosphorylation of DNA-PK on treatment with NVP-BEZ235, inhibition of DNA-PK was observed, though only at high concentrations of NVP-BEZ235.\textsuperscript{59}

Docking of NVP-BEZ235 into a PI 3-Kγ-based 3-D homology model of PI 3-Kα kinase domain demonstrated that 10 forms three H-bonds with the protein. The core quinoline nitrogen forms one H-bond with PI 3-Kα Val851, the quinoline substituent nitrogen forms a hydrogen bond with the backbone of Asp933 and the nitrile nitrogen interacts with the side-
chain of Ser774. Hydrophobic interactions with Met772, Tyr836 and Met922 also contribute to binding. Homology modelling with mTOR suggests similar binding, including replication of the H-bond to the Ser2165 (mTOR numbering) side chain.

Treatment of Pten-null cell-lines PC3M and U87MG with 10 demonstrated a dose-dependent effect on cell-proliferation, with a GI\textsubscript{50} of 10-12 nM. NVP-BEZ235 has a cytostatic effect, inducing growth arrest at G1 due to downregulation of cyclin D1. However 10 can increase the efficiency of anticancer drugs, such as temozolomide in combination. Treatment of tumour-bearing mice with both NVP-BEZ235 and NVP-BBD130 reduced neovascularisation, without effecting pre-existing vessels. Both NVP-BEZ235 and NVP-BBD130 have a good pharmacological profile, with high and prolonged cellular exposure. Treatment of PC3M with a 50 mg/kg dose of NVP-BEZ235 resulted in a plasma C\textsubscript{max} of 1.68 µM at 0.5 h and a C\textsubscript{24} of 0.03 µM. Within the tumour tissue a C\textsubscript{max} of 2.05 nM was observed after 1 h, which after 24 h was decreased to 0.23 nM. NVP-BEZ235 is currently in phase I/II clinical trials in solid tumours.

During synthetic efforts to develop dual tyrosine and PI 3-K inhibitors a number of mTOR inhibitors were developed. PP242 and PP30 share a pyrazolopyrimidine scaffold and inhibit mTOR with an IC\textsubscript{50} of 0.008 and 0.080 µM respectively. PP242 also demonstrates a minimum of 10-fold selectivity over all isoforms of PI 3-K, and in a screen against 219 protein kinases at a concentration 100-fold above that of the mTOR IC\textsubscript{50}, only demonstrated at 90% inhibition of one kinase and three at 75%. Due to the selectivity of PP242 and PP30 over PI 3-K\textalpha, the functions of mTOR within the PI 3-K/Akt can be explored and the different response to mTOR kinase domain inhibition and rapamycin can be explored. PP242 was shown to have a dose-dependent inhibition of proliferation, and inhibited phosphorylation of
4E BP1 at Thr36, Thr45 and Ser65 to a greater extent than rapamycin, suggesting that some functions of mTORC1 are insensitive to rapamycin treatment.\textsuperscript{61}

Development of the Torin series of inhibitors has resulted in a highly potent mTOR inhibitor. During SAR exploration of 6-(pyridin-3-yl)-N-(3-(trifluoromethyl)phenyl)quinolin-4-amine (14) it was observed that compound 10 contained a cyclic urea moiety which constrains substituents in the ideal conformation for biological activity. Luo \textit{et al} decided to incorporate a six-membered lactam to hold the trifluoromethyl substituted aniline in the optimal position which after further optimisation, and addition of an acetyl substituted piperizine gave compound (15), which was observed to improve biological activity against mTOR from 5 \(\mu\)M to 5.4 nM.\textsuperscript{62} Compound 15 was then docked into an mTOR homology model based on the crystal structure of PI 3-K\(_\gamma\). It was proposed that compound 15 forms a hydrogen bond between the quinoline nitrogen and Val2240 in the hinge, with the quinoline substituent positioning in an inner hydrophobic pocket consisting of Glu2190, Leu2192, Asp2195, Tyr2225, Asp2357, Phe2358, Gly2359 and Asp2360 and the substituent quinoline nitrogen is proposed to form a hydrogen bond with Tyr2225 side-chain. The phenyl piperizine substituent positioned below a loop consisting of residues 2183-2187 and the acyl carbonyl carbon is thought to form a hydrogen bond with the amine of Lys2186.\textsuperscript{62} Optimisation of the acyl substituent to a propyl amide increased activity to an IC\(_{50}\) of 0.29 nM against mTOR, and a cellular IC\(_{50}\) of 2 nM, this compound was named Torin1 (16) and subjected to further biochemical analysis.\textsuperscript{62}

Torin1 has 800-fold selectivity over PI 3-K and is highly selective over other PIKK enzymes with the exception of DNA-PK. Within LanthaScreen analysis 16 has an IC\(_{50}\) against mTOR of 4.32 nM and against DNA-PK of 6.34 nM, however, within a radiometric kinase assay
against DNA-PK it has an IC$_{50}$ of approximately 1 µM.\textsuperscript{62} Both human and mice microsome stability studies revealed a half-life of 4 min, and \textit{in vitro} studies demonstrated a half-life of 0.5 h, which is proposed to be due to high first-pass metabolism. Intraperitoneal dosing resulted in suppression of phosphorylation at Akt Ser473 for 2-3 h and phosphorylation of S6 (an indirect target of mTORC1 activity) was inhibited for 6 h in the lungs and 10 h within the lungs.\textsuperscript{62} A U87MG xenograft model, which is a Pten-null glioblastoma cell line, demonstrated 99% inhibition of growth inhibition after 10-days dosing, but growth returned after cessation of treatment, suggesting Torin1 is cytostatic.\textsuperscript{62}

Due to the pharmacokinetic problems, poor water solubility and low oral bioavailability of Torin1 further synthetic efforts were directed towards identification of a further compound with improved properties.\textsuperscript{63} It was proposed that by reducing the molecular mass of 16 a more water-soluble compound could be developed, which would potentially reduce the sites for metabolism.\textsuperscript{63} Removal of the piperizine moiety, and replacement of the substituent quinoline groups with amino-substituted pyridine resulted in compound 17 Torin2. Torin2 has an EC$_{50}$ of 0.25 nM and is estimated to be 10-fold more potent than Torin1. Torin2 also maintains 800-fold selectivity over PI 3-K.\textsuperscript{63} 17 is predicted to bind in a similar mode to compound 15, with the hinge hydrogen bond maintained. The pyridine nitrogen forms a hydrogen bond to Tyr2225. The pyridine amino substituent forms two hydrogen bonds with Asp2195 and Asp2357.
2. mTOR: An Anti-Cancer Target

Torin2 displayed improved pharmacodynamics over Torin1, with over 95% inhibition of phosphorylation of S6K Thr389 and Akt Thr308 after 6 h in lung and liver tissue. 17 also has a $t_{1/2}$ of 11.7 min in liver microsomes and on intravenous and oral dosing in mice had a $t_{1/2}$ of less than 2 h. Torin2 has improved oral bioavailability of 51%. 63 Lanthascreen assay analysis of Torin2 has an $IC_{50}$ of 2.81 nM (against mTOR) and 0.5 nM (against DNA-PK), though kinase assay activity of 17 against DNA-PK has not been reported.

Development of Torin1 has also supported the findings of Feldman et al who demonstrated that ATP-competitive inhibitors of mTOR inhibited a greater range of mTORC1 phosphorylations than rapamycin. 61 Treatment of MEFs (mouse embryonic fibroblasts) with Torin1 reducing protein synthesis by 50%. 64

KuDOS pharmaceuticals published two series of ATP-competitive inhibitors of mTOR in 2009. 65-66 The first series is based on either a pyrimidine or triazine scaffold, optimisation gave compound 18, with an $IC_{50}$ of 0.023 µM, and selectivity over PI 3-Kα, with an $IC_{50}$ > 10 µM. However, this series shown disappointing cellular activity, which was possibly due to poor cell permeability. 66 The second series was based on pyridopyrimidine scaffold, with optimisation leading to compound 19. 65 Compound 19 (also known as Ku-0063794) has an $IC_{50}$ of 0.016 µM. Cell-line U87MG was treated with 19 for 2 h and inhibition of phosphorylation of S6 at Ser235 and Akt Ser473 was measured with $IC_{50}$s of 0.10 and 0.15 µM observed for these targets respectively. Treatment of the T47D breast cancer cell line with 19 resulted in a GI$_{50}$ of 0.35 µM. 65 On treatment of 76 protein kinases with 1µM of 19, no significant inhibition was observed and at 10 µM only one kinase showed any significant degree of inhibition, the MAPK kinase-1, with 55% inhibition at 10 µM. This panel also included PI 3-Kα and β. 67
As with previous compounds Ku-0063794 has a greater effect on 4E BP1 phosphorylation than rapamycin and induces G₁ growth arrest. Treatment of HEK-293 (human embryonic kidney) cells with 19 resulted in almost complete loss of S6K activity, resulting in a decrease of phospho-S6. This effect was rapid, with maximal inhibition observed within 10 min. Inhibition of Akt phosphorylation at Ser473 was also observed, with a knock-on decrease in Akt target phosphorylations.67 Garcia-Martinez et al also proposes that Ku-0063794 is more selective than PP242. At 1 µM 22 out 76 kinases were inhibited by PP242 at > 50%, and 46 out of 76 were inhibiting at > 50% at a concentration of 10 µM, unlike Ku-0063794, which only inhibits one other kinase > 50% at 10 µM.67

Further development resulted in AZD8055 (20), a potent, selective and orally available ATP-competitive inhibitor of mTOR.68 AZD8055, is currently in phase I/II clinical trials for solid tumours, including hepatocellular carcinoma.49 20 has an IC₅₀ of 0.13 nM against truncated mTOR, and 0.8 nM against mTOR extracted from HeLa cells. A range of ATP concentrations resulted in a linear IC₅₀, indicating 20 is ATP-competitive, and has a Kᵢ of 1.3 nM. Counterscreening against class I and II PI 3-K, DNA-PK and ATM, demonstrated a 1000-fold selectivity for mTOR and a panel of 260 other kinases demonstrated no significant activity at 10 µM.49 In MDA-MB-468 cells an IC₅₀ of 24 nM and 27 nM was calculated for phosphorylation of Akt Ser473 and S6 Ser235.49 Xenograft studies with U87-MG bearing mice observed that after a 10 mg/kg oral dose phosphorylation of Akt at Ser473 had decreased to 5% of control level after 20 min, and inhibition of phosphorylation was greater than 50% for at least 8 h. Phosphorylation of S6 at Ser235 was decreased to 21% of control level after 20 min, though after 8 h 63% of control level of phosphorylation was observed.49

One of the most potent and selective mTOR inhibitors has been developed by Wyeth Pharmaceuticals, who have published a number of articles on the development of ATP-competitive inhibitors of mTOR.69-76 The Wyeth compounds e.g. 21-23 contain a pyrazolopyrimidine core, where potency was achieved with morpholine, piperidine and
aromatic substituents, but replacement of the morpholine moiety with a bridged morpholine groups in the 4–position achieved selectivity over PI 3-Kα. Docking of this series of compounds into an mTOR homology model, based on the crystal structure of PI 3-Kγ suggested that the morpholine group forms a hydrogen bond with a hinge residue Val2240 backbone NH. The pocket the morpholine groups occupy is defined by Tyr867 and Cys885 (PI 3-Kγ numbering), which limits the size of the substituent, tolerates an ethyl bridged morpholine, but not a dimethyl morpholine. Within mTOR, the bridged morpholine derivative can still interact with Val2240, however, within PI 3-K, this interaction cannot occur, resulting in a loss of PI 3-K activity. A single change in amino acid is thought to be the grounds for this selectivity. mTOR has a smaller Leu residue, which tolerates the bridged morpholine group, whilst PI 3-K has a Phe residue, which creates a smaller pocket, ensuring the bridged morpholine is not tolerated.

WYE-132 (24) is the most potent and selective compound developed in this study, with an mTOR IC₅₀ of 0.21 nM and PI 3-Kα IC₅₀ of 1180 nM, giving 5619 fold selectivity. 23 has high selectivity over ATR, with an IC₅₀ of >10000 nM and hSMG1 (IC₅₀ = 1250 nM) and in a panel of 230 protein kinases was largely inactive. 24 also has a LNCap cellular assay IC₅₀ of 2 nM and was shown to have metabolic stability with a half life of greater than 30 min in murine microsomes and 26 min in human microsomes.
The selectivity of WYE-132 was tested using biomarkers in a nude mouse MDA361 xenograft model. Treatment with WYE-132 showed complete inhibition of phosphorylation at the mTORC1 biomarker S6K Thr389, sustained suppression of the mTORC2 biomarker Akt Ser 473 and complete inhibition of the downstream biomarker S6 Ser 240/244 for 8 h at a dose of 25 mg/kg. No phosphorylation was observed at the PI 3-K biomarker Akt Thr308, demonstrating the selectivity of this compound. Within the nude mouse model a dose of 25 mg/kg resulted in complete inhibition of growth of the tumour but with no significant weight loss within the mice. WYE-132 has been demonstrated to induce a G₁ arrest, and like other compounds has been demonstrated to fully inhibit 4E BP1, unlike the rapalog CCI-779. Treatment of an oral 5 mg/kg dose of 24 in a MDA361 tumour resulted in a growth delay in a dose-dependent manner, and within MDA361, BT474, LNCap and H1975 cell-lines, which all have both PI 3-K/mTOR and Her-2 hyperactivation, a significant apoptotic response was observed.

Wyeth also published on an alternative series of mTOR inhibitors which replace the pyrazolopyrimidine core with a thienopyrimidine core e.g. 25 (IC₅₀ = 0.7 nM). Potency in this series of compound is good, as is selectivity, but cellular potency is slightly lower than in the pyrazolopyrimidine series. Further work identified a dimorpholine substituted triazine moiety as having activity against mTOR.
to increase selectivity for mTOR over PI 3-Kα, and side chains at 4-position of the ureidophenyl type of previous molecules such as 24 increased potency. Unfortunately, the bridged morpholine groups were seen to be the primary site of metabolism of these compounds. Synthetic efforts were then directed towards identification of alternative groups for the 2- and 6-position, that maintains potency and selectivity over PI 3-Kα, as well as identification of the optimal position of the 4-position. The optimal replacement for the bridged morpholine groups were found to be either a tetrahydropyran group or a (R)-3-methylmorpholine. For the 4-position substituent, a basic group was found to improve cellular activity, though selectivity was reduced in comparison with previous analogues (e.g. 24), a range of compounds were synthesised, including 26 with an IC$_{50}$ of 0.2 nM, 27 with an IC$_{50}$ of 0.7 nM and 28 with an IC$_{50}$ of 0.7 nM.

27 has the poorest selectivity over PI 3-Kα, only 131-fold, with both 26 and 28 having at least 400-fold selectivity for mTOR over PI 3-Kα. The highest cellular IC$_{50}$ were observed for 27 with values < 0.8 nM in both LNCaP and MDA468 cell-lines.

Along with these series of selective ATP-competitive inhibitors of mTOR, Wyeth have also published a number of dual PI 3-K/mTOR inhibitors, including PKI-402 (29). These dual PI 3-K/mTOR inhibitors have a triazolopyridimidine core and have a morpholine group on the 4-position of the pyrimidine. The structure of PKI-402 is show below. PKI-402 has an IC$_{50}$ of 1.4, 9.2 and 1.7 nM for PI 3-Kα, γ and mTOR respectively and an IC$_{50}$ of 8 nM in MDA-361 cell lines. Compound 29 was also shown to induce cleaved PARP enzymes, a marker of apoptosis.
Sutherlin et al. have also developed a dual mTOR/PI 3-K inhibitor. GNE-493 (30) is an orally available inhibitor and has good selectivity over other kinases.\textsuperscript{82} Compound 30 has an IC\textsubscript{50} of 3.4 and 30 nM for PI 3-K\textalpha{} and mTOR respectively and in nude mice has a half life of 3.6 h.\textsuperscript{82} GNE-493 was also shown to be 0.5-2 more potent within PC3 and MCF7 cell lines than a PI 3-K inhibitors alone.\textsuperscript{82}

The dual PI 3-K/mTOR inhibitor GSK2126458 (30), is currently in phase I clinical trials for solid tumours or lymphomas.\textsuperscript{49} 30 was originally developed as a potent PI 3-K inhibitor and has a PI 3-K\textalpha{} IC\textsubscript{50} of 0.04 nM, but is also active against other isoforms of PI 3-K. 30 was also found to have both mTOR and DNA-PK activity, with a \( K_i \) of 0.18 and 0.3 nM against mTORC1 and mTORC2 respectively, and a DNA-PK of 0.28 nM.\textsuperscript{83} GSK2126458 was crystallised with PI 3-K\( \gamma{} \), which demonstrates that the pyridyl nitrogen forms a H-bond with a conserved water molecule, the sulphonamide interacts with Lys833, the difluorophenyl group sits within a hydrophobic pocket and the quinoline nitrogen forms a hydrogen bond to Val882 within the hinge.\textsuperscript{83}

In cellular assays, 30 induces G\textsubscript{1} arrest and in vivo has good oral bioavailability and low clearance. Monitoring of pharmacodynamic response indicated that a single dose of 300 \( \mu \)g/mL resulted in a decrease in phosphorylation of Akt for 10 h, with a return to control
levels after 24 h. Within a BT474 xenograft model treatment with 30 resulted in dose-dependent decrease in tumour growth.

![GSK2126458](image)

D’Angelo *et al* have described the synthesis of a series of benzothiazole-based dual PI 3-K/mTOR inhibitors.\(^{84}\) Compound 31 has an IC\(_{50}\) against mTOR of 2.0 nM and a \(K_i\) against PI 3-K\(\alpha\) of 1.2 nM, though 31 also inhibits PI 3-K\(\beta\), \(\gamma\) and \(\delta\) with \(K_i < 5\) nM, DNA-PK with an IC\(_{50}\) of 3 nM and hVsp34 with a \(K_i\) of 31 nM.\(^{84}\)

Crystallisation of 31 with PI 3-K\(\gamma\) identified that the \(N\)-acetylbenzothiazole forms two hydrogen bonds with Val882 backbone, and a hydrogen bond with Tyr867 and Asp841 via a water molecule to the pyridine ring nitrogen.\(^{84}\) 31 displayed stability to both human liver microsome and rat liver microsomes of greater than 50 (µL/min)/mg, and in vivo, after intravenous administration had clearance of 0.007 (L/h)/kg and a half life of 19 h. Given orally to rats, 31 has an oral bioavailability of 103%.\(^{84}\) Pharmacodynamic analysis of 31 identified a dose dependant decrease in the phosphorylation of Akt Ser473, and significant inhibition was observed for 24 h after dosing.

![Compound 31](image)

However, further investigation revealed that in vivo compound 31 is metabolised to the deacetylated compound.\(^{85}\) To avoid this two approaches were taken. In the first, the \(N\)-acetyl amino benzothiazole moiety was replaced with a range of 6,6-heterocycles, which negated the requirement for the acetamide moiety of compound 31.\(^{85}\) The second approach replaced the benzothiazole moiety with a range of 6,5-bicyclic heterocycles, with the aim of identifying a more stable acetamide moiety.\(^{86}\)
The N-acetyl 2-aminobenzothiazole moiety forms two key hydrogen bonds with Val882 backbone, the benzothiazole nitrogen interactions with Val882 NH and the amino hydrogen atom interacts with the carbonyl of Val882.\textsuperscript{85} It is these interactions which any 6,6 heterocycle must reproduce to maintain activity. It was also postulated that substitution from either the 3- or 4-position of a 6,6-heterocycle could probe the ribose pocket and lead to a further increase in potency.\textsuperscript{85} After synthesis of a range of heterocycles a quinoline was found to maintain potency comparable to the N-acetyl 2-aminobenzothiazole analogue. Substitution with a morpholine group at the 4-position of the quinoline ring which lead to a further increase in potency, gave 32, with an IC\textsubscript{50} against PI 3-Kα of 0.6 nM, and \textit{in vitro} activity of 4.6 nM against PI 3-Kα and 3.9 nM against mTOR. Compound 32 is also active against all isoforms of PI 3-K, hVsp34 and DNA-PK.\textsuperscript{85}

X-ray crystallography of compound 32 bound in PI 3-K γ active site revealed that the quinoline nitrogen forms a hydrogen bond with Val882 backbone NH, the hydrogen bond formed between the pyridyl nitrogen Asp841 and Tyr867 in compound 31 is maintained, though the morpholine ring points out towards solvent, rather than toward the ribose pocket as predicted.\textsuperscript{85} 32 has good metabolic stability in both rat liver microsomes and human liver microsomes, \textit{in vivo} clearance was good (0.17 L/kg/h) and good bioavailability (92%).\textsuperscript{85} \textit{In vivo} pharmacodynamic modelling demonstrated that after 3 h, a 3 mg/kg dose resulted in 97% inhibition of Akt Ser473 phosphorylation. In a Pten-null U-87 MG glioblastoma model oral dosing of 10 mg/kg daily reduced growth to 28% of control.\textsuperscript{85}

![Chemical Structure of Compound 32](image)

Design of alternative 5,6-heterocycles to replace the benzothiazole moiety identified imidazopyridazines as having good potency. Compound 33 has a \( K_i \) of 1.4 nM against PI 3-Kα (with activity also observed against other isoforms), an IC\textsubscript{50} of 0.4 nM against mTOR and
an IC$_{50}$ of 13 nM for the phosphorylation of Akt within U87 MG. Incubation with hepatocytes and microsomes revealed the acetyl group of compound 33 was stable, with only trace amounts of deacetylation observed. In vivo 33 had low clearance 0.04 L/h/kg and an oral bioavailability of 46%. A xenograft model with U87 MG, demonstrated that 3mg/kg once a day dosing resulted in tumour stasis.

The mTORC1/2 inhibitor OSI027 is currently in phase I clinical trials for solid tumours and lymphomas. The structure of OSI027 has not currently been revealed, but synthetic efforts directed towards the discovery of OSI027 have recently been published. Crew et al describe the optimisation of a series of imidazo[1,5-a]-pyrazine compounds. Several other inhibitors of mTOR are currently in clinical trials but the structure of these compounds has not been disclosed. These include XL765, a dual mTOR (IC$_{50} = 157$ nM) and PI 3-Kα (IC$_{50} = 39$ nM) inhibitor in phase I clinical trials for solid tumours, non-small-cell lung cancer and malignant glioma and phase I/II for breast cancer, and GDC0980 a further dual inhibitor with an IC$_{50}$ of 17.3 nM (against mTOR) and 4.8 nM (against PI 3-Kα). GDC0980 is currently in phase I trials for non-Hodgkin’s lymphomas and solid tumours. An mTOR selective inhibitor currently in phase I trials is INK128, with an IC$_{50}$ against mTOR of 1 nM.

All ATP-competitive inhibitors of mTOR are either selective for mTOR or dual PI 3-K/mTOR inhibitors. Whether an mTOR selective inhibitor or a dual PI 3-K/mTOR will be optimal has yet to be established. Inhibition of both mTORC1 and mTORC2 is well tolerated within adult tissue. During mouse models of leukaemia with PP242, only a mild effect was observed on normal lymphocytes. However, selective mTOR inhibitors can potentially suffer from problems due to the S6K negative feedback loop. Lack of mTORC1 signalling results in continued signalling from IRS-1, which results in the conversion of PtdIns (4,5) P$_2$ to PtdIns (3,4,5) P$_3$ recruiting PDK1. As PDK1 phosphorylation is not inhibited by selective mTOR inhibitor, loss of mTORC1 signalling results in PDK1 phosphorylation of Akt, which if a suboptimal dose of an mTOR selective inhibitor is used, can result in full Akt activation.
Rodrik-Outmezguine et al have observed that treatment of a number of cell lines with AZ8055 resulted in inhibition of phosphorylation of Akt Ser473, S6K and 4E BP1 for greater than 24 h. Inhibition of Akt Thr308 was also observed, although after 4 h phosphorylation levels began to return to baseline levels, as well as the phosphorylation levels of Akt targets including PRAS40 and FOXO1/3, inhibition of Akt Thr308 is only temporary. Treatment of PP242 was also observed to have the same effect, and this effect was identified as being due to reactivation of Akt as a result of AZ8055 and PP242 activation of PI 3-K. However, cotreatment with either an Akt inhibitor or an HER kinase inhibitor, the phosphorylation of PRAS40 and FOXO1/3 was not observed. In cells treated with both a Akt inhibitor and AZ8055 an increase in the level of apoptotic cells was observed, as well as an increase in levels of cleaved PARP and caspase-3.

A PI 3-K/Akt inhibitor would avoid the activation of Akt but may prove more toxic to normal cells. In studies with PI-103, a toxic effect on normal lymphocytes was also observed, however, this could be due to the slightly promiscuous profile of PI-103. The current clinical trials should illuminate which class of inhibitor is optimal for tumours addicted to the PI 3-K/Akt pathway.
3. MDM2/p53: An Anti-Cancer Target

3.1 Inhibitors of Protein-protein Interaction

Inhibition of protein-protein interactions is an important area within medicinal chemistry. Protein-protein interactions control a number of cellular processes including mammalian immune response, recognition between cells, signal transduction and transcription. These interactions are not random but the result of the particular structures of each protein involved.\(^{89}\)

The development of small molecule protein-protein inhibitors faces a number of challenges. Unlike enzymes, where the active site is small and frequently shielded from solvent,\(^{90}\) protein-protein interactions occur over large areas (750 - 1500 Å on each protein)\(^ {89}\) and are relatively flat. Interactions can also occur over non-adjacent binding domains.\(^ {90}\) Small-molecules require pockets into which they can bind. In enzymes, small molecules can exploit the active site. However, with protein-protein interactions identifying a small pocket may be more difficult.\(^ {89}\) For some protein-protein interactions, although the area of the interaction may be large, the binding may be due to a small number of high affinity interactions between a small number of residues and any disruption of the binding could result in a biochemical effect.\(^ {89}\) These areas of high affinity residues are known as a ‘hot spots’, and were identified in 1995\(^ {91}\) by Wells and Clackson using mutagenesis studies.\(^ {92}\) These studies involved a process known as alanine scanning, in which residues at the face of the protein-protein interaction are mutated to alanine, to identify the individual contribution to the binding energy of each residue.\(^ {93}\) This analysis identified that residues contributed unevenly to the binding interaction, with some labelled ‘hot spots’ strongly contributing to the binding interaction. To be defined as a ‘hot spot’ a residue must cause an increase in free binding energy of 8.37 kJ/mol on mutation to an alanine.\(^ {93}\) The average number of ‘hot spots’ at the face of a protein-protein interaction is 9.5%.\(^ {93}\) They are usually hydrophobic regions with buried charged regions. ‘Hot spots’ are complementary to each other, with residues on one protein fitting into a pocket on the other protein.\(^ {91}\)

Therapeutic proteins and antibodies have shown success in inhibiting extracellular protein-protein interactions, however, as these are not usually cell permeable or orally bioavailable there is a need for small-molecules to disrupt cellular protein-protein interaction.\(^ {94}\) To
develop inhibitors of the protein-protein interaction compounds must mimic protein surfaces, such as α-helices and β-sheets, and are known as *surface mimetics* or *proteomimics*. These challenges ensure not all protein-protein interactions are druggable, and attempts to develop small molecules to inhibit interactions are very dependent on the unique structure of each protein; those with ‘hot-spots’ are most likely to be druggable. Each protein-protein interaction must be carefully chosen before beginning development. Blundell *et al* proposed that interactions between one rigid component, and one more flexible component, which only forms a secondary structure on binding to the partner, may prove to be the most druggable protein-protein interactions. The p53 MDM2 protein-protein interaction is an example of a protein-protein interaction of this type and is discussed in more detail in chapter 3.2. The use of peptides may aid the identification of druggable protein-protein interactions. If a small fragment of peptide from one of proteins is able to bind to the second this suggests a suitable pocket exists for the targeting of small molecules.

The ‘rules’ for designing small molecule inhibitors of protein-protein may be different to typical enzyme inhibitors. Ligand efficiency may be much lower as the binding region is a large hydrophobic region. Abbott’s compound ABT-263 (33) fails Lipinski’s criteria, with a molecular mass of well over 500 Da but is orally available and has good cellular activity. There is currently only one non-natural protein-protein inhibitor on the market. Maraviroc (34) is an anti-HIV agent developed by Pfizer; however, this molecule does obey Lipinski’s rule. Maraviroc is a chemokine co-receptor 5 antagonist, antagonism of CCR5 (a GPCR) by Maraviroc inhibits HIV binding, preventing virus entry into the cell. Targeting protein-protein interactions are becoming an increasing area of interest within drug discovery with the emergence of new techniques for drug discovery.
3.2 p53, MDM2 and MDMX

p53 is a tumour suppressor that was simultaneously discovered in 1979 by a number of groups. Named after its apparent mass of 53 kDa, p53 acts as a tumour suppressor through its ability to act as a transcription factor and instigator of apoptosis.\(^9\) p53 acts as the cells’ regulator of growth arrest, cell cycle senescence and apoptosis as a response to cellular stress. Under stress conditions, cellular levels of p53 rapidly increase to prevent proliferation of cells containing damaged DNA.\(^10\) It is highly conserved throughout, over both vertebrates and invertebrates, underlining its crucial cellular function.\(^11\) The importance of p53 as a tumour suppressor is underlined by one in two cancers carrying a mutation of p53, and remaining cancers negate p53 by inactivation of associated pathways.\(^12\)

**Figure 19** Structure of p53

The tumour suppressor p53 is a 393-residue protein, consisting of a number of domains (see Fig 19) including a transactivational domain, an unfolded acidic region which acts as a promiscuous binding site for proteins such as MDM2 and p300/CBP (which are discussed in more detail later). A short section of this domain (residues 15-29) is able to form an α-helix.
on binding to MDM2. The DNA-binding domain is a structured region which interacts with DNA in a sequence specific manner. It interacts with both the minor and the major groove of DNA, the minor groove via two loops which contain a Zn$^{2+}$, co-ordinated to His179, Cys176, Cys238 and Cys242 and the major via loop-sheet-helix motif. Although p53 is a monomer under low concentrations, under stressed conditions it can form a tetramer. p53 is transcriptionally active as a tetramer, monomeric p53 can bind to DNA but its affinity is 10-100 times lower than that of tetrameric p53. The tetramer is made up of four identical p53 chains – 393 residues in length. The tetramer is formed by residues 325-356, which form the tetramization domain. The quaternary structure is made up of a dimer of dimers, with each monomer consisting of a short β-strand (residues 326-335) and an α-helix (335-355) linked by Gly334. The monomer forms a V shape, with hydrophobic residues Ile332, Phe338 and Phe341 forming an hydrophobic cluster at the hinge of the V structure. Two monomers interact via β-sheets, forming an anti-parallel double strand, with the two helices forming a double helical bundle. The anti-parallel β-sheet has eight backbond H-bonds. The two dimers interact through the α-helices, forming a four-helix bundle. The tetramer is stabilised by hydrophobic interactions. The formation of tetramers also aids accumulation of p53 within the nucleus. The leucine-rich nuclear export signal located between residues 340-351 is on the monomer surface but is hidden when the tetramer is formed. For nuclear export to occur the tetramer must dissociate.
Murine double minute gene 2 (MDM2) is a 90 kDa protein, with human MDM2 containing 491 amino acids. MDM2 was first discovered in mouse chromosomes from abnormal chromosomes known as double minutes, and a human homolog was later discovered. In 1992, MDM2 was discovered to interact with p53, blocking the biological activity of p53. MDM2 achieves this by firstly blocking the transactivation domain of p53 and secondly acting as a p53 specific E3 ubiquitin ligase. The E3 ubiquitin ligase activity of MDM2 catalyses the ubiquitylation of p53, which in turn labels the protein for degradation by proteasomes. MDM2 is one of the target genes of p53 transcriptional activity and as MDM2 inhibits and marks p53 for degradation, these two proteins are locked in a negative feedback loop. The negative feedback mechanism allows p53 to be kept at low cellular concentration under unstressed conditions. When the stress pathway is activated, p53 is stabilised and cellular concentrations increase. As a response, MDM2 levels are also increased, which will bring the level of p53 back down to its previous low concentrations after the stress signalling has been removed. MDM2 has a number of different domains which are shown below.

Figure 20 Tetramer of p53 – each colour represents a monomer unit (Taken from reference 102)
Figure 21 Structure of MDM2

The proteins MDM2 and p53 interact via a hydrophobic region on MDM2, which lies towards the amino terminus of MDM2. The hydrophobic region forms a deep cleft made up of residues 19-102. These residues form two structurally similar sections which fold to form a pocket lined by 14 highly conserved hydrophobic and aromatic amino acids. Residues Gly58, Glu68, Val75 and Cys77 are important for p53 binding, as MDM2 with mutations at any of these sites does not interact with p53. p53 interacts with this region through an amphipathic α-helix found within the amino terminal domain, the area also responsible for p53 transcription abilities. The α-helix is formed by 15 amino acids, those crucial for binding are residues 18-26, whilst Thr18 is thought important in stabilising the α-helix. Phe19, Trp23 and Leu26 of p53 insert into the cleft formed by MDM2. The strength of the interaction between these two proteins is thought to have an IC₅₀ of between 60 -700 nM (dependent on length of the p53 peptide used) but the interactions are predominantly hydrophobic, with only 3 H-bonds thought to be formed; the most deeply buried being formed with Trp23 on p53.
In unstressed cells p53 is a very unstable protein with a half life of 5-30 minutes as a result of being constantly degraded by MDM2, and is therefore found in low cellular concentrations under normal conditions. As a response to DNA damage, hypoxia, telomere shortening or
oncogene activation p53 is stabilized via the blocking of p53 degradation. MDM2 inhibits p53 activity in two ways. The first is by physically blocking the domain p53 uses for transcriptional activation. p53 requires L22 and W23 for transcriptional activity but these amino acid residues are found within α helix through which p53 and MDM2 interact. The second approach by which MDM2 deactivates p53 is through its E3 ligase activity.

The protein MDM2 has both self and p53 ligase activity, due to its highly conserved C-terminal RING finger domain. The RING domain (Really Interesting New Gene) has a C2H2C4 motif, which chelates Zn$^{2+}$, important for ligase activity. Modification of proteins by ubiquitin conjugation is a cellular mechanism which marks proteins for degradation by protease. MDM2 monoubiquitinates lysine residues, predominantly within the C-terminal domain of p53, by transferring ubiquitin from E2 conjugating enzymes. When the MDM2-p53 complex is in a further complex with p300/CREB binding protein, which acts as a scaffold, p53 can then be polyubiquitinated. It is the polyubiquitination which marks p53 for degradation by cellular proteases. Before the proteasome can degrade p53, it must be exported from the nucleus into the cytoplasm of the cell. The exact mechanism of this export is unclear, but the MDM2 mediated ubiquitination is thought to be important. The ubiquitination of one or more lysines residues within a group of six lysines found between residues 370-386 at the C-terminal of p53 is thought to be a vital requirement. The nuclear export of p53 also requires the p53 C-terminal nuclear export signal (NES), found at residues 345 to 355. Nie et al propose that the ubiquitination of the lysine residues is vital as it induces a conformational change which can then activate or expose the NES, resulting in p53 nuclear export.

The relationship between p53 and MDM2 was further complicated by the discovery in 1996 of MDMX (also known as MDM4). MDM2 and MDMX are descended from a common ancestor gene and share significant sequence similarity. The N-terminal domain of MDMX has highest sequence similarity with MDM2 (53.6%). MDMX can also bind to the N-terminal of p53, therefore also blocking the translational activity of p53. However, the Leu54 (of MDM2) is replaced by a methionine in MDMX, resulting in a smaller binding site. The central domain of MDMX has 41.9% sequence similarity and the C-terminal domain has 51.3% sequence similarity, but MDMX does not have the E3 ligase activity of MDM2. Instead MDMX acts to stabilise and increase the ligase activity of MDM2. In vivo, MDM2 is not particularly stable, and by formation of a heterodimer with MDMX through the RING
domain on both proteins, MDM2 is stabilised. MDMX is in turn labelled with ubiquitin by MDM2 and marked for degradation.\textsuperscript{104,111}

Due to the role of p53 as a tumour suppressor, the interaction between p53, MDM2 and MDMX is important within cancer. p53 is either lost or mutated in either 50\% of cancers\textsuperscript{112} with MDM2 amplified in around 7\% of all cancers.\textsuperscript{113} However, within some cancer types this figure rises. Within brain tumours or nervous system cancers (including astrocytomas, oligodendriomas, glioblastomas, gliosarcomas, medulloblastomas, ependymomas and neuroblastomas) the figure rises to 10.4\%, within HCC it rises to 44.4\%, within soft tissue tumours 30.9\% and in Hodgkins disease to 66.7\%.\textsuperscript{114} However, these two mutations rarely happen simultaneously. In most tumours with amplified levels of MDM2, the cell has retained wild type p53, but because of the high levels of MDM2, the protein is rapidly degraded, allowing the cell to replicate unrestricted. If the MDM2 function was restricted, the p53 could function normally, placing the cell into senescence or beginning a programme of apoptosis. This makes the MDM2 a highly attractive target for the development of new anti-cancer therapeutics within medicinal chemistry.

3.3 p53 Cellular Function

The tumour suppressor p53 is activated as a response to genotoxic stress or oncogenic activation through two distinct pathways. These two pathways act to release p53 from the inhibitory activity of MDM2, allowing p53 to act as a transcriptional initiator. Through this, the cell cycle is halted allowing either cellular repair or apoptosis if the damage cannot be repaired.

The first pathway is activated as a response to genotoxic stress. The genotoxic stress includes irradiation, x-rays, ultraviolet radiation, ionising radiation (IR), cytotoxic chemotherapeutic drugs and carcinogens.\textsuperscript{115} The signal from genotoxic stress is transmitted to p53 through a pathway of ‘stress kinases’.\textsuperscript{100} As a response to IR and double strand breaks,\textsuperscript{116} ataxia telanglectasia mutated (ATM)\textsuperscript{117} kinase is activated. ATM phosphorylates p53 at serine 15 as well as MDM2 at Ser395. ATM also acts on the checkpoint kinase CHK2, phosphorylating CHK2 at threonine 68. This activates CHK2 allowing the DNA-damage signal to be amplified, resulting in the phosphorylation of a number of targets including p53 at serine 20 as well as other sites within p53.\textsuperscript{116,118}
In the case of DNA-damage caused by UV radiation, single strand breaks and bulky lesions, ATR is activated.\textsuperscript{116, 119} ATR can also phosphorylate Ser15,\textsuperscript{116} as well as activating CHK1. Activation of CHK1 results in the phosphorylation of p53 at Ser20. Therefore whether activated by DNA-damage resulting in single strand breaks or double strand breaks, p53 will be phosphorylated at both sites Ser15 and Ser20.

p53 can be further phosphorylated by other kinases including DNA-PK.\textsuperscript{120} One crucial site of phosphorylation is Thr18 by CK1. Phosphorylation at Ser15, Ser20 and Thr18 of p53 and sites on MDM2 are thought to destabilise the interaction between p53 and MDM2. Ser15, Ser20 and Thr18 lie on the α-helix which interacts with MDM2 – Thr18 is especially important as it can destabilise the α-helix – altering its structure.\textsuperscript{114} Phosphorylation of MDM2 at a site close to the RING domain by ATM or an ATM-dependant kinase also acts to switch the MDM2 ligase activity from p53 to MDM2/MDMX, leading to increased accumulation of p53.\textsuperscript{101}

Phosphorylation at multiple sites destabilises the interaction between p53 and MDM2, releasing p53 from the inhibitory activity of MDM2. This results in the increase in the half-life of p53 and its accumulation in the nucleus. In addition, p53 can also be stabilised as a response to oncogene activation.

Acute renal failure (ARF) is the result of the alternative transcription of the gene for the inhibitor of cyclin dependent kinase 4a.\textsuperscript{121} ARF (p14\textsuperscript{ARF} in humans)\textsuperscript{115} is a tumour suppressor and is activated by oncogenes including Ras.\textsuperscript{100} It acts by sequestering MDM2,\textsuperscript{115} physically releasing p53 from its interaction. ARF inhibits the E3 ligase activity of MDM2 by binding to the RING finger domain of MDM2,\textsuperscript{100} stabilising the cellular level of p53. Over the process of the development of a tumour, the boundary between these two methods of p53 activation can blur. Continuous replicative cycles as a result of oncogene activation results in replicative stress. The resulting DNA damage will activate the stress kinase cascade.

After p53 has been freed from the inhibitory activity of MDM2, it can then undertake its transcriptional activity. p53 acts as a transcription initiator, initiating the transcription of a large number of genes. p53 can undergo a range of post-translational modifications including phosphorylation, acetylation, methylation, ubiquitination, sumoylation and neddylation.\textsuperscript{122}
The degree of modification can control which genes are translated and the level of translation. Phosphorylation of p53 increases the affinity for transcriptional co-activators, including histone acetyl transferases (HATs) p300 and CREB binding protein (CRB). These HATs acetylate p53 at sites within the carboxyl terminal of p53, sites which are usually ubiquitinated, therefore contributing to the further stabilisation of p53 as well as increasing the site specificity of p53. p53 and p300 can then form a DNA-binding complex.

The C-terminal of p53 interacts with DNA through a 26 amino acid sequence. The 26 amino acid sequence contains nine basic residues which interact with DNA/RNA. Residues K120, S241, R273, A276 and R283 interact with the phosphate backbone in the major groove; residues K120, C277, R283 form H-bonds with DNA-bases and R248 interacts with the minor groove, forming multiple H-bonds. p53 binds with DNA in a sequence specific manner. p53 binds to areas of DNA known as responsive elements (REs). The RE sequence consists of RRRCWWGYYY and 0-21 spacer and RRRCWWGYYY where R = purine W = A or T and Y = pyrimidine. Genes whose transcription is initiated by p53 have a RE within the DNA close to or within the gene. p53 binding to one of these RE either activates or represses transcription. p53 binds to RE and for genes whose transcription is activated by p53 will recruit general transcription proteins through protein-protein interaction. However, for genes which are repressed by its binding, p53 either blocks the gene through overlap of the binding site, by squelching of the transcriptional activator or by recruiting p53 histone deacetylases. Proteins whose transcription is inhibited by p53 includes Bcl-2, an anti-apoptotic protein which will be discussed later.

The transcription of number of proteins is initiated by p53. These proteins include those responsible for initiating apoptosis, preventing angiogenesis, growth arrest and DNA repair. A few examples of p53 target genes and the cellular processes they are involved in are listed below.

- PUMA – Apoptosis
- DRAM – Autophagy
- p21 – Cell cycle arrest
- R2 – DNA repair
- LIF – Embryo Implantation
Some of these translation targets will be discussed in more detail. p21, which acts to prevent cell cycle progression by inhibiting cyclin E and CDK2, is required for progression from G1 to S phase. The expression of p21 is induced by very low cellular levels of p53. This allows a temporary pause of the cell cycle as a response to mild DNA damage or stress, allowing the issue to be resolved and the cell to survive.

Two further proteins also play a vital role in halting the cell cycle and DNA repair. GADD45 (Growth Arrest DNA Damage) is proposed to have a role in halting the cell cycle at the G1 to S phase transition – possible through an interaction with p21. Better understood is the role of GADD45 in G2 to M arrest. GADD45 can interact with the Cdc2 (also known as CDK1)/cyclin B complex, causing it to dissociate. As the CDK1/cyclin B complex induces the G2 to M transition, this dissociation halts the cell-cycle before it enters mitosis. G2 arrest is also instigated by the 14-3-3 protein, a further transcriptional target of p53 which acts in two ways. 14-3-3 acts upstream of cyclin B/Cdc2, preventing activation, which is required for the G2/M transition. CHK1 and 2 phosphorylate Cdc25, and phospho-Cdc25 can then bind to 14-3-3. The 14-3-3 sequesters Cdc25 in the cytoplasm, preventing activation of Cdc2 (by dephosphorylation). The phospho-Cdc2 binds to 14-3-3σ (one member of the 14-3-3 family), which also anchors it in the nucleus, preventing the inducement of the G2 to M transition. The halting of the cell cycle allows repair of DNA damage or the stress-signalling to abate. GADD45 is also implicated in DNA damage repair. GADD45 interacts with PCNA, a protein which is involved in the nucleotide excision repair pathway. However, if this DNA damage cannot be repaired, the cell commits to apoptosis, programmed cell death, to prevent the continuing proliferation of damaged genetic material.

There are two major routes to apoptosis, the intrinsic and extrinsic pathway. p53 plays a role within both of these pathways, as well as a family of proteins called the Bcl-2 (B
lymphoma 2) family. The Bcl-2 family contain both pro and anti-apoptotic members. The anti-apoptotic members block apoptosis and include Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1 whilst the pro-apoptosis members block inhibitors of apoptosis. The proapoptotic members can be divided further into two subfamilies: the BAX family compromising of BAX, BAK and BOK and the BH3-only subfamily compromising of BID, BIM, BAD, BIK, BMF, PUMA, NOVA; and HRK. Both the intrinsic and extrinsic pathways result in the activation of proteases known as caspases, but via a differing route. The extrinsic pathway is also known as the death receptor pathway whilst the intrinsic pathway is also known as the mitochondrial pathway.115

The extrinsic pathway is activated by members of the tumour necrosis factor (TNF) including TNFα, FAS/CD95 ligand and APO2 ligand. These activate death receptors including TNF/NGF receptor family115 resulting in activation of the cell surface receptor FAS. This interacts with FADD protein (Fas associated death domain) forming the complex DISC (death-inducing signalling complex). The complex activates caspase 3 and caspase 7 which in turn results in apoptosis.115 p53 initiates the transcription of a number of proteins within this pathway.115

The intrinsic pathway results in the release of cytochrome c from the mitochondria into the cytoplasm through the activity of the Bcl-2 family.115 Bcl-2 is found within the outer membrane of the mitochondria and is thought to safeguard the integrity of the mitochondrial membrane, but other anti-apoptosis members such as Bcl-XL and BCL-W only associate with the mitochondria after a cytotoxic event.129 The anti-apoptotic proteins are found in complex with the BAX family proteins BAX and BAK under unstressed condition. Both are both activated by BID, a member of the BH3 only family. As BID is, in turn, activated by TNFα and FAS ligand, a link is formed between the extrinsic and the intrinsic pathway.115

Bcl-associated protein (BAX)121 is found within the cytoplasm under normal conditions in complex with anti-apoptotic conditions,115 however, as a result of apoptotic signalling it is replaced in this complex by BAD and other BH3 only members which inhibit the BH3-only proteins by binding to them. The anti-apoptotic proteins include proteins PUMA and NOXA which are translated as a result of p53 activity.129,115 BAX then changes conformation and is incorporated into the outer membrane of mitochondria.129 Unlike BAX, BAK (Bcl-homologous antagonist-killer)121 is always found within the mitochondrial membrane but
during apoptosis changes conformation. Both BAX and BAK are responsible for making the membrane of the mitochondria permeable, forming channels which allows the efflux of cytochrome c.

![Diagram of cellular changes after an apoptotic event]

**Figure 24** Cellular changes after an apoptotic event

Once cytochrome c is released from the mitochondria it binds to Apaf-1 (Apoptotic protease activating factor 1) forming apoptosome. Apoptosome acts on procaspase 9, resulting in its activation which in turn activates other caspases beginning what is known as a ‘caspase cascade’ resulting in cell death. Caspases (Cysteine aspartic acid proteases) are selective proteases. There are 14 caspases, which organize all steps of apoptosis. They are found within the cell as inactive precursors, which are known as procaspases and are activated during apoptosis. The caspases act to break down cellular molecules and DNA, condense chromatin and result in the changes of an apoptotic cell.

A further aspect of the apoptosis pathway directly links p53 to apoptosis. During apoptosis a small portion of the free p53 migrates to the membrane of the mitochondria. p53 then interacts with Bcl-XL, freeing Bax from its complex with Bcl-XL. This results in Bax releasing cytochrome c from the mitochondria.
3.4 Drug-like Inhibitors of the MDM2-p53 Protein-protein Interaction

The tumour suppressor p53 protects the integrity of the genome and prevents the development of tumours. In 7% of all tumours the cell retains wild type functional p53 but is essentially p53-null due to an over expression of MDM2. The highest frequency of amplification occurs in soft-tissue cancer, osteosarcomas and oesophageal carcinomas. Therefore, small drug-like molecules which inhibit the protein-protein interaction between p53 and MDM2 are an attractive target for the treatment of cancer. The interaction between MDM2 and p53 is an ideal target for the development of small molecule inhibitors. The area of interaction is relatively small, calculated to be 660 Å² surface area on MDM2 and 809 Å² surface area on p53, and forms a ‘hot spot’, an area of intense interaction formed by an α-helices of p53 and a hydrophobic cleft on MDM2 (discussed in chapter 3.2).

Figure 25 X-ray Structure of MDM2 with p53 bound, crucial residues for the interaction are shown in purple

Inhibition of the protein-protein interaction was first demonstrated by the use of peptides such as the 12mer peptide (Ac-Met-Pro-Arg-Phe-Met-Asp-Tyr-Trp-Glu-Gly-Leu-Asn-NH₂) that demonstrated an IC₅₀ of 0.3 µM. Introduction of unnatural amino acids further increased potency, by inducing the correct conformation before binding to the MDM2 pocket. Experimentation with unnatural amino acids also identified that the replacement of tryptophan (which mimics Trp23) with 6-chlorotryptophan fills a small hydrophobic cavity unfilled by the wild type p53, thus leading to a 60-fold increase in potency between the two comparable peptides. However, peptides are not appropriate for use as drugs and extensive
medicinal chemistry efforts have resulted in a number of small-molecule inhibitors of the p53/MDM2 interaction.

3.4.1. Nutlins

One of the first series of potent, small molecule inhibitors of the MDM2 p53 protein-protein interaction were the Nutlins, developed by scientists at Hoffman la Roche.\textsuperscript{131} A screen of a diverse library of chemicals identified a class of \textit{cis}-imidazolines as lead compounds for the inhibition of the MDM2 p53 interaction. These compounds, named Nutlins (for the Nutley inhibitors) (35-37) have activity within the range of IC\textsubscript{50} 100-300 nM range.\textsuperscript{131}

![Figure 26](image)

The IC\textsubscript{50} values for the compounds were identified as 0.26 µM and 0.14 µM for Nutlin-1 and -2 respectively, with these two compounds tested as a racemic mixture. Nutlin-3 was separated into its two enantiomers using chiral HPLC. The IC\textsubscript{50} for these two enantiomers initially arbitrarily named Nutlin-3a (37) and Nutlin-3b were 0.09 µM and 13.6 µM respectively.\textsuperscript{131-132}

The crystal structure of Nutlin-2 bound to human MDM2 shows that it closely mimics the interaction of p53 and MDM2. The \textit{cis}-imidazoline scaffold mimics the α-helix backbone of p53. One bromophenyln group sits in the pocket occupied by Trp23 of p53; the second bromophenyln group sits in the Leu26 pocket, and the ethyl ether side chain points towards the Phe19 pocket.\textsuperscript{131}
3. MDM2/p53: An Anti-Cancer Target

The as Nutlins target the MDM2 p53 interaction stabilising p53, treatment of cells with these compounds should result in an increase in levels of p53, MDM2 and p21 (as both MDM2 and p21 are transcriptional targets of p53). However, this effect should only be observed in cells with wild type p53 and not those with mutated or deleted copies of the gene. Nutlin-1 was tested against human cancer cell lines with wild type p53 (colon cancer cell lines HCT116 and RKO) and also cell lines with inactive p53 (SW480 (colon), MDA-MB-435 (breast) and PC3 (prostate)) to act as a negative control. HCT116 was treated with Nutlin-1 for 8h. A dose dependent increase in levels of p53, MDM2 and p21 was observed. SW480 was treated under similar conditions and whilst basal levels of p53 were observed, no increase in MDM2 or p21 was observed. To ensure that the HCT116 response was a post-translational response to treatment with the Nutlin, the gene expression of both p53 and p21 was monitored. Treatment with Nutlin-1 had no effect on p53 expression but p21 increased in a dose-dependent manner, suggesting that the Nutlins were acting purely by inhibiting the MDM2 p53 protein-protein interaction to upregulated p53 cellular levels.

A recent patent has been published from the Nutlin series suggesting increase in activity and an improvement in the physical properties of the series. The Nutlin analogue RG7112 (38) is currently in clinical trials. The hydrogen atoms in the imidazolidine ring of the early Nutlin compounds have been replaced by methyl groups and the piperazine ring has a further N-propyl methyl sulfone group. This additional change may possibly be forming further H-bond interaction between the sulfone oxygen atoms and Tyr67 and Asn72.
3.4.2. Spirooxindoles

A series of spirooxindoles are also potent small molecule inhibitors of the MDM2 p53 protein-protein interaction. Crystal structures of the MDM2 p53 interaction showed that the indole side chain of Trp23 from the p53 peptide forms a hydrogen bond with a carbonyl of the MDM2 protein backbone.\textsuperscript{135} Ding \textit{et al} then identified compounds which could imitate this interaction. Oxindoles were found to mimic this interaction and a screen of natural products was undertaken to identify compounds which contained this motif. A number of alkaloids were identified, including spirotryprostatin A and Alstonisine, and although these did not inhibit the protein-protein interaction the spiro (oxindole-3,3'-pyrolidone) motif was used as a starting point.\textsuperscript{135} The spiro pyrrolidine ring provides a rigid scaffold from which group to mimic the Phe19 and Leu26 can be added. Molecules were designed using the docking program GOLD by altering the R\textsuperscript{1}, R\textsuperscript{2} and R\textsuperscript{3} around the ring. This process, followed by further optimisation resulted in compound 39, which has a $K_i$ of 86 nM in a fluorescence polarisation assay.\textsuperscript{135}

![Figure 28](image.png)

**Figure 28** Spirooxindoles core, following optimisation gave 39
To test the cell activity of the spirooxindoles series the compounds were tested against LNCaP human prostate cancer cells, which have wild type p53. Compound 39 had an IC\textsubscript{50} of 0.83 µM. The compounds were also shown to be selective for cell lines with wild type p53 and for cancer cells. Treatment of normal human prostate epithelial cell, with wild type p53, had an IC\textsubscript{50} of 10.5 µM.\textsuperscript{135}

Although the preliminary compounds showed good potency, comparison with the p53 inhibitory peptides suggested that additional interactions may be accessible to gain additional activity. X-ray crystallography identified a fourth interaction between MDM2 and Leu22 of p53.\textsuperscript{136} The 5’ carbonyl of 39 is predicted to lie within 2 Å of the position of carbonyl backbone of Leu22 in p53, suggesting that expanding the compound from the 5’ carbonyl may pick up further interaction with MDM2. As Leu22 is partially solvent exposed the additional functionalities may also act to improve solubility. Ding et al used the docking program GOLD to predict that the N, N-dimethylamine group of 39 can be replaced with 2-morpholin-4-yl-ethylamine group.\textsuperscript{136} The oxygen within the morpholine was proposed to lie close to the positively charged amine of Lys90 of MDM2. The resulting compound has a $K_i$ of 13 nM.\textsuperscript{136}

Further optimisation of the m-chlorophenyl ring resulted in incorporation of a fluorine in the 2-position. Combination of the two changes resulted in the development of MI-63 (40) with a $K_i$ of 3 nM. Compound 40 has four chiral centres, and as binding to MDM2 is stereospecific, identification of the most active stereoisomer (shown below) is important. A further isomer with the opposite stereochemistry has a $K_i$ of 4 µM.\textsuperscript{136}

![Figure 29 The structure of MI-63 (40)](image)

Other protein-protein interactions have a similar binding interaction to MDM2-p53 i.e one partner forms an α-helix which fits into a complementary pocket on the other partner.\textsuperscript{136} This
manner of binding is seen for the Bcl-2/Bcl-XL anti-apoptotic proteins with a pro-apoptotic protein such as BID, BAD, or BAX, and was studied to see if compound 40 inhibits this binding. No significant inhibition was observed at concentrations up to 50 µM, suggesting that the spirooxindoles are selective for the MDM2-p53 interaction. Compound 40 was also tested to ensure that it was only active in cells with wild type p53, which supports the believed mode of action, as well as minimally toxic to normal cells.\textsuperscript{136}

Although compound 40 is a potent inhibitor of the MDM2-p53 protein-protein interaction it has a poor pharmacokinetic profile and modest oral availability. MI-63 is therefore not a good drug candidate. The morpholine group is partially solvent exposed so there is no selectivity for the functionality at this position, and therefore the substituent was altered without loss of activity. Replacement with either a methylpiperazinyl or a methyl-piperadinyl group resulted in a $K_i$ of 1.5 and 20 nM respectively.\textsuperscript{137} Both the N-methyl-piperaziny1 and N-methyl-piperadinyl substitutions resulted in improved pharmacokinetic profile but the maximum concentration within the plasma and the area under the curve remained low.\textsuperscript{137} The morpholine, methylpiperadine and methylpiperazine are all protonated and positively charged at physiological pH, contributing to the low bioavailability. The synthesis of a butyl-1,2-diol group improved the $K_i$ to 0.6 nM, and also improved pharmacokinetics, as well as higher concentration within the plasma and improved area under the curve. The compound (41), known as MI-147, became the new lead compound.\textsuperscript{137} A further compound, MI-219 (42) was also synthesised, without the fluorine atom on the phenyl ring, instead it lies on the oxindole ring. MI-219 has an IC$_{50}$ of 162 nM, but in cellular assays is only slightly less growth inhibitory in SJSA-1 and HCT-116 cell lines than MI-147.\textsuperscript{137} MI-219 has improved oral bioavailability than MI-147 (from 21\% to 65\%) and a 25 mg/kg oral dose achieve a C$_{\text{max}}$ of 3751 ng/mL and an area under the curve of 7677 h·mg/L.\textsuperscript{137}

![Figure 30](image_url)
SJSA-1 cell lines, with wild type p53 MDM2 amplified, were treated with compound \textbf{41}. A dose dependent activation of p53 was observed, with increasing levels of MDM2 and p21. Compound \textbf{41} also induces a dose dependent increase in BAX, PUMA, and NOVA.\textsuperscript{137} A two day treatment of SJSA-1 cell line with 1.25 µM dose of compound \textbf{41} resulted in cell death in 50% of cells. Compound \textbf{41} was confirmed to be acting in a p53-dependant manner as minimal effects were seen on a SAOS-2 cell line which has deleted p53. In xenograft models animals were treated with either \textbf{41} alone or \textbf{41} in combination with irinotecan and was growth inhibitory. Compound \textbf{41} is potentially the most potent, orally available, cell permeable, specific inhibitor of the MDM2 p53 protein-protein interaction.\textsuperscript{137}

\textbf{3.4.3. 1,4-Benzodiazepine-2,5-diones}

From a screen of MDM2 binding of 338,000 compounds designed using directed diversity software, 1216 compounds were selected for further investigation by Grasberger \textit{et al}.\textsuperscript{138} From these 1216, 116 compounds had a benzodiazepinedione core. The benzodiazepinedione compounds have two chiral centres and were screened as a mixture of four stereoisomers, with the most active compounds used as a guide for further elaboration of this scaffold. Separation of the most active pair of enantiomers revealed the active compound to be the (S, S) diastereoisomer.\textsuperscript{138}

The benzodiazepinediones were screened using a novel affinity-based assay named ‘Therofluor’. The assay uses fluorescent dye to monitor the unfolding of protein on heating. Compounds which bind to the MDM2 protein increase the thermal stability of the protein and will increase the temperature at which the protein unfolds. The value was quantified by change in the mid-point transition temperature. The initial 116 benzodiazepinedione compounds had a change in the midpoint transition temperature of 1.0 - 4.9 °C,\textsuperscript{138} with any hit being a compound with a change of greater than three times the standard deviation. The change in stability of the MDM2 protein over a range of concentrations allowed a dissociation constant ($K_d$) to be calculated.\textsuperscript{139}

Further characterisation of the hits was under taken using a fluorescence polarisation (FP) assay. This assay used fluorescein-labelled p53 peptide which undergoes excitation at 485 nm and emits at 530 nm. On the binding of a compound to the MDM2, the p53 peptide will be
displaced and the polarisation will be altered. This change was expressed as a percentage with respect to the p53 control.\textsuperscript{139}

\begin{center}
\includegraphics[width=0.5\textwidth]{fig31}
\end{center}

\textbf{Figure 31}

Optimisation around the benzodiazepinedione ring resulted in compound 42, with a $K_d$ of 67 nM and an IC\textsubscript{50} of 420 nM.\textsuperscript{139} Incorporation of an ortho amino group on the benzylic ring improved the IC\textsubscript{50} value for the compound but reduced cellular potency, probably due to the compound behaving as a zwitterion.\textsuperscript{140} The amino group is thought to form a hydrogen bond with Val93 on MDM2,\textsuperscript{140} and can be replaced with a hydroxyl group which can form a similar H-bond. A methyl group on the benzylic carbon maintains good potency in the $R$-enantiomer, with a 3-fold increase in potency.\textsuperscript{141}

\begin{center}
\includegraphics[width=0.8\textwidth]{fig32}
\end{center}

\textbf{Figure 32} Structure of compounds 43, 44, 45 and 46

Groups on the 1-amino position are relatively solvent exposed, and were therefore varied to improve water solubility. A valeric acid group (43), whilst increasing solubility and potency can form a zwitterion which resulted in poorer cell activity when compared to a morpholin-4-ylethyl group (44), a 2-(2-methoxyethoxy)ethyl (45) and 1-methyl-4-propylpiperazine (46), although these compounds had somewhat reduced potency.\textsuperscript{140} This series are amongst the most potent inhibitors of the MDM2 p53 protein-protein interaction published. The benzodiazepinedione scaffold acts as an $\alpha$-helix mimic to orientate the aromatic groups into the correct positions to interact with MDM2.\textsuperscript{142}
3.4.4. Chromenotriazolopyrimidines

A further class of inhibitors of the MDM2/p53 protein-protein interaction has been published by Allen et al.\textsuperscript{143} From a high-throughput screen of 1.4 million compounds, compound 47 (shown below) was identified as binding to MDM2. Compound 47 was initially tested as a racemic mixture of syn and anti diastereoisomers and had an IC\textsubscript{50} of 3.88 µM.\textsuperscript{143} On chiral separation the active compound was shown to be the syn 6\textsuperscript{R}, 7\textsuperscript{S} configuration (48) with an IC\textsubscript{50} of 1.23 µM.\textsuperscript{143}

\begin{center}
\begin{tabular}{c c}
\textbf{47} & \textbf{48} \\
\end{tabular}
\end{center}

\textbf{Figure 33}

Compound 48 was cocrystallised with MDM2. The crystal structure shows the C7 bromophenyl group occupies the Leu26 pocket, forming a weak π-stacking interaction with His96 which lies 4.0-4.9Å away. The C6 bromophenyl group lies within the Trp23 pocket and the aromatic ring of the chromenone lies within the Phe19 pocket.\textsuperscript{143}

\begin{center}
\textbf{Figure 34}
\end{center}
3. MDM2/p53: An Anti-Cancer Target

However, compound 48 has a number of problems as a lead structure. It has a high molecular weight (536.22 Da), although the ligand efficiency is comparable with other protein-protein inhibitors. The isomers of compound 47 are poorly soluble in DMSO and at room temperature solutions of enantiomerically pure syn diastereoisomers racemise to the anti configuration. The racemisation only occurs at the 6-position and is thought to proceed via the mechanism shown below.

![Scheme 2](image)

To prevent racemisation the N11 position was methylated (49), preventing formation of the enamine, the intermediate in the racemisation process. N-methylation increases stability in solution, and improved organic solubility. Crucially, N-methylation maintained good potency with an IC$_{50}$ of 1.17 µM.

The N-methylated compound was then used as a starting point for further development. In an attempt to reduce the molecular mass the dibromo motif was replaced by either difluoro which was not tolerated, or dichloro which was equipotent, IC$_{50}$ = 1.21 µM for 89 Da reduction in molecular mass. The Trp23 pocket was probed by a series of larger substituents on the C6 aryl group. Of these, the linear groups with some π-character maintained some...
activity, with the nitrile analogue the most active at 2.08 µM. These substituents are thought to interact with aromatic residues Phe86 and Phe91 of MDM2.$^{143}$

Synthetic efforts were directed at optimising the substituents for the Leu26 pocket demonstrated that the fluorophenyl analogue was better tolerated as the C6 substituent, however, no improvements in potency were observed.$^{143}$ The aromatic ring of the chromenone was then probed. In the C1 position a methoxy group was shown to be favourable, with a 3.9-fold increase in potency observed for the racemic mixture. The $6R,7S$ enantiomer of the methoxy compound (50) has an IC$_{50}$ of 0.20 µM. The improvement in potency is thought to be due to the twist induced in the molecule due to clashes between the methoxy group and the N11 methyl group. This induces an improved in the position of the two bromophenyl groups within the Trp23 and Leu26 pockets.$^{143}$

At the C2 position of the chromenone a methyl substituent improved potency 2-fold, with an IC$_{50}$ of 0.44 µM (0.39 µM for the $6R,7S$ enantiomer (51)).$^{143}$ The crystal structure of 51 showed a small pocket which could be probed by a small group, as demonstrated by tolerance for an ethyl group. No further increase in potency was observed by substitution at either the C3 or C4 position around the ring. However, addition of the C1 methoxy group and C2 methyl group failed to have an additive effect with an IC$_{50}$ 3.94 µM.$^{143}$

![50](image1.png) ![51](image2.png)

Biochemical analysis demonstrated that the chromenotriazolopyrimidines are cell permeable, though the level varied between different compounds. Treatment of HCT116 cells with 51 showed a dose-dependent increase in p21 mRNA although not in p53 knockout cells indicating chromenotrizolopyrimidines acts via MDM2 inhibition.$^{143}$ However, compound 49 has high clearance (2.4 L/h/kg) and an oral bioavailability of only 23%. On incubating with rat liver microsomes less than 5% remained after 30 min and on intravenous administration significant levels of des-methyl enantiomers were detectable within the plasma.$^{144}$ Oxidative
N-demethylation and isomerisation were thought to occur, resulting in the formation of the inactive enantiomers therefore a more metabolically stable substituent was required.

A series of N-alkyl carboxylic acid were synthesised. Of these the N-pentyl carboxylic acid analogue (52) was shown to maintain good potency with an IC$_{50}$ of 0.76 µM for the racemic mixture and 0.35 µM for the (6R,7S) enantiomer. 52 showed low in vivo clearance and modest oral bioavailability of 54%.  

![Figure 35](image)

3.4.5. Imidazoindoles

Imidazoindole scaffold for MDM2-p53 inhibition was discovered simultaneously by a number of groups including Novartis and Popwicz et al. The imidazoindole scaffold is also the only scaffold to also have MDMX inhibitory activity. The imidazoindole scaffold compromises an imidazole ring with a phenyl substituent at the 4-position, a 2-carboxy-6-chloroindole substituent at the 5-position and a 4-chlorobenzyl substituent at position 1.

Novartis 101 (WW298) (53) has an IC$_{50}$ of 0.19 µM against MDM2 and 19.7 µM against MDMX. The indole group of WW298 sits within the Trp23 pocket with the indole N forming an H-bond to Leu54 of MDM2. However, in the Trp23 pocket of MDMX binding of WW298 induces an energetically unfavourable change in conformation. The phenyl ring occupies the Phe19 pocket, orientated perpendicular to the ring of Phe19 and the Leu26 pocket is filled by the chlorobenzyl group. Within the MDMX crystal structure the N,N-dimethylaminopropyl moiety folds over the Phe19 pocket, shielding the pocket from solvent...
and providing additional hydrophobic interactions. However, the crystal structure of WW298 with MDM2 is not currently available to confirm whether this occurs with MDM2. The N,N-dimethylaminopropyl pyrrolidine moiety also improves the water solubility of WW298.

Figure 36

Compound 54 (WK23) developed by Popwicz et al. is essentially the core of WW298. It lacks the dimethylamino pyrrolidine moiety, instead replacing the amide linker with a carboxylic acid group. WK23 has significantly lower activity, with an IC₅₀ of 1.71 µM. A crystal structure of WK23 bound to MDM2 demonstrates that it binds in a similar manner to WW298 (Figure 37).

Figure 37 WK23 bound to MDM2 (PDB 3LBK)

To date no MDM2 p53 protein-protein inhibitor has been approved for clinical use. R7112 (38) from Hoffman – La Roche is undergoing clinical evaluation, and R7112 is currently in phase I trials for haematological neoplasm (from May 2008 to March 2012) and advanced...
solid tumours (from December 2007 to November 2011)\textsuperscript{134} and is an orally available compound.\textsuperscript{132} A Johnson and Johnson MDM2 inhibitor with a non-p53 mechanism JNJ00676910 underwent clinical trials from November 2006 to February 2010 for advanced/refractory solid tumours. However, no results have been published and there is currently no evidence of this compound going forward into phase II trials.\textsuperscript{134} AT-219 is undergoing late preclinical development. It is an optimised member of the spirooxindoles family of compounds.\textsuperscript{132}
4. The Development of ATP-competitive Inhibitors of mTOR

4.1 Pyrimidines as mTOR Inhibitors

A high-throughput screen of pyrimidine-based compounds carried out by KuDOS Pharmaceuticals identified a number of O\(^1\)-substituted pyrimidines as having inhibitory activity against mTOR. Three series were identified and are shown below.\(^{147-151}\) The compounds were originally synthesised within a CDK2 project of the NICR drug discovery laboratories and all compounds had exhibited some degree of activity against this target. The structures of compounds and their inhibitory activity against both mTOR and CDK2 are shown below (Table 1).

![Series A](image1)

![Series B](image2)

![Series C](image3)

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Series</th>
<th>X</th>
<th>R</th>
<th>Kinase Inhibition (IC(_{50}) µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mTOR</td>
</tr>
<tr>
<td>55</td>
<td>A</td>
<td>H</td>
<td>H</td>
<td>&gt;50</td>
</tr>
<tr>
<td>56</td>
<td>A</td>
<td>NO</td>
<td>H</td>
<td>2.6</td>
</tr>
<tr>
<td>57</td>
<td>A</td>
<td>CHO</td>
<td>H</td>
<td>3.1</td>
</tr>
<tr>
<td>58</td>
<td>A</td>
<td>NO(_2)</td>
<td>H</td>
<td>1.5</td>
</tr>
<tr>
<td>59</td>
<td>A</td>
<td>CN</td>
<td>H</td>
<td>1.5</td>
</tr>
<tr>
<td>60</td>
<td>A</td>
<td>NO</td>
<td>COMe</td>
<td>&gt;50</td>
</tr>
<tr>
<td>61</td>
<td>A</td>
<td>NO</td>
<td>COEt</td>
<td>11</td>
</tr>
<tr>
<td>62</td>
<td>B</td>
<td>NO</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>63</td>
<td>C</td>
<td>NO</td>
<td>SO(_2)NH(_2)</td>
<td>4.7</td>
</tr>
<tr>
<td>64</td>
<td>C</td>
<td>NO</td>
<td>OH</td>
<td>3.1</td>
</tr>
</tbody>
</table>

In the O\(^1\)-cyclohexylmethoxy series substituents are required at the 5-position for activity against both mTOR and CDK2. Modest activity is observed for those compounds bearing
electron withdrawing, H-bond accepting substituents at the 5-position. However, with the exception of compound 59 no selectivity for mTOR is observed. For the acyl analogues the propionyl derivative (61) shows significant selectivity for mTOR, in contrast to the acetyl and unsubstituted derivatives (60) and (56), although the latter is much more potent against mTOR than 61.

Compound 62 shows that a benzyl substituent is tolerated for mTOR activity but not for CDK2. The N²-anilino series also maintains good potency against mTOR. However, these compounds are highly selective for CDK2 over mTOR, suggesting that pursuing compounds of this type would be unwise. The most promising starting points from the HTS were NU6027 (56) and NU6227 (59) that showed the best potency for mTOR and offered the possibility of selectivity over CDK2.

![Chemical structures](image)

**Figure 38**

The CDK2 inhibitory activity of compounds 55-64 has been described previously. All maintain a crucial triplet of hydrogen bonds with the CDK2 protein, forming a donor-acceptor-donor motif. The 5-nitroso-6-aminopyrimidines were found to form a pseudo-cycle between the nitroso group and one proton on the 6-amino group (Figure 39) and therefore act as a purine-mimetic. Detailed structure-activity relationships have been deduced for CDK2 inhibition by 2,6-diamino-4-alkoxypyrimidines and so synthetic efforts were first directed towards generating a SAR for mTOR inhibition. This is particularly important given the lack of an available crystal structure of the ATP-binding site of mTOR. A detailed understanding of the SAR should aid the development of a potent inhibitor, but also aid in the separation of mTOR inhibitory activity from that of CDK2.
4.2 Synthesis of NU6027 and NU6227

As a prelude to further studies, compounds NU6227 and NU6027 were prepared using the described synthetic route. The first step in both synthetic schemes requires generation of cyclohexylmethoxide formed by heating sodium in cyclohexylmethanol. On addition of 4-chloro-2,6-diaminopyrimidine (65) the 4-chloro group is displaced by the cyclohexylmethoxide to generate 4-(cyclohexylmethoxy)pyrimidine-2,6-diamine (55). The reaction utilises the electron-deficient nature of pyrimidines to facilitate an $S_N Ar$ addition-elimination reaction displacing the chloride with the alkoxide.

The 5-nitroso substituent was installed by nitrosation with sodium nitrite in aqueous acetic acid at 80 °C in 86% yield. The electron-donating nature of the substituents on the pyrimidine counteracts the electron deficient nature of the pyrimidine ring, allowing electrophilic substitution to occur at the activated 5-position of the pyrimidine under mild conditions.

The synthetic route to NU6227 is shown below (Scheme 4). Bromination at the 5-position was achieved using N-bromosuccinimide in acetic acid at 60 °C in 80% yield. Previously the nitrile moiety had been incorporated using CuCN after protection of the amino groups. Protection of the amino groups by acetylation prevents chelation to the copper but may also have activated the ring, by reducing the electron-donating nature of the amino groups.
Protection of the amino groups was achieved in a 53% yield using a 1:1 mixture of acetic acid and acetic anhydride. Incorporation of the nitrile group was achieved using CuCN in DMF for 5 h. Ethylenediamine was then added to chelate the remaining copper, allowing it to be removed on aqueous work-up, but also as a useful side reaction resulted in the removal of the acetyl protecting groups, giving the desired final compound in 70% yield.

Scheme 4 Reagent and conditions; a) NBS, AcOH, 1 h, 60 °C, 80%; b) AcOH, Ac₂O, overnight, reflux, 53%; c) i. CuCN, DMF, 5 h, reflux; ii. Ethylenediamine, overnight, 70%

4.3 Modifications to the $O^4$-Position of NU6227

4.3.1. Synthesis of $O^4$-Alkyl Substituted Analogues of NU6227

To develop the $O^4$ structure activity relationship a series of $O^4$-alkyl analogues were synthesised. The 5-cyano analogue has some selectivity for mTOR over CDK2 and as a number of 5-nitroso analogues had previously been synthesised, the substitution at the 5-position was fixed as the nitrile group. A range of substituents was selected covering alkyl and cycloalkyl groups, all smaller than a cyclohexylmethyl group.

![Figure 40]

The desired compounds were synthesised using a similar synthetic route to that of NU6227 (Scheme 5). Previous work suggested that whilst the 5-nitroso substituted compounds were sufficiently electron deficient to allow incorporation of a 4-methoxy substituent, followed by displacement with the desired 4-substituent (Scheme 6), this does not occur with 5-nitrile analogues. As a result the $O^4$-substituent must be incorporated in the first step of the synthetic scheme. Consequently, five linear syntheses were undertaken, rather than one divergent route.
4. The Development of ATP-competitive Inhibitors of mTOR

Scheme 5 Reagents and conditions: a) Na, ROH, 20 min – 5 h, 98-160 °C; b) NBS, AcOH, 15–60 min, RT-60 °C; c) AcOH, Ac₂O, reflux, 17-48 h; d) i. CuCN, DMF, 2-7 h, 120 °C; ii. Ethylenediamine, overnight

Scheme 6 Reagents and conditions: a) Na, MeOH, 1 h, 50 °C, 96%; b) NaNO₂(aq), AcOH(aq), 20 min, 80 °C, 84% c) Morpholine, 10 min 120 °C, 76%

Each sodium alkoxide was generated from sodium in the neat alcohol with conventional heating, followed by the addition of 4-chloro-2,6-diaminopyrimidine (65). However, a search of the literature suggested that microwave heating may also be appropriate for the S_NAr reaction, and a slight improvement was achieved in this way (Table 2). For alcohols that did not react readily with sodium, microwave heating proved impractical as the metal cannot remain in the reaction mixture when heating in the microwave.

Scheme 7 Reagents and conditions; a) Na, isopropanol
Bromination was achieved in an analogous manner to NU6227 (59) using N-
bromosuccinimide. However, for the isopropyl, sec-butyl and isobutyl analogues the reaction
required less vigorous conditions. The desired products were obtained in 15 min at room
temperature in good yields (58% for the isopropyl analogue, 72% for the sec-butyl analogue
and 86% for the isobutyl analogue). Indeed, heating the isopropyl, sec-butyl and isobutyl
analogues gave only poor yields (~20%).

Acetylation of the amino groups was achieved using a 1:1 mixture of acetic acid and acetic
anhydride, though further addition of acetic anhydride and longer reaction times were
required to increase conversion to the product, compared to those required for the
cyclohexylmethyl compound (67). Displacement of the bromo substituent was achieved using
CuCN in DMF. It was found that reducing the temperature to 120 °C (from 160 °C) gave
cleaner conversion to the product. Reaction conditions are summarised below.

![Scheme 8 Reagents and conditions; a) i. CuCN, DMF ii. Ethylenediamine](image)

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Conditions and yields for conversion to nitrile product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>( O^4 )-substituent</td>
</tr>
<tr>
<td>81</td>
<td>Ethyl</td>
</tr>
<tr>
<td>82</td>
<td>Isopropyl</td>
</tr>
<tr>
<td>83</td>
<td>sec-butyl</td>
</tr>
<tr>
<td>84</td>
<td>isobutyl</td>
</tr>
<tr>
<td>85</td>
<td>cyclopropylmethyl</td>
</tr>
</tbody>
</table>
Compounds 82 and 85 required additional purification by semi-preparative HPLC before achieving appropriate purity for biological testing. The impurity was likely to be bromo-substituted compound 74, which is generated from remaining starting material on addition of ethylenediamine. To try and avoid this problem, further equivalents of CuCN were added to try to ensure complete conversion of 74 to 75, but this approach was not successful.

4.3.2. 4-Unsubstituted Analogues of NU6227

Previously a number of 4-substituted pyrimidines had been prepared (see chapter 4.3.1 and Payne154). The synthesis of the unsubstituted analogues was explored to expand the SAR. Compound 86 maintains all substituents found in NU6227, other than the 4-position substituent. The analogous 5-nitroso analogue cannot be prepared as the nitrosation reaction requires a highly activated pyrimidine ring, and nitrosations without either the 4-alkoxy or 6-amino groups have previously proved impossible.153 Feuer et al suggest that the reaction occurs via initial nitrosation on the amino group, followed by a rearrangement to give the 5-nitroso compound, a variation of the Fischer-Hepp reaction.156

Scheme 9 Reagents and conditions; a) p-methoxybenzylamine, DMF, 3 h, 100 °C, 91%; b) TFA, DCM, 18 h, reflux, 77%

The synthesis of compound 88 is possible from bromo compound 87. Displacement of the 2-bromo substituent was achieved employing para-methoxybenzylamine, which was used as a convenient source of the amino group. A number of methods of deprotection of the amine groups was then attempted. The deprotection was first attempted using 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (89) in DCM. The use of the oxidant DDQ requires a 2-electron transfer from the electron rich methoxybenzyl group to the DDQ via a charge transfer complex (Scheme 10).157 However, treatment of compound 88 with DDQ failed to remove the PMB group. Hydrogenation using 10% Pd/C in acidic methanol also failed to
remove the PMB group. Finally 88 was treated with 70% TFA in DCM at reflux overnight to give 86 in a 77% yield.

Scheme 10

4.4 Structure-Activity-Relationships Around the Amino Groups

4.4.1. Synthesis of Pyrimidine Derivatives Lacking a 2-Amino Group

The three hydrogen bonds formed by the 2,6-diamino groups and the $N^3$ of the pyrimidine ring has been shown to be essential for CDK2 inhibitory activity. A 2-unsubstituted analogue of NU6227 and NU6027 would establish whether this is also true for mTOR inhibitory activity. If mTOR inhibitory activity does not require the 2-amino group, mTOR inhibitory activity could be separated from CDK2 activity and the 2-position may provide an interesting position for further substitution.

Luo et al generated a series of 6-aminosubstituted-4-chloropyrimidines from 4,6-dichloropyrimidine using microwave heating. $^{158}$ para-Methoxybenzylamine can be used as a convenient source of the amino group, and due to the electron-rich nature of the $p$-methoxybenzyl group, it can be easily removed, leaving the desired amino group in place. Compound 91 was formed by treating 4,6-dichloropyrimidine with para-methoxybenzylamine, in the presence of Hüning’s base in isopropanol, in 83% yield. Despite the dichloro-motif no formation of the di-amino compound was observed. Only 0.9 eq of the $p$-methoxybenzylamine was used and the pyrimidine ring is deactivated toward $S_{\text{NAr}}$ reactions after the first displacement.
Scheme 11 Reagents and conditions; a) \( p \)-methoxybenzylamine, DIPEA, isopropanol, 20 min, 120 °C, 83%; b) Na, cyclohexylmethanol, 180 °C, 30 min, 76% or NaH, cyclohexylmethanol, THF, 18 min, 120 °C, 72%; c) NBS, AcOH, 1 h, 69%; d) i. CuCN, DMF, 3 days, 120 °C; ii. Ethylenediamine, 3 days, 28%; d) TFA, DCM, 18 h, reflux, 50%

Two alternative reaction conditions were developed for the formation of compound 92. The first reaction uses the published methodology, i.e. sodium in neat cyclohexylmethanol with conventional heating, giving 92 in 76% yield. The second uses microwave heating. Previous work had suggested that formation of \( O^4 \)-alkoxy derivatives with microwave heating is feasible, though impractical for derivatives where formation of the sodium alkoxide requires heating. A series of \( O^4 \)-benzyl derivatives were also synthesised using microwave heating from sodium hydride and the appropriate benzyl derivative in THF\(^{153}\). Therefore, this methodology was also applied to the formation of compound 92 in 72% yield.

Bromination of 93 was achieved with NBS in AcOH using the conditions previously optimised for the formation of the \( O^4 \)-alkyl derivatives (81-85). Slightly longer reaction times were required, as the lack of the 2-amino group results in a less-activated ring towards electrophilic aromatic substitution. The nitrile group was incorporated using CuCN in DMF at 120 °C to give 94. The PMB group was removed from compound 94 using 30% TFA in DCM at reflux to give compound 95 in 50% yield.
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Scheme 12 Reagents and conditions: a) \( p \)-methoxybenzylamine, DIPEA, isopropanol, 20 min, 120 °C, 83%; b) Na, cyclohexylmethanol, 180 °C, 30 min, 76% or NaH, cyclohexyl methanol, THF, 18 min, 120 °C, 72%; c) NaNO\(_2\) \( (aq)\), AcOH \( (aq)\), 1 h, 80 °C, 79%; d) TFA, 60 °C, 1 h

A similar synthetic route to scheme 11 was developed for the synthesis of the 2-unsubstituted analogue of NU6027 (56) (Scheme 12). Compound 96 was synthesised from compound 92 using sodium nitrite in aqueous acetic acid in an analogous reaction to the synthesis of NU6027. Deprotection of the PMB group was attempted using neat TFA, a reaction which had previously been successful for more electron-rich pyrimidine systems.\(^{159}\) However, multiple products were observed by TLC and the major component was only isolated in poor yield (Scheme 12). The \(^1\)H NMR spectrum of the isolated compound was initially thought to correspond to that of the expected product 97. However, mass spectrometry showed an \( m/z \) of 208, which corresponded to a chemical formula of \( C_{11}H_{16}N_2O_2 \), presumed to be 6-hydroxypyrimidine 98 (Scheme 13).

Scheme 13 Reagent and conditions; a) TFA, 1 h, 60 °C

Compound 98 can be formed \( \textit{via} \) a diazonium reaction; thus the reaction of TFA with the 5-nitroso group of 96 liberates nitrous acid forming the diazonium intermediate. Reaction with water will produce the 6-hydroxy compound 98.
In light of the above results a milder deprotection of the PMB group was attempted (Table 4). Oxidative conditions were attempted including CAN, both in a buffered reaction with sodium tetraborate/borax buffer, in an analogous reaction to Marko et al., and in a dual phase reaction as described by Fürstner et al. The ‘dual phase’ reaction was achieved using a mixture of chloroform and water, with either 1,2-dimethoxyethane or camphorsulfonic acid as phase-transfer agent to aid the reaction (Table 4).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Conditions</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>0 °C</td>
<td>Pyrimidinone compound observed after 15 min</td>
</tr>
<tr>
<td>1.5 eq TFA in DCM</td>
<td>0 °C</td>
<td>Slow conversion to pyrimidinone compound</td>
</tr>
<tr>
<td>DDQ</td>
<td>RT</td>
<td>No reaction</td>
</tr>
<tr>
<td>CAN, aqueous acetonitrile</td>
<td>RT</td>
<td>Loss of nitroso group</td>
</tr>
<tr>
<td>CAN, buffered solution</td>
<td>RT, pH 8</td>
<td>Mixture of products</td>
</tr>
<tr>
<td>CAN, dual phase conditions, DME</td>
<td>RT</td>
<td>No reaction</td>
</tr>
<tr>
<td>CAN, dual phase conditions, DME (5% of solvent total)</td>
<td>RT, vigorous stirring</td>
<td>No reaction</td>
</tr>
<tr>
<td>CAN, dual phase conditions, DME and camphorsulfonic acid</td>
<td>RT, vigorous stirring</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

The failure of the PMB route required an alternative approach to compound 97. As the nitroso group had proved especially sensitive, it was decided to remove the para-methoxybenzyl group before nitrosation.
Scheme 14 Proposed reagents and conditions; a) 4-methoxybenzylamine, DIPEA, isopropanol, 83 min, 120 °C, 83%; b) sodium, cyclohexylmethanol, 30 min, 160 °C, 76%; c) trifluoroacetic acid, DCM, 24 h, reflux 87%; d) NaNO₂ (aq), AcOH(aq), 80 °C

Aminopyrimidine 99 was prepared from PMB protected 92 (prepared as discussed for Scheme 11) using 30% TFA in DCM at reflux overnight in good yield. Attempts to nitrosate 99 using the previous conditions failed, instead forming pyrimidinone 98. The failure of the reaction may be explained by the lack of activation of amino pyrimidine 99 relative to the diamine 56. Alternative conditions were then investigated. Previous work had identified nitrosation conditions appropriate for electron-deficient pyrimidines. Compound 99 was treated with sodium nitrite in trifluoroethanol with stoichiometric equivalents of TFA at reflux. This reaction proceeded at a slower rate than previous nitrosation reactions and a small percentage of the 5-nitroso 97 was observed by LCMS, but the predominant observed product was compound 98.

Marchal et al used isoamyl nitrite in DMSO as nitrosating reagent on a series of alkoxy, amino pyrimidines. However, for the example which correlates with the desired ecompound, (4-methoxy-6-aminopyrimidine) they also observed failure of the nitrosation. Instead, they isolated the pyrimidinone formed by diazotation and hydrolysis. The result obtained by Marchel et al demonstrated that the nitrosation cannot be performed with a free amino group in place. The nitrosating agent always forms nitrous acid, and without an activating group para- to the 5-position, this preferentially forms the diazonium ion instead of nitrosating the pyrimidine ring.
It was proposed that an alternative protecting group, which is removed under different conditions to PMB, may allow access to compound 97, therefore, the synthesis of 97 via an allyl protected amino group was attempted. The S\textsubscript{N}Ar reaction on 4-6-dichloropyridimidine with allylamine proceeded cleanly at room temperature for 24 h, with good conversion to give 100. The second displacement of the chloro from the pyrimidine heterocycle with cyclohexylmethoxide was undertaken as previously with NaH, cyclohexylmethanol in THF and microwave irradiation at 120 °C. However, conversion to the product was incomplete, and using medium pressure column chromatography separation of the starting material and product was not possible. Increasing the time of heating from 15 min to 20 min resulted in total conversion to the product. Compound 101 was then nitrosated using standard conditions, in a 66% yield.

![Chemical structure](image)

**Scheme 15** Proposed reagents and conditions; a) allylamine, Et\textsubscript{3}N, THF, 24 h, 98%; b) cyclohexylmethanol, NaH, THF, 20 min, 120 °C, 75%; c) NaNO\textsubscript{2}(aq), acetic acid(aq), 75 min, 80 °C, 66%; d) deprotection

Deprotection of the allyl group to give the free amine group and the desired 5-nitroso 97 was then required. The deallylation was initially attempted using Wilkinson’s catalyst and DABCO. These conditions isomerize the allyl double bond, allowing extraction using DABCO as a nucleophile.\textsuperscript{163} Allyl 102 was treated with Wilkinson’s catalyst and DABCO in refluxing ethanol. However, the allyl group remained in place but the nitroso group had been removed giving allyl 101.
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Scheme 16 Reagent and Conditions; a) DABCO, Rh(PPh₃)₃Cl, EtOH, 80 °C, 4 h

Ueda et al used palladium triphenylphosphine and N,N-barbituric acid to remove allyl groups during the total synthesis of (+)-haplophytine.¹⁶⁴ Treatment of 102 with Pd(PPh₃)₄, 1,3-dimethylbarbituric acid in refluxing DCM failed to remove the allyl group. However, replacing the DCM with the higher boiling ethanol resulted in the generation of a number of products. ¹H NMR identified these two products as 101 and 99, one lacking the 5-nitroso group 101 and the other lacking the 5-nitroso and the allyl group 99.

Figure 41

Karpf et al removed allyl groups in their azide free synthesis of oseltamivir using 10% palladium on carbon and ethanolamine in ethanol.¹⁶⁵ However, under these conditions, the nitroso group was rapidly removed. In view of these unsuccessful results and time-constraints it was decided to discontinue work on the synthesis of 97.

4.4.2. Synthesis of 6-Hydroxylamino Pyrimidines

The high-throughput screen which identified pyrimidine-based compounds as having mTOR inhibitory activity also identified a number of 6-amino acyl derivatives with mTOR inhibitory activity e.g. 60 and 61. A range of acyl and carbamates derivatives were therefore synthesised. These derivatives may be unstable under assay conditions, and HPLC monitoring showed decomposition over time.¹⁵⁴ A more stable substituent was required to
probe the region around the 6-amino position, such as the methyl (103) and the benzyl (104) aminooxy analogues.

Figure 42

Too et al proposed that with electron-withdrawing methoxy or benzyloxy groups on the 6-amino groups an alternative tautomer may be observed, whereby the amino tautomer (105) interconverts with the imino tautomer (106), which would disrupt the triplet of hydrogen bonds to the protein. However, with the 5-nitroso group forming a hydrogen bond to the 6-amino proton the equilibrium may favour the desired tautomeric form.

The synthetic route to access compounds 103 and 104 is shown below.

Scheme 17 Proposed reagents and conditions; a) R = Me methylamine hydrochloride, DIPEA, DMSO, 16 h, 130 °C, 52%; R = Bn O-benzylhydroxylamine, DIPEA, DMSO, 6 h, 130 °C, 66%; b) Na, cyclohexylmethanol; c) NaNO₂(aq), AcOH(aq)

Too et al prepared aminooxy-substituted purines by treating chloro-substituted purines with 10 equivalents of the appropriate alkyl substituted hydroxylamines in the presence of Hünig’s base. To avoid using such a large excess of reagent, conditions were adapted from the synthesis of compound 91 to synthesise 109. However, multiple products were formed.
Therefore, optimisation of this reaction was undertaken. The conditions and results are summarised below.

![Chemical structure](image)

**Table 5** Results and conditions for the synthesis of 109

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Heating type</th>
<th>Equivalents of O-benzyl hydroxylamine</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>microwave</td>
<td>1</td>
<td>140</td>
<td>75 min</td>
<td>27 %</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>microwave</td>
<td>1</td>
<td>160</td>
<td>90 min</td>
<td>39%</td>
</tr>
<tr>
<td>Trifluoro-ethanol</td>
<td>microwave</td>
<td>1</td>
<td>170</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>DMSO</td>
<td>microwave</td>
<td>1</td>
<td>170</td>
<td>40 min</td>
<td>29%</td>
</tr>
<tr>
<td>DMSO</td>
<td>microwave</td>
<td>1</td>
<td>180</td>
<td>15 min</td>
<td>17%</td>
</tr>
<tr>
<td>DMSO</td>
<td>microwave</td>
<td>1</td>
<td>170</td>
<td>30 min</td>
<td>29%</td>
</tr>
<tr>
<td>DMSO</td>
<td>conventional</td>
<td>1.5</td>
<td>130</td>
<td>8 h</td>
<td>42%</td>
</tr>
</tbody>
</table>

Conventional heating for longer times at a low temperature was shown to be higher yielding, along with increasing the equivalents of O-benzylhydroxylamines. These conditions were then applied to the synthesis of compound 108, though the reaction time was slightly longer for the methyl compound and slightly lower yielding (52%). However, on attempting formation of cyclohexylmethoxy compound 111, the sensitive nature of hydroxylamine 109 again proved problematic. Treatment of compound 109 with either sodium in cyclohexylmethanol or sodium hydride with cyclohexylmethanol in THF at 120 °C with both microwave and conventional heating resulted in decomposition of the starting material and the formation of by-products. Under similar conditions the analogous methyl compound was also seen to decompose. Multiple products may be formed as a result of nucleophiles attacking the benzyl and methyl group. Therefore, incorporation of the hydroxylamine
functionality at a later step may be advantageous. An alternative synthetic route was devised which reversed the order of substitution. The synthetic route is shown below (Scheme 18).

Scheme 18 Proposed reagents and conditions; a) Na, Cyclohexylmethanol, 20 min, 100 °C, 62%; b) R = Me, methoxyamine hydrochloride, DIPEA, DMSO, 130 °C; R = Bn, O-benzylhydroxylamine, DIPEA, DMSO, 130 °C; c) NaNO₂(aq), AcOH(aq)

Conventional introduction of the cyclohexylmethoxide group (180 °C, Na) resulted in disubstitution forming 113. Optimisation of this reaction was therefore undertaken. Optimal conditions were found to 0.9 eq. of sodium at 100 °C, resulting in the formation of compound 112 in a 62% yield. However, attempts to incorporate the amino-oxy functionality proved unsuccessful. In view of their instability it was decided to discontinue work on these compounds.

Table 6

<table>
<thead>
<tr>
<th>Equivalents of sodium</th>
<th>Temperature (°C)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excess</td>
<td>180</td>
<td>113</td>
</tr>
<tr>
<td>Excess</td>
<td>120</td>
<td>113</td>
</tr>
<tr>
<td>0.9</td>
<td>100</td>
<td>112</td>
</tr>
</tbody>
</table>

Unfortunately, modification to either of the amino groups proved either unsuccessful, or abolished activity. Alternative areas of the molecule were then addressed to develop further the SAR.
4.4 Modifications to the 5-Position

Potent compounds identified from the high throughput screen all maintained an electron-withdrawing substituent capable of accepting a hydrogen-bond at the 5-position. Compounds bearing electron-withdrawing substituents, which cannot form hydrogen bonds, have also been synthesised, including the acetylene derivative shown below, but this modification resulted in a loss of biological activity against mTOR.

![Figure 43](image)

To examine further the structure-activity relationships around the 5-position a series of analogues was therefore designed. Compound 117, which introduces a CH$_2$ unit between the aromatic ring and the nitrile group, was synthesised. Hockova et al alkylated at the 5-position by treating 2,6-diaminopyrimidinone with bromoacetonitrile in the presence of base.$^{167}$ Using this synthetic route compound 117 was synthesised in an unoptimised yield of 9% (Scheme 19).

**Scheme 19** Reagents and conditions; a) bromoacetonitrile, NaHCO$_3$, DMF, 4 days, 9%; b) (bromomethyl)cyclohexane, K$_2$CO$_3$, DMF, 15 h, 100 °C, 22%

Formation of the desired final compound requires alkylation of the pyrimidine oxygen. The attempted conditions are shown below (Table 7). The alkylation reaction with (bromomethyl)cyclohexane proved successful, although purification of compound 117 proved difficult, requiring multiple rounds of medium pressure chromatography, followed by recrystallisation giving compound 117 in 22% yield.
Table 7

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Conditions</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>DEAD, PPh₃, Cyclohexylmethanol, THF</td>
<td>RT</td>
<td>No reaction</td>
</tr>
<tr>
<td>DEAD, PPh₃, Cyclohexylmethanol, DMF</td>
<td>RT</td>
<td>No reaction</td>
</tr>
<tr>
<td>DIAD, PPh₃, Cyclohexylmethanol, DNF</td>
<td>100 °C</td>
<td>No reaction</td>
</tr>
<tr>
<td>(Bromomethyl)cyclohexyl, K₂CO₃, DMF</td>
<td>100 °C</td>
<td>22</td>
</tr>
</tbody>
</table>

Both the N-alkylated product and O-alkylated product are possible results of this reaction. The $^{13}$C NMR spectrum of the major product suggested O-alkylation. The predicted values for the methylene carbon adjacent to either the pyrimidine N or the exocyclic O are 44 or 72 ppm respectively. The observed value for the methylene carbon was 70.3 ppm, suggesting that the desired O-alkylated compound has been formed. Unlike the compounds where the nitrile group is directly attached to the aromatic ring, which have a strong absorption peak in the IR at around 2000 cm⁻¹, compound 117 had a weak CN absorption, as expected for an alkyl nitrile as this is known to be a weaker absorption.

Compound 118 extends the nitrile further from the 5-position and retains a hydrogen bond acceptor at a similar position to that of the nitroso group in NU6027.

Figure 44

Quiroga et al cyanoacetylated a series of pyrimidinone compounds by forming a mixed anhydride from cyanoacetic acid and acetic anhydride. Pyrimidinones are required starting materials, as the use of pyrimidines as the starting material resulted in acylation at the exocyclic amino group.¹⁶⁸
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Scheme 20 Reagents and conditions; a) Ac₂O, NCCH₂CO₂H, 80 °C; b) (bromomethyl) cyclohexane, K₂CO₃, DMF, 15 h, 100 °C

Scheme 21

The mixed anhydride was formed from acetic anhydride and cyanoacetic acid at 80 °C. Pyrimidinone (115) was then added to the reaction mixture to form compound 119. As the cyano-substituted portion of the acetic anhydride is more electron-deficient, attack from the pyrimidinone ring should occur on this moiety. However, the crude product obtained from this reaction proved to be very insoluble, and no purification or NMR analysis was possible. Therefore the synthesis of this molecule was delayed until the biological results of compound 117 were obtained. Subsequently, as 117 showed a loss of activity, no further synthetic efforts were directed towards 118.

It has been proposed that the biological potency of derivatives with electron-withdrawing groups at the 5-position is due to the acidification the two amino protons, leading to the formation of stronger hydrogen bonds with the protein. A fluorine group at the 5-position of the pyrimidine ring would also act to acidify the 2-amino protons. The 5-fluoropyrimidine derivative 119 might identify whether the 5-substituent is required to act as a hydrogen-bond acceptor or to acidify the 2-amino substituents.
Fluorinating agents have become an area of interest due to their increasing use within medicinal chemistry. A class of reagents known as N-F reagents are becoming increasingly popular, as they are a stable and easy to handle source of ‘F’. Selectfluor™ is one of the commonest reagents of this class, and there are examples within the literature of electrophilic substitution of aromatic rings using Selectfluor™, including the fluorination of pyrimidine compounds. The structure of Selectfluor™ (120) is shown below.

![Figure 45](image-url)

**Figure 45**

Compound 55 was treated with Selectfluor™ in acetonitrile at temperature up to 80 °C. LCMS monitoring indicated the formation of a new product. However, the reaction did not go to completion and on work-up and purification, no new product was obtained.

![Scheme 22](image-url)

**Scheme 22** Proposed reagents and conditions; a) Cyclohexylmethanol, sodium, 160 °C, 2h, 20 %; b) Selectfluor™, MeCN, 80 °C

A further group of reagents within the N-F class of reagents, which also includes Selectfluor™, are the N-fluoropyridinium salts, which also act as a source of electrophilic fluorine. On treating 55 with 1-fluoro-2,4,6-trimethylpyridinium salt, LCMS monitoring of the reaction suggested a new product was formed. However, after work-up and attempted purification only starting material was isolated (Scheme 23).
Furuya et al have reported the fluorination of aromatic species from boronic acids,\textsuperscript{172} using silver triflate and Selectfluor\textsuperscript{TM}. The reaction proceeds via transmetallation of the aromatic species from the boronic acid to silver, which is then followed by fluorination of the aromatic ring. To follow the synthetic procedure developed by Furuya et al, the 5-boronic acid analogue 121 was required. The bromo derivative 66 was accessed from 4-cyclohexylmethoxy-2,6-diaminopyrimidine on treatment with NBS (Scheme 24). However, attempts to form the boronic acid, via the boronic ester, using bis(pinacolato)diboron, palladium acetate and potassium acetate\textsuperscript{173} in DMF rapidly resulted in protodeboronation. In view of these results the synthesis of 119 was abandoned.

Cabaj et al have synthesised compound 122 using Selectfluor\textsuperscript{TM}, but no experimental procedure was given. The compound is described as ‘moderately stable’ and only a crude yield is provided, suggesting that 5-fluoropyrimidines, with three electron-donating substituents are not especially stable.
4.5 Pyridine AnalOGues of mTOR inhibitory Pyrimidines

To separate mTOR inhibitory activity from CDK2 inhibitory activity, and to develop the structure-activity relationships for the pyrimidine ring, two pyridine-based analogues were synthesised. Each of these analogues probes the role of both of the pyrimidine nitrogens.

The first pyridine analogue 134 is capable of maintaining the triplet of hydrogen bonds, shown to be essential for CDK2 inhibitory activity but replaces the N3 nitrogen with CH. The proposed synthetic route utilises a reaction described by Manley et al, which attaches secondary amides α to pyridine N-oxides (Scheme 25).
The first step of the reaction is formation of the 4-cyclohexylmethoxypyridine (126). It was proposed to achieve formation of this product via a Mitsunobu reaction between cyclohexylmethanol and 4-pyridone (126) (the preferred tautomer of 4-hydroxypyridine (135)).

On treating 4-pyridone in THF with DEAD, PPh$_3$ and cyclohexylmethanol the highest isolated yield was 16%. Further analysis of the product also revealed that the compound was not the desired $O$-alkylated product, but instead the $N$-alkylation product. Although there are numerous examples within the literature of a Mitsunobu reaction performed on 2-pyridone, there are few examples of the Mitsunobu reaction with 4-pyridone. Comins et al suggest that THF is an appropriate solvent to form the $O$-alkylated product in a Mitsunobu reaction with 2-pyridone. However, in our hands 4-pyridone in THF forms the $N$-alkylated product. The pKa of 2-pyridone (11.62) is similar to that of 4-pyridone (pKa of 11.09), so should not influence the reaction outcome. However, Schlegal et al suggest that a weak interaction can occur between the 2-hydroxy and pyridine nitrogen in 2-hydroxypyridine, stabilising the hydroxy form slightly, and it could be this form which reacts in the Mitsunobu reaction. However, in view of the disappointing yield an alternative method of formation of 4-cyclohexylmethoxypyridine was investigated.
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![Proposed interaction between 2-hydroxy group and nitrogen of 2-hydroxypyridine]

**Figure 47** Proposed interaction between 2-hydroxy group and nitrogen of 2-hydroxypyridine

Alkylation of 4-pyridone with (bromomethyl)cyclohexane in the presence of a base was investigated. Two products are possible from this reaction, the N-alkylated product and the O-alkylated product. By altering the reaction conditions the reaction could be tuned to provide the desired O-alkylated product (Table 8). All the reactions used 1 equivalent of 4-pyridone, 1.5 equivalents of base, 5 equivalents of cyclohexylmethyl bromide and if used, 2 equivalents of 18-crown-6 and followed the procedure as described for 1-(cyclohexylmethyl)pyridin-4(1H)-one (137).

![Scheme 26 Reagent and conditions; a) (bromomethyl)cyclohexane, base and solvent]

**Scheme 26** Reagent and conditions; a) (bromomethyl)cyclohexane, base and solvent

18-Crown-6 was used as an additive to the reaction as previous work within the laboratory suggested that this would increase the yield of the O-alkylated product. Crownethers ‘shield’ the cation allowing delocalisation of the negative charge. But in our hands this did not aid formation of the desired product. Both CsF and Cs₂CO₃ were used as a larger, softer cation may allow delocalisation of the negative charge, therefore resulting in O-alkylation. Caesium carbonate did result in formation of the greatest percentage of the desired product (127) but still a 1:1 ratio of product and unwanted isomer was formed. Both Hopkins *et al* and Comins *et al* suggest that silver salts favour formation of O-alkylated products in alkylation reactions of 2-pyridone, and Hopkins *et al* also demonstrated selective O-alkylation by using pentane as a solvent. In our case, silver salts gave particularly poor yields of any alkylated products.
Identification of the two isomers was possible via the $^{13}$C NMR spectrum and by comparison with the 4-methoxypyridine $^{13}$C spectrum. The NMR spectrum of the $N$-alkylated product has a signal at 179 ppm, which corresponds to the pyridone carbonyl carbon, whilst the $O$-alkylated product has a signal at 166 ppm which corresponds with the pyridine C-O carbon.
To confirm the identity of the two possible isomers a crystal structure of the putative O-alkyl product was obtained. The N-alkylated product did not give appropriate crystals for X-ray crystallography.

Figure 49 X-ray structure of the O-alkylated product (127)

To avoid the formation of the undesired N-alkylated an alternative route to the desired 4-cyclohexylmethoxy.pyridine was developed. Utilising a similar approach to pyrimidine derivatives the chloro group can be displaced from 4-chloropyridine by sodium alkoxide. To
To utilise the synthetic route developed by Manley et al. the pyridine N-oxide must first be formed. This was achieved using m-CPBA at room temperature in good yield. 4-Methoxypyridine was also used as a model compound for this reaction and was converted to pyridine N-oxide in good yield. Manley et al. formed imidoyl chloride (139) in situ from a secondary amide on treatment with oxalyl chloride and 2,6-lutidine. It is the imidoyl chloride species which then reacts with the pyridine N-oxide.

\[ \text{Scheme 27} \]

N-benzylacetamide (138) was synthesised by treating benzylamine with acetic anhydride in 27% yield. 138 was then treated with oxalyl chloride and 2,6-lutidine in DCM at 0 °C, and after 15 min 4-methoxypyridine N-oxide was added and the reaction was warmed to RT. Unfortunately, during the model reactions no reaction was observed, and so an alternative method to incorporate the desired 2,6-diamino motif was required. Treatment of pyridine N-oxides with phosphorous oxychloride results in chlorination α to the nitrogen atom. The phosphorous oxychloride firstly reacts with the N-oxide which results in the loss of a chloride ion. This chloride ion can then attack the ring, which has been activated towards nucleophilic attack by formation an intermediate N-oxy species, which makes the ring more electron-deficient.
The new proposed scheme is shown below (Scheme 28). Each reaction of the N-oxide will result in the addition of one chloro group. Therefore, the molecule must be re-oxidised to incorporate the second chloro group. After both chloro-groups have been incorporated they can be displaced using p-methoxybenzylamine, which can act as a protected form of the amino groups for the synthetic route.

Scheme 28 Proposed reagents and conditions; a) Na, cyclohexylmethanol, 3.5 h, 200 °C, 53%; b) m-CPBA, DCM, 2 days, 97%; c) POCl₃, MeCN, 30 min, 160 °C, 56%; d) m-CPBA, DCM, 94%; e) POCl₃, MeCN

The optimal conditions for the chlorination were found to be with POCl₃ in MeCN and microwave heating at 160 °C for 30 min giving compound 142 in a 56% yield. Conventional heating resulted in lower yields and required overnight heating. Re-oxidising to the N-oxide was achieved using mCPBA in good yield 94%. However, the second chlorination failed to occur, both with microwave and conventional heating and continual heating resulted in the formation of a number of by-products.

An alternative synthetic route to the desired pyridine analogue was required. Braxmeier et al. has synthesised 4-benzyloxy-2,6-diaminopyridine (148) in three steps from chelidamic acid, by utilising a Hofmann rearrangement to convert amide groups to amine groups (Scheme 29). The benzyloxy pyrimidine derivatives are equipotent as mTOR inhibitors, so to allow direct comparison with the literature compounds, it was decided to synthesise compound 149.
The first step of the synthetic scheme is an alkylation of both carboxylic acid groups and the pyridine oxygen, followed by conversion to the amide, prior to the Hofmann rearrangement. In our hands these alkylation conditions resulted in the formation of multiple products, making it difficult to identify the desired products.

Scheme 29 Reagents and conditions; a) BnBr, K$_2$CO$_3$, acetone, 3 days, reflux, 71%; b) ammonia, methanol, 3 h, 93%; c) 5 M KOH$_{(aq)}$, Br$_2$, 5 h, 90 °C, 76%.$^{180}$

However, alternative procedures to dialkyl 4-alkoxy-pyridine-2,6-dicarboxylate rely on formation of the esters before alkylation.$^{181}$ Conversion of chelidamic acid (150) to ethyl ester (153) followed by alkylation of the pyridine ring proved to be more reliable, with the alkylation achieved in particularly high yield (96%) (Scheme 31). The solvent was changed for the alkylation reaction, using DMF rather than acetone suggested by Kruizinga et al.$^{182}$ Conversion of the ester groups to amide was achieved using 7 M ammonia in methanol in good yield (Scheme 30).

The Hofmann rearrangement was achieved using bromine in 5 M KOH, which procedes via a nitrene species, which can rearrange to an amine, via an isocyanate. The desired 2,6-diamino functionality was now in place. The amino groups were then protected using acetic acid and acetic anhydride at room temperature in 91% yield. The pyrimidine-based synthesis of nitrile analogues suggested that the motif must be protected to prevent chelation to the copper cyanide.$^{152}$ As the pyridine ring has two potential sites of electrophilic addition it was decided the ring should be deactivated slightly to prevent over-substitution. Therefore, the acetyl protection was incorporated before the bromination. Bromination was achieved using NBS in acetic acid in a 77% yield. However, on treating the brominated pyridine with CuCN in DMF at 120 °C no product was formed. Increasing the temperature only resulted in decomposition.
The reaction was then repeated at higher temperatures using microwave irradiation, with N-methylpyrrololidine as solvent. Under the conditions, only slight traces of the product were observed, with the majority of the starting material decomposing. The reaction was repeated with further equivalent of CuCN, with similar results, as well as with the deprotected compound, but no compound was isolated.

Nitrile groups can also be incorporated using a palladium-catalysed method. Maligres et al used Pd(dba)$_2$ to catalyse the formation of aromatic nitriles from aromatic bromines using Zn(CN)$_2$. However, under these conditions no reaction occurred. Replacing the catalyst with Pd(PPh$_3$)$_4$ also failed to give any product. It was therefore decided to halt the attempted synthesis of this molecule.

Scheme 30 Proposed reagents and conditions; a) SOCl$_2$, EtOH, 2 h, reflux, 46%; b) benzyl bromide, K$_2$CO$_3$, DMF, 30 min, 80 °C, 96%; c) 7 M methanoic ammonia, 3.5 h, 88%; d) Br$_2$, 5 M KOH$_{(aq)}$, 2 h, 90 °C, 71%; d) Ac$_2$O, AcOH, 3 days, 92%; e) NBS, AcOH, 30 min, 77%; f) i. CuCN, DMF; ii. ethylenediamine

Whilst this work was ongoing the direct pyridine analogue of NU6027 was also synthesised. Using the previously developed methodology, compound 160 was synthesised using the route shown below. Formation of 160 proceeded cleanly in 30 min using standard nitrosating condition of NaNO$_2$ in 30 % acetic acid in water to give the direct pyridine analogue of NU6027.
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The alternative pyridine analogue of the ‘hit’ compounds was also required. Szczepankiewicz et al synthesised 2-alkoxy-3-carbonitrile-4,6-diaminopyridines from a bromo-substituted pyridine ring, with a sodium alkoxide to displace the bromo group.\textsuperscript{155} Szczepankiewicz et al synthesised the bromo-substituted pyridine ring (162) in a one-step cyclisation step as described by Carboni et al by treating malononitrile with gaseous hydrogen bromide.\textsuperscript{184} For safety reasons, the reaction was replicated using a liquid solution of 45% HBr in acetic acid, resulting in multiple products by TLC, and it was not possible to isolate the desired intermediate.

\begin{scheme}
\begin{align*}
\text{Proposed reagents and conditions; } & \text{a) } 48\% \text{ HBr in AcOH} & \text{b) } \text{Na, cyclohexylmethanol} \\
\text{160} & \text{161} & \text{162}
\end{align*}
\end{scheme}

Silver et al treated malononitrile with zinc chloride in the presence of ethanol to synthesise 3-carbonitrile-4,6-diamino-2-ethoxypyridine.\textsuperscript{185} A proposed mechanism of formation of the pyridine ring is shown below.
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The synthesis of compound 162 by treating malononitrile with ZnCl$_2$ in the presence of cyclohexylmethanol was attempted. A number of products was observed by TLC, and although the LCMS suggested that the correct product had been formed, it was not possible to isolate the desired compound. The reaction was repeated using ethanol as described in the literature, but multiple products were formed.

Scheme 33

Junek et al. treated 2-amino-1,1,3-tricyanopropene (163) with sodium alkoxide, synthesising a pyridine ring with the desired functionality.$^{186}$ 2-Amino-1,1,3-tricyanopropene is also known as malononitrile dimer and is proposed as the intermediate in the synthesis of the substituted pyridine described by Carboni et al., suggesting that this reaction may proceed by a similar mechanism, which is shown below.$^{184}$

Scheme 34 Proposed reagents and conditions; a) ZnCl$_2$, cyclohexylmethanol, 100 °C
Nitrile 163 was treated with sodium methoxide for 48 h at reflux forming two products, as described by Junek et al. The two compounds had similar $R_f$ to those described in the paper (0.3 and 0.6 in 4:1 diethyl ether to petrol). The major compound, with an $R_f$ of 0.3, was isolated in 20%. It is this compound which Junek et al identified as being the desired isomer. The minor compound has an $R_f$ of 0.6 and was produced in 5% yield.

NMR experiments were undertaken to confirm the identity of the compounds. The proton NMR spectrum was insufficient to identify the compounds. A 1D nOe (Nuclear Overhauser Effect) experiment was undertaken to observe interactions between the protons within the molecule. By the irradiation of a known proton (in this case the aromatic proton), an effect should be observed on protons which lie close within space to this proton. For the desired isomer an interaction should be seen between the aromatic proton and both amino groups, and for the unwanted isomer an interaction should be seen between one amino group and the methyl group. On running the 1D nOe experiment on the major compound an interaction was seen between the aromatic proton and both amino groups. These results suggest the major compound is the desired isomer. However, when the same experiments were run on the minor compound no interaction was seen. As a result a new $^1$H NMR experiment was run, which
revealed significant decomposition of the compound, suggesting this compound is unstable in deuterated chloroform. This degradation would explain the apparent lack of interaction between protons in the nOe experiment.

Figure 50 $^1$H NMR spectrum and 1D nOe experiment performed on compound 165
A further experiment was therefore run to confirm the identity of 165. A $^{15}$N HMBC looks at the interaction between nitrogen atoms and the protons. The interaction between a proton and the nitrogen atoms can occur only over a distance of four bonds. With the desired compound the aromatic proton will interact with three nitrogen atoms, however the other isomer will only interact with 2 nitrogen atoms. After running the $^{15}$N HMBC experiment on the major product the aromatic proton was seen to interact with 3 nitrogen atoms, confirming that the major product is the desired isomer (165).

**Figure 51**

**Scheme 37** Reagents and condition: a) Na, cyclohexylmethanol, 48 h, 120 °C, 4%
After successfully forming the methoxy analogue (165), synthesis of the direct pyridine analogue of NU6227 was undertaken using cyclohexylmethanol. The reaction mixture was heated at 120 °C for two days resulting in a complex mixture which required prep. HPLC. The 1D nOe experiment was not conclusive as irradiation at the frequency of the aromatic proton resulted in signal in both the diamino protons and the cyclohexylmethoxy CH$_2$ protons. However, the $^{15}$N HMBC showed that the aromatic proton interacts with three nitrogen nuclei, both amino nitrogens and the pyridine nitrogen. As this interaction occurs over a maximum of three bonds the aromatic proton must lie between the amino groups for this to occur. This suggests that the synthesised compound is the desired substance.

The synthesis of both pyridine isomers of the modestly active pyrimidine compounds unfortunately failed to maintain activity against mTOR; therefore no further investigation of this heterocycle was undertaken.

4.6 Biological Results

4.6.1. Assay details

Biological analysis was carried out at the Northern Institute for Cancer Research by Lan Zhen Wang. Testing was undertaken with a KLISA assay using a recombinant human mTOR protein, recombinant p70S6K-GST protein, ATP, an anti p70S6K-T389 antibody and a secondary antibody with immobilised horse radish peroxidase.

A solution of p70S6K protein was added to a 96 well plate and incubated for 1 h at room temperature. Excess solution was then poured away and the plate dried. A solution of tris buffered solution (TBS) was used three times to wash the wells. The mTOR standard (protein and ATP) and the compound (dissolved in DMSO) were then added to the wells with a buffer solution; the plate was then covered and shaken for 30 seconds by a plate shaker, then incubated for 30 min at 30 °C. The kinase stop solution was then added and the plates washed. The anti p70S6K-T389 antibody solution was added and the plate incubated at room temperature for 1 h. The plate was washed and the horse radish peroxidase conjugate antibody added, then again incubated for 1 h and washed. A 3,3',5,5'-tetramethylbenzidine (TMB) solution was added and the mixture was incubated for 10 min at room temperature, followed by the ELISA stop solution (2.5 M H$_2$SO$_4$). The plate was read at 450 nm.
Table 9 Biological results * indicates compounds first synthesised by other chemists

<table>
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<tr>
<th>Number</th>
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4. The Development of ATP-competitive Inhibitors of mTOR

<p>| | | | | |</p>
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<td>162</td>
<td>C</td>
<td></td>
<td>CN</td>
<td>NH₂</td>
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</table>

None of the new compounds that have been synthesised showed improved activity compared to the parent compounds. All newly synthesised compounds were weakly active, with an IC₅₀ against mTOR > 10 µM. The 5-nitroso or nitrile substituent has been demonstrated as important for activity, as is the triplet of hydrogen bonds.

The series of O₄-alkyl substituents demonstrated that the further the alkyl group is from the cyclohexylmethyl group, in both size and shape, the greater the loss of activity. A series of aromatic O₄-derivatives have been synthesised and demonstrated a tolerance for large lipophilic groups – suggesting the alkyl groups are too small to fill the lipophilic pocket in which the O₄-substituent sits. Complete removal of the substituent at the 4-position, interestingly, has increasing biological activity over smaller alkoxy derivatives, suggesting that the oxygen atom itself is not forming an interaction with the protein, but is only acting as a link between the pyrimidine ring and the alkyl substituent. No substituent at the 4-position appears to be preferred over a small alkoxy substituent.

Insertion of a CH₂ group between the pyrimidine ring and the nitrile group in compound 117 abolished activity. The binding site around the 5-position may be very narrow, and does not tolerate the additional chain length. Or, the additional CH₂ group positions the nitrile group in the incorrect alignment to form a hydrogen bond with the protein. Removal of the 2-amino groups also abolished activity, underlining the importance of the triplet of hydrogen bonds, for not only CDK2 inhibition but also for mTOR inhibitory activity.

For both pyridine-based compounds, activity was significantly reduced. For compound 157, activity was approximately halved. Though the triplet of hydrogen bonds is maintained, a
nitrogen atom at the 3-position of the pyrimidines is deleted, which may be forming an interaction with the protein. However, the change from pyrimidine to pyridine heterocycles will also result in a change in $pK_a$. This may result in a change of protonation state under assay conditions, which removes the acceptor moiety of the donor-acceptor-donor motif. Compound 160 completely abolishes mTOR inhibitory activity. Removal of the acceptor moiety of the triplet of hydrogen bonds resulted in a complete loss of activity.

Interestingly, though mTOR activity is significantly reduced by the modification from the pyrimidine to the pyridine heterocycle, CDK2 activity does not follow the same trend. Compound 157, which retains the triplet of hydrogen bonds maintains biological activity, but compound 160, which lacks the triplet of hydrogen bonds has significantly reduced biological activity.

![Series A and Series B](image)

**Table 10** Biological activity against CDK2

<table>
<thead>
<tr>
<th>Number</th>
<th>Series</th>
<th>X</th>
<th>CDK2 inhibition % inhibition at 100 µM (IC$_{50}$ (µM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>A</td>
<td>NO</td>
<td>(5.2)</td>
</tr>
<tr>
<td>162</td>
<td>B</td>
<td>CN</td>
<td>43</td>
</tr>
</tbody>
</table>
4.7 Future Work for the Development of Pyrimidine-based mTOR Inhibitors

A 5-fluorinated pyrimidine was desired to determine the role of the 5-substituent, specifically the requirement to form an intermolecular hydrogen bond, or as an electron withdrawing group. Due to time constraints it was not possible to explore fully the alternative routes to access compound 122. One approach would involve the building of the pyrimidine ring with the 5-fluoro substituent in place.

Joule et al proposed that the use of ethyl-2-cyanoacetate would allow the 6-amino group to be incorporated during the building up of the pyrimidine heterocycle. Protons which lie between the nitrile group and the ester groups are acidic, with a pKa value of ca. 13. Deprotection followed by quenching the anion with a source of F should result in generation of compound 172. Formation of the pyrimidine from guanidine would allow incorporation of the 2-amino group. After chlorination of the oxo group with POCl₃ and displacement with cyclohexylmethoxide the desired compound 119 would be obtained.

\[
\begin{align*}
171 & \xrightarrow{a} 172 & 173 & \xrightarrow{b} 174 & \xrightarrow{c} 175 \\
\text{EtO} & \xrightarrow{N} \text{CN} & \text{EtO} & \xrightarrow{F} \text{CN} & \text{HN} & \xrightarrow{NH} \text{NH}_2 & \text{HN} & \xrightarrow{NH} \text{NH}_2 & \text{Cl} \\
\end{align*}
\]

Scheme 38 Proposed reagents and conditions; a) KOH, Selectfluor, THF; b) Na, methanol; c) POCl₃ d) Na, cyclohexylmethanol

Parks et al have reported the regiospecific displacement of fluorine atoms by nucleophiles from tetrafluoropyrimidine. Using a similar approach, the desired fluoro compound could be synthesised. The displacements are thought to occur first at the 4- and 6-position, followed by the 2-position. Therefore, adapting the synthetic route shown below, compound 122 could be synthesised. Hünig’s base was added by Parks et al to all reactions to neutralise any formed HF.
4. The Development of ATP-competitive Inhibitors of mTOR

Nowak et al. have described the synthesis of pyrazolopyrimidine-based potent mTOR inhibitors such as 179, with an IC_{50} of 4 nM.\textsuperscript{72} The 5-nitroso compound (180) has been demonstrated to form a hydrogen bond between the 5-nitroso substituents and the proton on the 6-amino group forming a pseudocycle. Therefore a pyrimidine with a 5-nitroso substituent may act as a mimetic for a pyrazolopyrimidine, and a scaffold–hopping approach will identify active substituents for the pyrimidine ring such as 180.

A S_NAr reaction between sodium methoxide and trichloropyrimidine will result in substitution at the 2-position, the most reactive position. Two further S_NAr reactions with morpholine and N-benzyl protected 4-aminopiperidine, will incorporate the desired functionality at both the 4- and 6-position. Removal of the methyl group with the Lewis acid boron tribromide, will also result in removal of the Boc protecting group. Triflation of the resulting hydroxyl group will generate the appropriate substituent for a Suzuki reaction,
which followed by nitrosation and treatment with benzoyl chloride should result in the formation of the desired compound.

Scheme 40 Proposed reagents and conditions; a) Na, MeOH; b) Morpholine, DIPEA, THF; c) 4-amino-1-Boc-piperadine, DIPEA, THF; d) 1 M BBr$_3$ in DCM, DCM; e) Tf$_2$O, Et$_3$N, THF; f) (3-hydroxyphenyl)boronic acid, Pd(PPh$_3$)$_4$, Na$_2$CO$_3$, dioxane, water; g) NaNO$_2$, AcOH, water; h) Benzoyl chloride, Et$_3$N, THF

4.8 Conclusion

mTOR is an attractive anti-cancer target due to its function within the PI 3-K/Akt pathway, which frequently suffers from aberrant signalling within cancer. Our work on ATP-competitive inhibitors of mTOR began with a series of pyrimidines with modest activity against mTOR. Synthetic efforts have identified the importance of the pyrimidine heterocycle through the synthesis of two pyridine analogues. The synthesis of a 2-unsubstituted analogue
of NU6227 has also identified the importance of the triplet of hydrogen bonds formed with the protein. Modifications to the $O^4$-substituent with a series of alkyl derivatives reduced activity, alkyl groups such as the sec-butyl which had comparable shapes and size to the cyclohexylmethyl group were shown to be the most active of the newly synthesised analogues, whilst smaller groups, such as the ethyl analogue, were shown to be less active than complete removal of the 4-substituent. A series of substituted benzyl analogues have also been synthesised and showed a flat SAR, suggesting that the $O^4$-substituent sits within a large lipophilic pocket, which is not fully utilised by the alkyl substituents. The initial screen of compound which identified the 2,6-diaminopyrimidine scaffold, demonstrated that 5-substituents capable of forming a hydrogen bond gave activity. An additional methylene group between the pyrimidine ring and the hydrogen bond forming substituent essentially abolished activity. Therefore, despite the extensive synthetic efforts no compound was synthesised with improved activity over the initial compound within the screen.

Since work began at the NICR on the development of ATP-competitive inhibitors of mTOR a number of highly potent and selective inhibitors of mTOR have been published, and a number of these have progressed into clinical trials. Many of these inhibitors are, like the NICR series, based on nitrogen containing heterocycles. The compound space has been covered extensively in this area, suggesting that continued synthetic efforts may not result in the synthesis of a novel compound.
5. Isoindolinone-Based Inhibitors of the MDM2-p53 Protein-protein Interaction

5.1 Development of Isoindolinone Based Inhibitors of the MDM2 p53 Protein-protein Interaction

The Northern Institute for Cancer Research programme to discover inhibitors of the MDM2-p53 protein-protein interaction was initiated by the identification of a number of isoindolinone-based compounds, by a preliminary screen of compounds using an in vitro binding assay. Compounds 189, 190 and 191 were identified as weak inhibitors with IC₅₀s of around 200 µM, were shown to have growth inhibitory activity in NCI 60 cell line screen and were classed as COMPARE negative against known classes of antitumour agents.

![Figure 52 Structure of the initial isoindolinone hits](image)

A programme of library synthesis was undertaken, guided by a virtual screening to suggest desired compounds. Compounds 189 and 191 were docked into MDM2 to identify a single, low energy binding mode using the program easyDOCK. Both stereoisomers of the isoindolinones were docked into the 1YCR crystal structure of MDM2. However, due to the lipophilicity of both the compounds and the MDM2 binding site, along with the open shape of the compounds a large number of potential binding modes were identified.

A number of further compounds were synthesised, some suggested from ‘virtual screening’ by the selection of a single binding mode from those identified, others suggested by the availability of starting material during the synthesis of the isoindolinones. However, those suggested by the ‘virtual screening’ were found to be no more potent than those synthesised without docking. These results suggested that there may be multiple experimental binding modes for isoindolinones and MDM2, and that the use of multiple binding modes within computational chemistry may increase possibility of accurately predicting the experimental binding mode. This approach was used to identify a more diverse group of seed compounds which were used by to aid the identification of the experimental binding mode.
Six seed compounds were selected and synthesised with activities ranging from 27 to 92 µM. These compounds were again docked into the MDM2 crystal structure using both the easyDOCK and GOLD program which again generated a large number of solutions. 24 high scoring, unique binding modes were chosen as a starting point for further work. These 24 modes were generated from the six seed compounds, one per stereoisomer of each compound per docking program. In each mode the position of the isoindolinone was preserved and unique compounds generated by varying R\textsuperscript{1} or R\textsuperscript{2} using commercial reagents.

![Figure 53 Structure of the isoindolinone core](image)

The interaction between the ligand and MDM2 protein was further explored using the program Skelgen. This explores the interaction of a ligand with that of the active site of a protein, defined as a rectangular box with selected fragments included. Ligands were selected which formed at least one interaction with the target residues within the MDM2 protein. The target residues included Leu54 O, Phe55 O, Gln59 NE2, Gln72 O and OE1, Val93 O, His96 ND1 and Tyr100 OH. The parameters suggested 57 ‘virtual hits’ and of the 57, 43 were synthesised. These 43 compounds covered 14 of the 24 potential modes, with substituents selected which appeared in only one binding mode chosen to aid identification of the preferred binding mode.

Of the 43 synthesised compounds, five showed no appreciable inhibitory activity, approximately half showed inhibitory activity in the region of 100-400 µM, a further 12 showed activity in the 50-100 µM range, similar to the initial six seed compounds. Two compounds had activity that improved on the most potent of the seed compounds. From these compounds a number of binding modes were proposed, dependent on the R\textsuperscript{1} and R\textsuperscript{2} substituents, the most important being those associated with the two most active compounds.

The first binding mode has the isoindolinone lying in the Trp23 pocket, with an N-ethylacetamide group (R\textsuperscript{1}) forming an H-bond interaction to Gln72 within the Phe19 pocket.
A *tert*-butylbenzyl group ($R^2$) overlays the surface. The second binding mode has the isoindolinone overlying the surface with the Trp23 pocket occupied by the 3-phenyl group, syringic alcohol (3,5-dimethoxy-4-hydroxybenzyl alcohol) ($R^2$) in the Leu26 pocket which forms an H-bond to Trp100 and an $N$-benzyl group ($R^1$) in the Phe19 pocket.

A combinatorial approach was then undertaken using the most favourable substituents which are summarized below. This resulted in the design of 36 isoindolinones, with the number reduced as synthesis proved problematic or preliminary biological results proved disappointing. The 4-SEM group was found to be a poor substituent, suggesting that previously the group was forming a non-specific hydrophobic interaction. 4-Chlorophenyl was the preferred aromatic substituent, with the presence of this substituent suggesting that the compounds are making a favourable interaction with the hydrophobic Trp23 pocket. This correlates with the second mode described. The most active compounds identified are shown below.

![Figure 54](image1)

**Figure 54** The isoindolinone scaffold with favoured substituents in each position

![Figure 55](image2)

**Figure 55**
Selected isoindolinones had also been shown to behave in a dose-dependent manner, with p53 related gene transcription increasing with increasing dose within a MDM2 amplified SJSA human sarcoma cell line.\textsuperscript{191} However, combinatorial chemistry and molecular docking were not providing sufficient increases in potency. Additional information about how the isoindolinone binds to MDM2 would validate the structure-activity relationships and suggest areas for further modification. Due to the moderate potency of these protein-protein inhibitors it can be difficult to gain further structural information. Computational methods are limited when interaction is due to hydrophobic interactions rather than H-bonds. Crystallography can also potentially prove difficult due to the weak infinities, poor solubility of the inhibitors and the flexibility of the protein. NMR studies would also prove difficult as the protein must be saturated and a micromolar affinity would result in line broadening.\textsuperscript{191}

A different approach uses 2D HSQC-NMR chemical shifts to determine the location of a ligand binding by observing changes in the chemical shift. The binding mode is determined by comparing the experimentally observed chemical shifts with those predicted for each of the suggested binding modes.\textsuperscript{191}

The 12 isoindolinones that were subjected to NMR analysis were divided into groups that differed in only one area of their chemical structure, either the isoindolinone $N$-substitution or substitution in the 3 position. The 3-position aromatic was fixed as a $p$-chlorophenyl moiety. The active site of the MDM2, in which the isoindolinone binds, can be summarised as 4 main sub-pockets.\textsuperscript{191}

- The pocket in which Trp23 of p53 binds is a shallow pocket, with the Trp23 forming a H-bond with Leu54 on MDM2
- The Leu26 pocket formed by the residues Ile99 and Ile103 of MDM2
- The Phe19 pocket around residue Ile61
- A deep pocket in which the Trp23 side chain lies
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

![Figure 56](image)

**Figure 56** Surface representation of MDM2 (1YCR) with the 4 sub-pocket highlighted.

1) Trp23 sub-pocket 2) Leu26 sub-pocket 3) Phe19 sub-pocket 4) Trp23 side chain sub-pocket (Taken from reference 190)

For the NMR studies 38 residues surrounding the isoindolinone binding site were monitored. The 38 residues were the most likely to be affected by the binding:

- helix α2 (residues 50 to 63 and 67 to 75)
- helix α1' (residues 82 to 86)
- helix α2' (residues 91-106)

Cluster analysis was then performed on each residue per group of compound, with closely clustered peaks suggesting similar binding. As each group only differs chemically in one area, the area of unclustered peaks will be the area of different binding. This was used to show which amino acid residue lies near each area of the isoindolinone.\(^{191}\)

As a result of these NMR studies the isoindolinones are thought to bind with either the isoindolinone or the 4-chlorophenyl group pointing into the deep pocket of MDM2, the Ile99 pocket is thought to prefer aromatic groups, while both Leu54 and Ile 61 pockets are not selective for the type of group.\(^{191}\) NMR studies with compound 184 identified a significant shift in the signal for the Leu54 region, which was thought to be due to an interaction between Leu54 and the hydropropoxy chain. It was proposed that by introducing rigidity to the propyl chain activity could be improved. Replacement of the propyl chain with a cyclopropyl derivative increased activity to 3.0 µM.\(^{192}\)
5. Isoindolinone-Based Inhibitors of the MDM2-p53 Protein-protein Interaction

**Figure 57** Structure of (R)-NU8165 (194) bound to MDM2 deduced from HSQC NMR studies (left) and modelling of a cyclopropyl derivative (Taken from reference 193)

Further SAR studies with the 3-hydroxyisoindolinone identified small lipophilic groups at the 4-position of the N-benzyl group as improving potency. Inclusion of a chlorine atom at this position resulted in a compound 17 times more potent than an initial parent compound. However, it was then found that inclusion of a nitro group at this position increased potency further, with activity 30 times greater than that of the parent. 192

Combining the optimal nitrobenzyl substituent with a racemic cyclopentyl ether side-chain was discovered to have an additive effect with an IC$_{50}$ of 0.70 µM, and on synthesis of the cis-derivative gave an IC$_{50}$ of 0.30 µM. A range of alkoxy derivatives were then incorporated to probe the SAR around this region, that identified the 1,1-bis(hydroxymethyl)cyclopropyl derivative as the optimal substituent at this position. 192 Interestingly, in NMR studies with cyclopropyl derivative (195) the 2-cyclopropylpropanol chain is seen to interact with an area, which is not exploited by either the Nutlin series or the benzodiazepinedione series. 192
NMR studies were undertaken comparing isoindolinone, Nutlin-3 and p53 peptide binding to MDM2. The ‘footprint’ of isoindolinone, Nutlin-3 and p53 binding was then characterised. A 2D NMR $^1$H $^{15}$N-heteronuclear single-quantum coherence (HSQC) titration was undertaken with a range of concentration of each inhibitor. The binding of ligands resulted in a shift in signal in the primary sequence of MDM2, apart from the C-terminal 10 residues. The average chemical shift is 0.045 to 0.083 ppm. Nutlin-3 has an average shift of 0.076 ppm, p53 peptide has an average shift of 0.22 ppm and compound 195, the most potent isoindolinone compounds analyzed had an average shift of 0.075 ppm. The greatest observed shift was seen in the hydrophobic pocket, which is in direct contact with the ligands. The greatest shift was observed between the helix 2α (residues 51-63), the area surrounding β-strands β3 and β1’ (residues 66-77) and the region connecting β2’ to α2’ (residues 89-104). The N-terminal also has larger shift, due to the change in conformation from the closed to open position. Nutlin-3 was seen to induce a larger chemical shift on the beta sheet β3, while isoindolinone 195 has a greater effect on resides at the opposite side of the pocket helix α2’ and the back of Trp23 pocket. This underlines that whilst all MDM2/p53 inhibitors are acting as p53 α-helix mimetics; there are differences between binding modes of the inhibitors.

As the nitro group is undesirable within drug molecules, due to the possibility of forming toxic metabolites, a number of classical and non-classical isosteres for the nitro group have previously been investigated. A table of the previously synthesised compounds and their activity is shown below. Compounds all have the structure type shown below
Table 11 Classical and non-classical isosteres attempted for replacement after the nitro benzyl group

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<th>Compound number</th>
<th>Structure of R group</th>
<th>IC$_{50}$ of activity against MDM2 (µM)</th>
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<td>0.23 ± 0.11</td>
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<tr>
<td>196</td>
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<td>8.8 ± 4.2</td>
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<td><img src="image" alt="Structure" /></td>
<td>11 ± 0.8</td>
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5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>IC50 ± SEM (nM)</th>
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<td>208</td>
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<td>2.3 ± 0.059</td>
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<td>209</td>
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<td>1.8 ± 1.3</td>
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<td>1.7 ± 0.73</td>
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</tr>
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<td>1.0 ± 0.39</td>
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<td>214</td>
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<td>218</td>
<td><img src="image" alt="Structure" /></td>
<td>15 ± 1.4</td>
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<tr>
<td>219</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

*a* indicates n = 1

Previous SAR studies around the ‘A’-ring showed that substitution around the isoindolinone further improved potency. A chloro-substituent in the 4-position was found to be optimal (220), increasing potency to 143 nM. A tert-butyl group in the 6-position was also shown to
be favourable, with an IC$_{50}$ value of 152 nM. Some of the previously synthesised compounds are shown below.

**Table 12** Substitution patterns around the ‘A’ ring and the resulting activity against MDM2

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>‘A’-ring and substituent</th>
<th>IC$_{50}$ of activity against MDM2 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>H</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td>220</td>
<td>4-Cl</td>
<td>0.095 ± 0.096</td>
</tr>
<tr>
<td>221</td>
<td>4-Me</td>
<td>0.30 ± 0.17</td>
</tr>
<tr>
<td>222</td>
<td>5-t-Bu</td>
<td>0.89 ± 0.29</td>
</tr>
<tr>
<td>223</td>
<td>5-Br</td>
<td>0.90 ± 0.071</td>
</tr>
<tr>
<td>224</td>
<td>6-t-Bu</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td>225</td>
<td>6-F</td>
<td>0.85 ± 0.090</td>
</tr>
<tr>
<td>226</td>
<td>6-Br</td>
<td>1.0 ± 0.068</td>
</tr>
<tr>
<td>227</td>
<td>5,6 Cl</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>228</td>
<td>4-Cl,5-F</td>
<td>0.31 ± 0.15</td>
</tr>
</tbody>
</table>

Interestingly, the addition of the chlorine in the 4-position reduces the difference between the different benzyl substitutions. When the ‘A’-ring is unsubstituted the difference between the benzyl NO$_2$ analogue and the bromo benzyl analogue is 5-fold. However, when the 4-chloro is placed on the ‘A’-ring the difference is less than 2 fold. These results are summarised below.
Table 13 Comparison activity of substituted isoindolinone with varying benzyl substitution

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>‘A’-ring substitution</th>
<th>Benzyl substitution</th>
<th>IC$_{50}$ of activity against MDM2 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>H</td>
<td>NO$_2$</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td>212</td>
<td>H</td>
<td>Br</td>
<td>1.2 ± 1.2</td>
</tr>
<tr>
<td>209</td>
<td>H</td>
<td>CN</td>
<td>1.8 ± 0.67</td>
</tr>
<tr>
<td>220</td>
<td>4-Cl</td>
<td>NO$_2$</td>
<td>0.095 ± 0.096</td>
</tr>
<tr>
<td>229</td>
<td>4-Cl</td>
<td>Br</td>
<td>0.17 ± 0.003</td>
</tr>
<tr>
<td>230</td>
<td>4-Cl</td>
<td>CN</td>
<td>0.18$^a$</td>
</tr>
</tbody>
</table>

$^a$ indicated n = 1

The values for all of the above compounds are for the racemic mixtures. When the most active compound 220 was separated into its two enantiomers, one was found to have an activity of 44 nM, with the alternative enantiomer shown to be significantly less potent. The inactive enantiomer was successfully crystallised, and the structure of the active enantiomer is that shown below. Treatment of SJSA-1 cell line with the active enantiomer of 220 produced a concentration dependent inducement of MDM2, p53 and p21, treatment with the inactive enantiomer and resulted in a response at the highest dose of 20 µM. The dose-dependent inducement of p21, p53 and MDM2 verifies that isoindolinones are acting via inhibition of the p53 MDM2 protein-protein interaction.$^{192}$

![Active isomer of 220](image)

Figure 58 Active isomer of 220

A final step forward in potency was discovered when attempting to add water-solubilising groups. A succinic ester group was added to the isoindolinone core via the diol side chain. Initially, the succinic ester group was added as a cleavable pro-drug, but the compound (231) displayed an increase in potency, with an activity of 11 nM.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Figure 59 The structure of 231

The two compounds (220, 231) were the leads for the development of inhibitors of the MDM2-p53 protein-protein interaction described herein.

5.2. Isoindolinone Formation

The isoindolinone series of compounds were synthesised using methodology previously developed within the laboratory (Scheme 41). The first step of the synthetic route is a Friedel-Crafts reaction between the appropriate phthalic anhydride and chlorobenzene to give the benzoyl benzoic acid (233). The Friedel-Crafts reaction can be omitted when \( X = H \) as the required 2-(4-chlorobenzoyl)benzoic acid is commercially available. For compounds which require substitution around the ‘A’ ring of the isoindolinone, the initial step is synthesis of the benzoyl benzoic acid. The Friedel-Crafts reaction between the appropriate phthalic anhydride and chlorobenzene is mediated by aluminium chloride, generating the reactive acylium ion (236) via the mechanism below (Scheme 42). Stoichiometric amounts of AlCl₃ must be used as it remains complexed to the oxygen and must be removed by hydrolysis at the end of the reaction.

Scheme 41 Reagents and conditions; a) PhCl, AlCl₃, 90 °C, 2h; b) i. SOCl₂, DMF, THF, 4 h; ii. Appropriate amine, Hünig’s base, THF, overnight; c) i. SOCl₂, DMF, THF, 4 h; ii. Appropriate alcohol, K₂CO₃, THF, overnight
In the case of substituted phthalic anhydrides, two possible isomers of the acylium ion may be formed, resulting in a mixture of isomers. For 3-chlorophthalic anhydride the major product formed is 3-chloro-2-(4-chlorobenzoyl)benzoic acid. The predominant formation of one isomer suggests that the chlorine atom plays a significant role in the reaction. One proposal is that the chlorine atom stabilises the acylium ion via conjugation (Scheme 42).

Newman et al observed the same effect when performing a Freidel-Crafts reaction with 3-chlorophthalic anhydride and benzene, with only the carbonyl alpha to the chloro atom reacting. Allahdad et al suggests that the aluminium chloride preferentially reacts with the most electron rich carbonyl group. For 3-chlorophthalic anhydride the most electron-rich carbonyl group lies meta to the chloro group. If AlCl₃ reacts with the meta carbonyl group, the acylium ion will be formed on the carbonyl group alpha to the chlorine atom. Both of these two different theories support formation of the acylium ion alpha to the chloro, which gives rise to the 3-chloro-2-(4-chlorobenzoyl)benzoic acid as the major product.

**Scheme 42** Proposed mechanism of formation of the acylium ion

**Scheme 43** Potential stabilisation of acylium ion

**Scheme 44** Reagents and conditions; a) AlCl₃, 2 h, 90 °C, 91%
The first work-up to the Friedel-Crafts reaction of 3-chlorophthalic anhydride and chlorobenzene an acidic solution resulted in the isolation of both possible isomers as a precipitate. However, on subsequent attempts the precipitate did not always occur and a further extraction into organic solvent was required, but re-extraction into organic solvent also resulted in the extraction of further impurities which were inseparable from the desired product. A simple alteration to the work-up avoided this problem. The benzoyl-benzoic acid was isolated as the sodium salt that precipitated reliably in good yield and purity (91%). At this point the two isomers cannot be separated; the second step of the reaction is performed on the mixture of isomers. After formation of the isoindolinones the two isomers can then be separated.

\[ \text{Scheme 45} \] The equilibrium between the open and cyclised form of ortho-benzoylbenzoic acids

The second step of the synthetic route requires formation of the isoindolinone heterocycle via the formation of a ψ-acid chloride,\(^\text{199}\) as the reactive intermediate. Ortho-benzoylbenzoic acids are found in an open state (229) as a solid but in solution are in equilibrium between the open form and a cyclic form (230) (Scheme 45).\(^\text{200}\)

\[ \text{Scheme 46} \] Reagents and conditions; a) SOCl\(_2\), DMF, THF, 4 h

Treatment of the cyclised form the benzoylbenzoic acids with the Vilsmeier reagent, synthesised from two equivalents of thionyl chloride and catalytic \(N,N\)-dimethylformamide, results in the synthesis of ψ-acid chloride (241)(Scheme 46). The 3-hydroxy group can attack the Vilsmeier reagent, which results in the formation of the chloride ion, which can then
attack the acetal, liberating DMF and forming the pseudo-acid chloride. The ψ-acid chloride (231) is an unstable intermediate and must be used immediately. Treatment with the appropriate benzylamine (either as the hydrochloride salt or the free amine) in the presence of Hünig’s base generates the isoindolinone.

Scheme 47 Mechanism of formation of ψ-acid chloride

The amine has a choice of two sites to attack, either the carbonyl carbon or the carbon substituted with the chlorine atom. Bhatt et al. describes these two routes as pathway A or pathway B. Pathway A is a $S_N^2$ reaction at the chloro-substituted atom, replacing the chloro atom with the nucleophile, whilst pathway B has the nucleophile attacking at the carbonyl, and ring opening the lactone.

Scheme 48
Route A is preferred by weaker nucleophiles whereas route B is preferred by stronger nucleophiles including amines.\textsuperscript{201} Sloan \textit{et al} also found that the reaction of primary amines resulted in the formation of amides, rather than displacement of the halide.\textsuperscript{202} Sloan \textit{et al} suggest that all nucleophiles attack initially at the chloro-substituted carbon but stronger nucleophiles form a looser transition state whilst weaker nucleophiles form tighter transition states.\textsuperscript{202} The looser transition state allows for delocalisation of the partial positive charge towards the carbonyl carbon, resulting in formation of the amide. The formation of this transition state may also be applied to the formation of the isoindolinone. A proposed mechanism is shown in Scheme 49. Formation of the isoindolinone is only possible via route B. Secondary attack on the ketone, formed by opening the lactone will result in the formation of the five-membered isoindolinone ring. If this step is attempted using the mixture of isomers from the previous step, two isomeric products will be synthesised. However, at this point the two isomers are separable by column chromatography.

\textbf{Figure 60} Proposed looser transition state formed during the nucleophilic attack, allowing delocalisation of the transition state

\textbf{Scheme 49} Mechanism of formation of the 3-hydroxyisoindolinone from the ψ-acid chloride of the benzoyl-benzoic acid

Treatment of the benzoyl benzoic acid (\textbf{239}) with thionyl chloride and DMF for 4 h results in the formation of the pseudo acid chloride. This must be immediately treated with a nucleophile, for formation of an \textit{N}-benzyl substituted isoindolinone a substituted benzyllamine is used, in the presence of Hünig’s base. The reaction is then stirred overnight.
Substitution of the ‘A’-ring can play a significant role in the yield of this reaction. Synthesis of the 4-chloro isoindolinone can be low yielding.

Formation of the isoindolinone gives rise to a characteristic $^1$H NMR spectrum. The diastereotopic benzyl methylene protons are expected to exhibit two pairs of doublets, at approximately $\delta$ 4-5. Each methylene protons only couples to the other methylene proton with geminal coupling constants of around 15 Hz.  

![Benzyllic protons](image)

Figure 61

The standard final step of the synthetic route is formation of the ether linkage. The optimal substituent at this position has been shown previously to be the (1-hydroxymethylcyclopropyl)methoxy ether group. The ether linkage is formed by using two equivalents of thionyl chloride with catalytic $N,N$-dimethylformamide in anhydrous THF. These reagents generate the Vilsmeier reagent in situ, resulting in chlorination at the 3-position of the isoindolinone (243) (Scheme 50). The resulting halide species must be used immediately to generate the ether, by treatment with 1,1 bis-(hydroxymethane)cyclopropane with potassium carbonate. The lone pairs of the oxygen of the 3-hydroxyisoindoline attacks the Vilsmeier reagent which rearranges to reform the iminium ion and kick out the chloride ion, which then attacks the hemi-aminal carbon to displace DMF. The ether can then be formed either via an $S_N1$ reaction or an $S_N2$ reaction. In the $S_N1$ reaction the chloride ion is displaced forming an iminium species, which is then attacked by the lone-pairs of the hydroxyl group of 1,1-bis(hydroxymethyl)cyclopropane. In the $S_N2$ reaction, the chloride ion...
is displaced by the hydroxy groups of 1,1-bis(hydroxymethyl)cyclopropane. The formation of the ether is thought to occur \textit{via} the $S_N2$ pathway.

\textbf{Scheme 50} Mechanism of formation of the ether linkage

An alternative method for the formation of the ether linkage uses BF$_3$.OEt$_2$ and 10 equivalents of the required diol. Lejkowski \textit{et al} \cite{203} use this method for the formation of an allyl ether group alpha to the oxygen in a tetrahydropyran ring. This methodology proved useful for the synthesis of compounds without the chlorine in the 4-position, with reactions taking approximately 2.5 hours, in good yields. The formation of 245 was achieved in 2.5 h in 62\% yield using the BF$_3$.OEt$_2$ chemistry. However, for the 4-chloroisoindolinone (246), the reaction was much slower, taking over 24 h, and the longer exposure to the harsher reagent resulted in a number of by-products were also generated. For comparison of reaction times between the two different reagents see Table 14.
Table 14 Reagents and conditions for formation of 248

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Time (h)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCl₂/DMF + diol (2 eq)</td>
<td>16</td>
<td>24%</td>
</tr>
<tr>
<td>BF₃.ΟΕ₄ (2.5 eq + 2.5 eq) + diol (10 eq)</td>
<td>42</td>
<td>86%</td>
</tr>
</tbody>
</table>

The mechanism of formation of the ether differs when using the BF₃.ΟΕ₄ from the chlorination method. The Lewis acid is thought to generate a cation at the 3-position, which is then quenched with the diol. Carey et al.²⁰⁴ used BF₃ to generate a trityl cation, and the cation on the 3-position of the isoindolinone could be considered to be equivalent to the trityl cation.

A distinctive pattern is again observed for the ¹H NMR resonances from the ether side-chain. Each of the CH₂ moieties within the side chain give a pair of doublets, with one proton coupling to the other within each CH₂ unit, again displaying geminal coupling. The OCH₂ resonances typically lie between δ 2-3 whilst the CH₂OH unit typically shows peaks between δ 3-4, and is occasionally observed to couple to the hydroxyl proton.
Formation of the succinic ester group

To improve the solubility of the isoindolinones a succinic half ester group was added to the ether side chain. The succinate group was initially added to the isoindolinone as a prodrug water-solubilising group. However, the addition of the succinate group was found to improve potency from an IC$_{50}$ of 143 nM (220) to 11 nM (231). Therefore, for selected compounds the succinate esters have been prepared for comparison.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Table 15 IC_{50} values for the succinic ester derivatives

<table>
<thead>
<tr>
<th>Number</th>
<th>X</th>
<th>R</th>
<th>IC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>NO₂</td>
<td>H</td>
<td>0.95</td>
</tr>
<tr>
<td>230</td>
<td>CN</td>
<td>H</td>
<td>0.18</td>
</tr>
<tr>
<td>229</td>
<td>Br</td>
<td>H</td>
<td>0.17</td>
</tr>
<tr>
<td>231</td>
<td>NO₂</td>
<td>C(O)CH₂CH₂CO₂H</td>
<td>0.020</td>
</tr>
<tr>
<td>249</td>
<td>CN</td>
<td>C(O)CH₂CH₂CO₂H</td>
<td>0.097</td>
</tr>
<tr>
<td>250</td>
<td>Br</td>
<td>C(O)CH₂CH₂CO₂H</td>
<td>0.118</td>
</tr>
</tbody>
</table>

The succinate group is added using succinic anhydride, with catalytic 4-dimethylaminopyridine (DMAP) and pyridine in refluxing THF. The DMAP activates the succinic anhydride to attack from the isoindolinone hydroxyl group. The addition of the succinate group proceeds cleanly in around 15 h in refluxing THF.

Scheme 52 Mechanism of formation of the succinic ester

5.3 Modifications to the N-benzyl Group

The most potent compound identified within the isoindolinone series contains the 4-nitrobenzyl group (210) with an IC_{50} of 143 nM. However, a nitro functionality is undesirable in a drug as it can be metabolised to a number of toxic metabolites. The metabolism can proceed via a nitro radical anion, the nitroso, the nitroxyl radical, the hydroxylamine and then
the primary amine. Hydroxylamines may cause methaemoglobinaemia and mutagenicity. Carcinogenic effects of nitro groups may be a result of nitro radicals, nitroso derivatives and hydroxylamines.\textsuperscript{205} A number of replacements for the nitro group have been synthesised previously. However, none of these have displayed equivalent potency to the nitro compound. Therefore the aim of this work was to find a nitro group replacement which is appropriate for use in a drug.

**Table 16** Key alternatives for the nitro group

<table>
<thead>
<tr>
<th>Number</th>
<th>X</th>
<th>Y</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>Cl</td>
<td>NO$_2$</td>
<td>0.095 ± 0.096</td>
</tr>
<tr>
<td>230</td>
<td>Cl</td>
<td>CN</td>
<td>0.18\textsuperscript{a}</td>
</tr>
<tr>
<td>229</td>
<td>Cl</td>
<td>Br</td>
<td>0.17\textsuperscript{a}</td>
</tr>
<tr>
<td>251</td>
<td>H</td>
<td>CCH</td>
<td>0.50\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} n = 1

**5.3.1 Ethynyl Replacement of the Nitro Group**

An isoindolinone with an ethynyl group in the 4-benzyl position has previously been synthesised within the group. The ethynyl compound (250), with no substituent at the 4-position, showed a 2-fold loss in potency compared with 220.
As addition of the chloro group at the 4-position of the isoindolinone has been shown to improve potency, synthesis of the chloro analogue of the ethynyl isoindolinone was desired. The synthetic route of the ethynyl compound is shown below (Scheme 53).

Scheme 53 Reagents and conditions: a) AlCl₃, chlorobenzene, 90 °C, 2h, 91%; b) i. SOCl₂, DMF, THF, 4 h; ii. 4-iodobenzylamine hydrochloride, Hünig’s base, THF, overnight, 36%; c) Pd(PPh₃)₂Cl₂, CuI, Et₃N, TIPS-acetylene, THF, 15 h, 88%; d) i. SOCl₂, DMF, THF, 4h ii. 1,1-bis-(hydroxymethyl)-cyclopropane, K₂CO₃, THF, overnight, 24%; e) 1M TBAF in THF, THF, 75 min, 41%

The 4-iodobenzyl substituted isoindolinone (252) was synthesised following the published synthetic route from 3-chlorophthalic anhydride and 4-iodobenzyl amine hydrochloride, with the ethynyl functionality incorporated using a Sonogashira coupling reaction to give 247 in a 88% yield.

The Sonogashira reaction cross-couples an aryl iodo species and triisopropylsilyl protected acetylene using a palladium catalyst and a copper (I) species. The reaction was achieved using 0.03 equivalents of Pd(PPh₃)₂Cl₂ and 0.02 equivalents of copper iodide. To improve conversion to the ethynyl product, the number of equivalents of TIPS-acetylene was increased from 1.1 to 1.3. Although this made little difference to the overall yield (95% conversion to 100% by LCMS) this resulted in significantly simpler purification as both the starting material and the product were close running during purification with medium
pressure column chromatography on silica. When using 1.3 equivalent of TIPS-acetylene the reaction proceeded cleanly and was readily purified in good yield (88%).

The catalytic cycle of a Sonogashira reaction is shown below. The first step of the catalytic cycle is formation of the Pd (0) species from Pd (II). The Pd (0) species then undergoes oxidative insertion with the aryl-iodo species, returning to Pd (II). The TIPS-acetylene initially forms a copper species via deprotonation with triethylamine. Chelation of the copper to the alkyne activates the triisopropylacetylene to deprotonation, without the presence of the copper the alkyne would not be deprotonated by Et$_3$N ($pK_a$ of 10.7)$^{196}$ as acetylene has a $pK_a$ of $\sim$25$^{196}$ and usually requires a strong base to deprotonate. The acetylene-copper species then undergoes transmetallation resulting in formation of a palladium acetylene species, with the acetylene group and the aryl group are trans to each other, which isomerizes to give the cis species and then undergoes reductive elimination, giving rise to the aryl acetylene species and regenerating the Pd (0) species.$^{206}$

\[ \text{Pd (II)} \rightarrow \text{Pd (0)} \rightarrow \text{Cu} \rightarrow \text{Pd (0)} \]

\[ \text{Ar} \equiv \text{TIPS} \rightarrow \text{Pd (0)} \rightarrow \text{Ar} \equiv \text{TIPS} \]

\[ \text{TIPS} \equiv \text{Pd} \equiv \text{Ar} \rightarrow \text{Cu} \equiv \text{TIPS} \]

\[ \text{L} \rightarrow \text{L} \]

**Figure 63** The catalytic cycle for a Sonogashira reaction

Once the ethynyl group had been incorporated, the TIPS protecting group was left in place until the final step of the synthesis. The ether linkage was formed using the standard reaction conditions to give 248. Deprotection was achieved using a 1M solution of TBAF in THF at room temperature to give the desired product. The first batch of 253 was synthesised using the 3-chloro-2-(4-chlorobenzoyl)-benzoic acid which contained an inseparable impurity, which persisted through the synthetic route, purification by HPLC was required to obtain the 95% purity required for testing. However, on resynthesis starting from the sodium salt of 3-
chboro-2-(4-chlorobenzoyl)-benzoic acid no addition HPLC purification was required and the final compound 253 was achieved in a higher yield (88%).

All isoindolinones are synthesised as racemic mixtures. Due to the favourable biological results compound 242 was resynthesised for separation of the enantiomers by chiral HPLC using a Daicel Chiralpak AD-H column, using 92.5:7.5 hexane:ethanol. Chiral separation gave two enantiomers, peak 1 (NCL-00018225, 254) with $[\alpha] = -9.16^\circ$ and peak 2 (NCL-00018226, 255) with $[\alpha] = +11.4^\circ$. NCL-00018225 is expected to be the R-enantiomer by analogy with NU8354 and NCL-00008406, and has the structure shown below.

![Structure of compounds 254 and 255](image)

**Figure 64**

As the ethynyl compound has already been shown to be potent, the succinate ester was also synthesised via a DMAP catalysed esterification. The TIPS protecting group was left in place to protect the ethynyl group until the final step of the synthesis route before removing via 1M TBAF (Scheme 54).

![Scheme 54](image)

**Scheme 54** Reagents and conditions; a) Succinic anhydride, pyridine, DMAP, THF, reflux, overnight, 94%; b) 1M TBAF in THF, THF, 2 h 45 min, 81%
Succinic ester 257 is acid sensitive. An aliquot of the compound, left in deuterated chloroform, which is relatively acidic, resulted in decomposition, with loss of the succinate group. Acidic chloroform was catalysing hydrolysis of the ester linkage. Succinic ester groups are easily cleaved which might explain the compounds relative sensitivity. The compound was therefore resynthesised before testing.

5.3.2 Trifluoromethylsulfone Replacement of the Nitro Group

An alternative non-classical isostere for the nitro group is a trifluoromethylsulfonyl group. The trifluoromethylsulfonyl group has been used by Abbott pharmaceuticals as a replacement for the nitro group within their series of inhibitors of the Bcl-2 anti-apoptotic family protein-protein interaction.\textsuperscript{207-208} The trifluoromethylsulfonyl group (259), which is slightly more electron withdrawing than the nitro group, was tolerated as a replacement for the nitro (258), whilst the methyl sulfonyl group (261) and the trifluoromethyl group (260) were not.\textsuperscript{207-208} A trifluoromethyl analogue of the isoindolinone has previously been synthesised and was not tolerated, a trifluoromethylsulfonyl may prove to be an ideal replacement for the nitro group.

![Figure 65](image.png)

**Figure 65** Replacement of the nitro group with trifluoromethylsulfonyl group in the Abbott pharmaceuticals compounds

The trifluoromethylsulfonyl analogue of the isoindolinone was synthesised using the published route.\textsuperscript{192} The required benzylamine was not commercially available, so it was
prepared from 4-(trifluoromethylsulfonyl)benzonitrile by reduction with a 1M solution of BH$_3$.THF complex in THF as described Leach et al.\textsuperscript{209} The compound was stored and used as a hydrochloride salt, as on standing as the free amine, the benzylamine converted from a solid to an oil.

Scheme 55 Reagents and conditions; a) AlCl$_3$, chlorobenzene, 90°C, 2 h, 91%; b) i. SOCl$_2$, DMF, THF, 4 h ii. 265, Hüning’s base, THF, X = Cl 35%, X = H 87%; c) X = Cl i. SOCl$_2$, DMF, THF 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropane, K$_2$CO$_3$, THF, overnight, 29%; X = H BF$_3$.OEt$_2$, 1,1-bis(hydroxymethyl)cyclopropane, DCM 0°C-RT 2.5 h, 69%; d) i. 1M BH$_3$.THF in THF, THF, reflux, 4 days ii. 1M HCl$_{(aq)}$, 51%

For compound 245 the ether linkage was formed using BF$_3$.OEt$_2$ in good yield (69%). As the ether formation using BF$_3$.OEt$_2$ reaction has previously been shown to proceed slowly when the 4-Cl is in place, the Vilsmeier reaction was instead used to chlorinate, followed by displacement with 1,1-bis(hydroxymethyl)cyclopropyl to form 246, which previous experience had shown to take 24 h, though with lower yields.

5.3.3. 3-Fluoro-4-Halo Benzyl Analogues

3,4-Difluorobenzyl may mimic both the space-filling capacity of the nitro group and the electron-withdrawing nature of the nitro group. A single fluoro atom is less electron withdrawing than a nitro group. The $\sigma$ value for a 4-fluoro group is 0.06 whilst for a 4-nitro group the $\sigma$ value is 0.78.\textsuperscript{210} However if the values for a 3-fluoro group (0.34) and 4-fluoro
(0.06) are combined (0.40) the value becomes closer to that of the nitro group.\textsuperscript{210} Previous work has shown that a number of 4-halo compounds have been moderately potent; therefore a series of 3-fluoro-4-halo compounds may aid identification of a compound with similar potency to the 4-nitro compound. As a result of the favourable potency of the 4-ethynyl analogue, a 3-fluoro, 4-ethynyl analogue was also synthesised. The combined Hammett values for each analogue are shown below. 3-Substitution has not previously been tolerated around the benzyl ring, a fluoro group should be sufficiently small to be tolerated.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Substituent & 3-Substituent Value & 4-Substituent Value & Total value \\
\hline
4-NO\textsubscript{2} & - & 0.78 & 0.78 \\
3-F,4-F & 0.34 & 0.06 & 0.40 \\
3-F,4-Cl & 0.34 & 0.23 & 0.57 \\
3-F,4-Br & 0.34 & 0.23 & 0.57 \\
3-F,4-I & 0.34 & 0.18 & 0.52 \\
3-F, 4-CCH & 0.34 & 0.23 & 0.57 \\
\hline
\end{tabular}
\caption{Literature $\sigma$ values for 3 and 4 substituents\textsuperscript{210} and calculated combined values}
\end{table}

The 4-chloro-3-fluoro analogue was particularly interesting in view of the activity of compound 253 as the literature has shown these groups can be interchangeable. The two EGFR kinase inhibitors Gefetinib and Erlotinib share significant structural similarities.\textsuperscript{7}
The 3,4-difluoro analogue was synthesised from 3,4-difluorobenzylamine following the previously described isoindolinone synthetic route as shown in Scheme 5.6.

Scheme 5.6 Reagents and Conditions; a) AlCl₃, PhCl, 90 °C, 2 h, 91%; b) i. SOCl₂, DMF, THF, 4 h; ii. 3,4-Difluorobenzylamine, Hünig’s base, THF, overnight, 39%; c) i. SOCl₂, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropane, K₂CO₃, THF, overnight, 23%

For the remaining compounds in the series the required benzylamine was not commercially available. Both the 4-bromo-3-fluorobenzonitrile and 3-fluoro-4-iodobenzonitrile were commercially available and could be reduced following the previously developed methodology. Unfortunately, the 4-chloro-3-fluorobenzonitrile was considerably more expensive than for the bromo and iodo analogues. Therefore, it was decided instead to access the benzylamine via the 4-chloro-3-fluorobenzyl bromide.

A search of the literature suggested that benzyl bromides could be converted to benzyl amines using methanolic ammonia at high temperatures.211 A table of the conditions used is shown below.

Scheme 5.7 Reagents and conditions; a) 7 M methanoic ammonia, methanol, conditions and yield as described below
Table 18 Summary of conditions used in attempted synthesis of 271

<table>
<thead>
<tr>
<th>Concentration of benzyl bromide</th>
<th>No. of equivalents</th>
<th>Heating type</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.45 M</td>
<td>16</td>
<td>Conventional</td>
<td>80</td>
<td>3 h</td>
<td>-</td>
</tr>
<tr>
<td>0.23 M</td>
<td>32</td>
<td>Conventional</td>
<td>80</td>
<td>3 h</td>
<td>-</td>
</tr>
<tr>
<td>0.15 M</td>
<td>16</td>
<td>Conventional</td>
<td>80</td>
<td>5 h</td>
<td>28%</td>
</tr>
<tr>
<td>0.23 M</td>
<td>16</td>
<td>-</td>
<td>RT</td>
<td>2.5 days</td>
<td>15%</td>
</tr>
<tr>
<td>0.23 M</td>
<td>16</td>
<td>Microwave</td>
<td>120</td>
<td>5 min</td>
<td>~ 35%</td>
</tr>
</tbody>
</table>

The best yield (28%) was achieved using conventional heating at 80 °C for 5 h. LCMS monitoring of the microwave irradiated reaction suggest that 35% conversion was achieved, though the product was not isolated.

The reactions all resulted in the formation of multiple products alongside the desired benzylamine. It was hypothesised that this is due to desired product (271) reacting with the starting material synthesising further compounds, as (270) may be highly reactive, due to the electron withdrawing substituents on the benzyl ring.

![Scheme 58](image)

Scheme 58 Reagents and conditions; 7 M methanoic ammonia, methanol, RT-120 °C, 3 h - 2.5 days

An alternative route was therefore required. To reduce the reactive nature of the synthesised benzylamine, it was proposed to initially form a tertiary amine, which should reduce the reactivity of the amine group, the primary amine could then be released under mild conditions. Both a modified Gabriel with sodium diformylamide, an alternative reagent to
potassium phthalamide, which requires mild hydrolysis conditions and a Staudinger reaction have been attempted.\textsuperscript{212-215}

![Reaction Scheme]

Table 19

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Conditions</th>
<th>Observed outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Sodium diformylamide, 18-crown-6, MeCN; ii. 5% ethanoic HCl</td>
<td>Reflux, then room temperature</td>
<td>No product observed</td>
</tr>
<tr>
<td>i. NaN\textsubscript{3}, DMF; ii. PPh\textsubscript{3}, H\textsubscript{2}O</td>
<td>100 °C, then room temperature</td>
<td>Major isolated product PPh\textsubscript{3}O</td>
</tr>
</tbody>
</table>

While the displacement reactions with both sodium diformylamide and sodium azide were in progress it was observed that the TLC fraction of the starting material, which was stood at room temperature in solvent (EtOAc), began to degrade forming multiple products, suggesting that the starting material itself may not be especially stable, leading to the disappearance of starting material by TLC but without conversion to the product.

While the above work was in progress, the commercial price for 4-chloro-3-fluorobenzonitrile significantly reduced, and it was purchased and reduced to the desired product using 1 M BH\textsubscript{3}.THF in 63% yield using the optimised conditions developed in the synthesis of 277.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Scheme 59 Reagents and Conditions; a) i. SOCl₂, DMF, THF 4 h; ii. 271, Hünig’s base, THF, overnight, 37%; b) i. SOCl₂, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropane, K₂CO₃, THF, overnight, 18%

Once 271 had been synthesised in sufficient yield, isoindolinone 273 was synthesised from the sodium salt of 3-chloro-2-(4-chloro-benzoyl)-benzoic acid and 271, using the standard conditions, followed by the chlorination at the 3-hydroxy position and displacement with 1,1-bis(hydroxymethyl)cyclopropane to give isoindolinone 274. Unfortunately LCMS analysis showed that 274 contained a minor impurity which require multiple rounds of medium pressure column chromatography and reverse phase chromatography before an appropriate level of purity was obtained leading to a slightly lower than anticipated yield.

Both the 3-fluoro-4-iodo and 4-bromo-3-fluorobenzonitrile compounds were commercially available and previous work suggested that the nitrile group could be reduced using borane. However reductions of both the 3-fluoro-4-iodobenzonitrile (275) and 4-bromo-3-fluorobenzonitrile (276) with BH₃·THF, the reaction proceeded very slowly (up to 5 days) so a series of alternative reduction agents were investigated (Table 20).

Table 20 Alternative reagents and conditions for reduction of 275

<table>
<thead>
<tr>
<th>Reaction No.</th>
<th>Reagent</th>
<th>Conditions</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LiAlH₄</td>
<td>0 °C for 15 min then warmed to RT</td>
<td>Multiple spots by TLC</td>
</tr>
<tr>
<td>2</td>
<td>LiAlH₄</td>
<td>0 °C for 2 h</td>
<td>Multiple spots by TLC</td>
</tr>
<tr>
<td>3</td>
<td>Li(Et)₃BH</td>
<td>RT to 70 °C</td>
<td>No significant change from starting material</td>
</tr>
</tbody>
</table>
Nitriles can also be reduced using hydrogenation with H$_2$ and palladium on carbon. Reduction of 275 using an ‘H-Cube’, an hydrogenation flow reactor was attempted using atmospheric pressure of H$_2$ at 70 °C. However, no reaction was observed.

The BH$_3$.THF reduction was reinvestigated. A concentrated solution of the benzonitrile (500 mg in 1 mL) was used with 4 eq. of BH$_3$.THF in an attempt to minimise the reaction times. After refluxing overnight, 3-fluoro-4-iodobenzonitrile in THF was reduced to the desired benzylamine (277) in good yield (82%). These conditions were then reproduced for the 4-bromo-3-iodobenzonitrile analogue, again giving good yields (84%). All the benzylamines within the 3-fluoro-4-halogen series were prepared, stored and used as the free amine and appeared stable.

![Scheme 60 Reagents and conditions; a) 1M BH$_3$.THF, THF, reflux, overnight X = Br 84%, X = I 82%)](image)

Compound 277 was then used to synthesise 3-hydroxyisoindolinone (279) using the previously discussed synthetic route giving the product (281) which required multiple rounds of medium pressure column chromatography before reaching the desired purity for biological testing. The iodo, fluoro analogue (280) was prepared under the same conditions to give 282 in a 36% yield.

![Scheme 61 Reagents and Conditions; a) i. SOCl$_2$, DMF, THF, 4 h; ii. 277 or 278, Hünig’s base, THF, overnight; X = Br 51%, X = I 33% b) i. SOCl$_2$, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropyl, K$_2$CO$_3$, THF, overnight, X = Br 19%, X = I 36%)](image)
The 4-ethynyl benzyl analogue (253) has proved to be potent so the 4-ethynyl-3-fluoro analogue was synthesised to explore the effects of the electronic of the ring. The 3-fluoro-4-iodo compound (280) was used as a precursor in a Sonogashira reaction, as described for the 4-iodo compound (223). Fluoro groups are poor leaving groups in palladium catalysed reactions, so selectivity problems were not anticipated. Compound 280 was instead as the precursor to 284 rather than compound 282 to allow incorporation of a range of alternative ether groups.

Scheme 62 Reagents and conditions: a) Triisopropylsilylacetylene, Et$_3$N, Pd(PPh$_3$)$_2$Cl$_2$, CuI, THF, 15 h, 95%; b) i. SOCl$_2$, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropyl, K$_2$CO$_3$, THF, overnight, 26%; c. 1M TBAF in THF, THF, 1 h, 62%

Compound 283 was synthesised using the previously optimised Sonogashira conditions from triisopropylsilylacetylene and compound 280 in excellent yield (95%). Vilsmeier conditions were used to incorporate the ether linkage and the TIPS group removed using 1 M TBAF in THF to give 285.

5.3.4. Benzoxadiazoles as Isosteres for the Nitro Group

The benzoxadiazole group has been reported as a replacement for a nitrophenyl group, as it maintains a number of atoms in similar positions from the nitro group.

Benzo[c]oxadiazol-5-ylmethanamine (288) is not commercially available but 5-(bromomethyl)-2,1,3-benzoxadiazole (276) is available and the patent literature suggest that compound 288 could be formed from 286 via a Gabriel reaction. In this case hydrazine was not used to liberate the amine, instead, Ohlmeyer et al used an excess of methylamine to open the phthalamide ring and liberate the free amine.
Scheme 63 Reagents and conditions; a) potassium phthalamide, DMF, 16 h; b) 40% MeNH$_2$(aq), EtOH, 25 h, 53%

Treatment of bromide 286 with potassium phthalamide gave 287. A $^1$H NMR showed that 286 formed the phthalamide 287 on treatment with potassium phthalamide in DMF. The crude material was worked up and used immediately in the second step. However, on addition of methylamine, the intermediate 287 rapidly disappeared within 30 min, but only a low yield of 25% product was obtained. A repeat reaction with LCMS monitoring demonstrated that after 1 h phthalamide 287 had completely disappeared but the product (288) had not been formed as the major product. Phthalamide 287 is rapidly converted to ring opened amide 289, however, the attack from the second molecule of methylamine is much slower, with the reaction requiring approximately 24 h.

Scheme 64

The reaction gave a modest yield of amine 288 (53%), which was then used to synthesise compound 290 (Scheme 65).
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Scheme 65 Reagents and conditions; a) i. SOCl₂, DMF, THF, 4 h; ii. 277, Hünig’s base, THF, overnight, 15%; b) BF₃·OEt₂, DCM, 0 °C-RT, 3 days, 36%

Synthesis of 290 from sodium 3-chloro-2-(4-chlorobenzyoyl)benzoate occurred in poor yield, due to being an unoptimised reaction and the product required multiple rounds of medium pressure column chromatography. This resulted in the loss of further material before compound 290 could be used in the next synthetic step.

As only around 100 mg of compound 290 had been synthesised it was decided rather than using the SOCl₂/DMF methodology for incorporation of the ether linkage, the BF₃·OEt₂ synthetic route would be used instead. The use of the BF₃·OEt₂ methodology would lead to a longer reaction time but these reagents usually have a higher yield, necessary in this case. Treatment of 290 with BF₃·OEt₂ for 3 days gave 291 as a colourless oil, which required both medium pressure column chromatography and semi-preparative HPLC before reaching the required purity for biological testing.

5.3.5. Replacement of the 4-Nitro Group with 4-Azide

A further proposed replacement of the nitro group is an azido group. The azido group is only thought to be featured in one drug on the market (AZT (292), an antiretroviral drug). However, azido groups can be used in labelling studies. Parang et al have used azido groups to observe protein cross linking with azido labelled ATP. Irradiation of the azido group with UV light results in release of N₂ and formation of a nitrene. The reactive nitrene species then react with C-H bonds within the protein, labelling the residues at the binding site. Singlet nitrenes are thought to react via addition and rearrangement with aryl CH and triplet nitrenes insert in alkyl C-H bonds via hydrogen abstraction.
Zhu et al.\textsuperscript{221} describe the CuI catalysed synthesis of aryl azides from aromatic iodides. The formation of the azide requires L-proline (0.2 eq) and sodium hydroxide (0.2 eq) alongside sodium azide (1.2 eq) and copper iodide (0.1 eq). The sodium hydroxide and L-proline form the sodium salt of L-proline which then forms a complex with the copper, resulting in the active species via 293.\textsuperscript{221} The copper species can then couple the azide with the aromatic iodide species. Ma et al.\textsuperscript{223} suggest that the formation of the aryl azide species occur via an Ullman-type reaction. However, traditional Ullman reactions require high temperatures, but with Zhu et al copper-catalysed reaction the temperature is lower as the reaction is promoted by the use of amino acids.\textsuperscript{223} Ma et al propose the reaction occurs via oxidative insertion of the iodoaryl species to the copper/amino acid complex. The formation of this amino acid/copper complex activates the copper to oxidative insertion and is also thought to stabilise the product of the oxidative addition, which allows the coupling reaction to occur.\textsuperscript{223}
To form the azido isoindolinone the copper catalysed reaction was initially attempted with 4-iodobenzylamine hydrochloride (294). The reaction was heated in DMSO for 20 h at 60 °C. However, the progress of the reaction was difficult to observe, even using amino TLC plates. IR analysis of the crude mixture suggested that the aromatic azido product had been formed, as a strong peak at around 2000 cm\(^{-1}\) was observed. However, on purification the product was not isolated.

**Scheme 67**

The basic nature of the benzylamine prevent monitoring and purification of the benzylamine it was decided to form the isoindolinone with the 4-iodobenzyl group in place. This should reduces the basicity of the azido-incorporated product, which should make monitoring and purifying the reaction easier, but will also mean that the potentially sensitive azido group will not be carried through a number of steps through the formation of the isoindolinone and incorporation of the ether but can instead be added in the final step. However, attempts to synthesise the desired product (295) proved unsuccessful, after heating overnight in the presence of CuI and L-proline, only starting material was observed.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Scheme 69 Proposed reagents and conditions; a) i. SOCl₂, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl) cyclopropane, K₂CO₃, THF, overnight, 52%; b) L-proline, NaOH, CuI, DMSO, 60 °C

Given the unreactivity of the isoindolinone to this methodology and inherent safety issues of using azide species with copper, which would prevent scaling up of reactions it was decided to access this molecule via an alternative route. Griffin et al prepared 4-azidobenzyl alcohol from 4-aminobenzyl alcohol via a diazonium intermediate, which could then potentially be converted to 4-azidobenzylamine via the bromide.

Scheme 70 Reagents and conditions; a) 5M HCl(aq), NaNO₂, NaN₃, 0-5 °C, 1 h, 86%; b) PBr₃, Et₂O, 45 min, 0 °C-RT, 73%; c) i. NaN(CHO)₂, 18-crown-6, MeCN, 95 °C, 24 h; ii. 5% ethanolic HCl, 3 days, 96%

Using the literature method 4-azidobenzyl alcohol 299 was successfully synthesised. The benzyl alcohol was then converted to the benzyl bromide using PBr₃ following the procedure developed by Takatori et al which used 0.4 eq of PBr₃ at 0 °C-RT. However, in our hands 0.8 eq of PBr₃ was required to give full conversion to the benzyl bromide in good yield (73%). The modified Gabriel reaction, using sodium diformylamide, with the milder conditions than the traditional Gabriel reaction was used to convert the benzyl bromide to the benzylamine. Treatment of 4-azidobenzyl bromide in MeCN with 2.7 eq of sodium diformylamide with 18-crown-6 (0.1 eq) at 95 °C for 24 h resulted in complete conversion to the intermediate, and hydrolysis of the formyl groups was achieved using 5% ethanoic HCl
overnight, with an overall yield of 96%. As the azido group is light sensitive all reactions with either sodium azide or with molecules which incorporated the azido group were carried out in the dark and were stored at 4 °C in the dark. However, the azide group did appear to be robust enough to allow formation of the benzylamine, and compounds were stable for up to 1 year when stored at 4 °C in the dark.

**Scheme 71** Proposed reagents and conditions; a) i. SOCl₂, DMF, THF, 4 h; ii. 284, Hünig’s base, THF, overnight, 78%; b) i. SOCl₂, DMF, THF, 4 h; ii. 1,1-bis(hydroxymethyl)cyclopropane, K₂CO₃, THF, overnight

4-Azidobenzyl amine was then used to synthesise hydroxy isoindolinone 290. When repeating these conditions to synthesis the 4-chloro analogue (292), the ¹H NMR spectrum of the isolated product did not correlate with the structure of the isoindolinone. However, as this reaction was only attempted once, the incorrect product simply may have been isolated.

When attempting to form compound (291) using the SOCl₂/DMF synthetic route, no product was observed, instead two new compounds which were not isoindolinones as they lacked the characteristic isoindolinone pair of doublets for the benzyl protons.

We doubted whether the sensitive azide group would survive the BF₃.OEt₂ conditions for forming the ether. Therefore, the reaction with BF₃.OEt₂ was attempted on a small scale, and after stirring at room temperature overnight with 2.5 eq of BF₃.OEt₂, no reaction or significant degradation was observed. In previous examples with the 4-H isoindolinone the
BF$_3$.OEt$_2$ reaction quickly proceeds to completion, but with the more hindered 4-Cl reactions, several additions of 2.5 eq and a longer reaction time is required. As the azido group does not appear as sensitive to BF$_3$.OEt$_2$ a further 2.5 eq was added to try and push the reaction. Unfortunately, after stirring the reaction for a further 5 h no reaction was observed.

The lack of success in these reactions prompted the search for alternative methods. Compound 293 was synthesised to be used as a model compound for alternative methods in these reactions. The alternative methodologies used can be broadly divided into two classes, the first is alternative methods of chlorinating the 3-OH group of the isoindolinone, the second looks at alternative methods of activating the 3-OH to displacement by the 1,1-bis(hydroxymethyl)cyclopropane.

Scheme 72 Reagents and conditions; a). i. SOCl$_2$, DMF, THF, 4 h; ii. 4-nitrobenzylamine hydrochloride, Hünig’s base, THF, overnight, 63%

The first question to address is why the SOCl$_2$/DMF method of chlorination, which is used in the formation of the azido isoindolinone and tolerated, when used to chlorinate the 3-hydroxy isoindolinone results in some degradation but no reaction? When forming the isoindolinone the SOCl$_2$/DMF reagents are used in the first step. For the second step of the reaction, the Hünig’s base is always added before the benzylamine, so any HCl generated will be neutralised before the addition of the benzylamine. For the azido analogue this could be vital as azides are known to be sensitive to acid.$^{222}$ In the second step, however, the azido functionality is present in the starting isoindolinone, and it must survive the SOCl$_2$/DMF conditions before the base (K$_2$CO$_3$) is added. The first alternative chlorination method attempted was the use of a commercial source of the Vilsmeier reagent. Thus, isoindolinone (293) was treated with (chloromethylene)dimethyliminium chloride (Vilsmeier reagent) in THF and the standard experimental procedure followed. This gave compound 195 in a poor...
yield (22%). Reaction of the azido analogue 301, under the same conditions resulted in no reaction.

![Scheme 73](image)

Scheme 73 Reagents and conditions; a) i. (chloromethylene)dimethyliminium chloride, THF, 4 h; b) 1,1-bis(hydroxymethyl)cyclopropyl, K$_2$CO$_3$, THF, overnight, 22%

An alternative chlorination, using Ghosez reagent (1-chloro-$N,N$-2-trimethylpropenamine) (305) was also attempted. Ghosez reagent have been used for the synthesis of acid chlorides from carboxylic acids and maintains essentially neutral conditions. Bendall et al also described the use of Ghosez reagent for the chlorination of primary, secondary and tertiary alcohols. The mechanism of Ghosez reagent is shown below (Scheme 74). The first step of the mechanism is attack of the hydroxyl group on the carbon alpha to the chloro atom, the lone pair of electron on the nitrogen then forms the iminium ion, breaking the C-Cl bond. The chloride ion can then attack the alkyl group to form the alkyl halide species.

![Scheme 74](image)

Scheme 74

3-Hydroxyiosindolinone (304) was treated with Ghosez reagent at -78 °C. No reaction was observed at room temperature or at 60 °C. The failure in this case may be due to the hindered nature of the isoindolinone OH, in comparison to the literature examples.
Therefore, an alternative less bulky chlorination methodology may prove more effective. Oka et al used oxalyl chloride to chlorinate tertiary alcohols, where each alkyl group is a phenyl ring.\textsuperscript{227} 3-Hydroxy isoindolinone (304) was treated with oxalyl chloride giving 195 in 62\% yield. These same conditions were applied to the azido analogues with the addition of 1 eq. of Hünig’s base. However, on addition of the oxalyl chloride to the solution of isoindolinone (301) the reaction mixture rapidly changed colour from pale yellow to black and significant decomposition of the isoindolinone was observed by TLC.

\textbf{Scheme 75} Reagents and conditions; a) i. (COCl)\textsubscript{2}, DCM, 2 h; ii. 1,1-bis(hydroxymethyl) cyclopropane, Hünig’s base, DCM, 15 h, 62\%

Alternative methods of activating the 3-hydroxy to displacement were explored. Firstly, triflation of the 3-OH group was attempted using \(N\)-phenyltriflimide. However on heating the 3-hydroxyisoindolinone (304) with \(N\)-phenyltriflimide and Hünig’s base, no reaction was observed.

\textbf{Scheme 76} Proposed reagents and conditions; a) i. \(N\)-phenyltriflimide, Hünig’s base, THF, RT-70 °C, ii) 1,1-bis(hydroxymethyl)cyclopropane, Hünig’s base, THF, overnight

An alternative methodology for activating hydroxyl groups to displacement is to convert them to isoureas,\textsuperscript{228} commonly by reacting the hydroxyl group with \(N,N\)-dialkyl carbodiimide in the presence of Cu(OTf)\textsubscript{2} or CuCl.\textsuperscript{229} Attack of a nucleophile will generate urea, thus driving the reaction.\textsuperscript{228} Formation of an isourea followed by attack of a nucleophile
is commonly used to form esters\textsuperscript{228,230-232} however Liu suggests that it can be used to form ethers.\textsuperscript{229} The reaction proceeds \textit{via} attack of the \textit{N,N}-dialkyl carbodiimide by the hydroxy species forming the isourea, which activates the hydroxy species to attack by a nucleophile forming the urea and in this example the ester species (Scheme 77).

Scheme 77

Treatment of 3-hydroxyisoindolinone 304 with diisopropylcarbodiimide and Cu(OTf)_2 in MeCN with microwave heating failed to give a new product on quenching aliquots with methanol. The reaction order was switched, first reacting 1,1-bis(hydroxymethyl)cyclopropyl with DIC, however no new product was formed. In view of these unsuccessful results, and the time restraints it was decided to discontinue work on the synthesis of the azido analogue.

5.3.6. Biological Results for Benzyl Analogue

All final compounds from the benzyl series were submitted for biological testing. For assay details see chapter 5.9.

Table 21 Biological results for modifications to the benzyl group * indicate compounds synthesised by other chemists

<table>
<thead>
<tr>
<th>Number</th>
<th>Rotation</th>
<th>X</th>
<th>R</th>
<th>Ar</th>
<th>Biological Activity IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>195*</td>
<td>(+/-)</td>
<td>H</td>
<td>H</td>
<td>(\text{Ar} \text{NO}_2)</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Formula</td>
<td>R1</td>
<td>R2</td>
<td>Inhibition (µM)</td>
<td>Notes</td>
</tr>
<tr>
<td>-----</td>
<td>----------------</td>
<td>----</td>
<td>----</td>
<td>-----------------------</td>
<td>---------</td>
</tr>
<tr>
<td>220</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.095 ± 0.096</td>
<td></td>
</tr>
<tr>
<td>251</td>
<td></td>
<td>H</td>
<td>H</td>
<td>0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>253</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.15 ± 0.084</td>
<td></td>
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<tr>
<td>257</td>
<td>ClC(O)CH&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>Cl</td>
<td>H</td>
<td>0.053&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>254</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.041 ± 0.022</td>
<td></td>
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<tr>
<td>255</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>2.4 ± 1.5</td>
<td></td>
</tr>
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<td>245</td>
<td></td>
<td>H</td>
<td>H</td>
<td>43% inhibition at 5 µM</td>
<td></td>
</tr>
<tr>
<td>246</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>306</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.13 ± 0.016</td>
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<tr>
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<td>Cl</td>
<td>H</td>
<td>0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>258</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.41 ± 0.021</td>
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<tr>
<td>274</td>
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<td>Cl</td>
<td>H</td>
<td>0.20&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>281</td>
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<td>Cl</td>
<td>H</td>
<td>0.19 ± 0.069</td>
<td></td>
</tr>
<tr>
<td>282</td>
<td></td>
<td>Cl</td>
<td>H</td>
<td>0.53 ± 0.083</td>
<td></td>
</tr>
</tbody>
</table>
The ethynyl compound 253 has similar potency to the nitro group. It is structurally dissimilar to the nitro group, suggesting that the nitro group is not forming a direct electrostatic interaction which a replacement must replicate. Instead, it is the size and electron-withdrawing nature of the nitro group which must be replicated. The 4-ethynyl des-chloro analogue (251) was relatively potent (IC\textsubscript{50} = 0.50 µM), few analogues have potency in the nanomolar range without the ‘A’-ring 4-chloro group, but a greater increase in potency is observed when going from the (251) to (253) than from (195) to (220). This suggests that compound 253 shifts slightly in relation to compound 220 which positions the 4-chloro of the isoindolinone ‘A’-ring in a slightly more optimal position.

The crystal structure of an isoindolinone bound to MDM2 (see chapter 5.7.1) suggests that the 4-chloro forms a halogen bond to the protein. This acts to pull the isoindolinone into a slightly different position in comparison to des-chloro analogues. In this position it would appear that the ethynyl substitution is positioned in a more optimal arrangement, than in comparison to the des-chloro analogue.

The proposed shift in position is supported by the biological activity of compound 257. When converting 220 to the succinic ester analogue 231 an increase in potency of around 130 nM is observed, however, when adding the succinic ester to compound 253 an increase in potency of only around 30 nM is observed. In optimally positioning the ethynyl group within the pocket, the succinic group is shifted from its optimal position.

Separation of the enantiomers (254 and 255) demonstrates that the biological activity resides in the (-) isomer. The residual activity observed for the opposite enantiomer suggests that potency is strongly controlled by the benzyl group and substituent choice. The absolute stereochemistry of 254 is yet to be determined. It is anticipated to be the $R$-enantiomer by comparison with NU8354 and NU8604.
The trifluoromethylsulfone substituent appears to be too large to be tolerated within the MDM2 pocket. However, the increase in potency is still observed on addition of the 4-chloro group to the isoindolinone ‘A’-ring. By comparing the Cresset Fieldview modelling of the negative field (areas which like to interact with positive areas or electron-deficient of the protein) of the nitro (220) and the trifluoromethylsulfone (246) compounds, we can see that these compounds are similar. However, observing the compounds for an alternative angle we can see that this is not the case. The negative field for the benzyl group is disrupted by the presence of the trifluoromethyl group.

![Figure 66](image1.png)

**Figure 66** Cresset Fieldview modelling of the nitro (220) (left) and trifluoromethylsulfone (246) right, the blue surface represents the negative field.

![Figure 67](image2.png)

**Figure 67** Cresset Fieldview modelling of nitro (220) (left) and trifluoromethylsulfone (246) (right) compounds showing the negative field.

The series of 3-fluoro-4-halo or ethynyl compounds shows some interesting results. For both the 4-chloro and the 4-ethynyl compounds, which both have similar levels of potency to
compound 220, addition of the fluoro group reduces activity. However, for compound 281, which without the fluoro is slightly less potent than the nitro analogue, although within the range of error, there is little change in the potency. The addition of the fluoro group can adjust the electronic of the benzyl ring sufficiently but any change is within the margin of error. The 3,4-fluoro motif appear to be small to fulfil the shape filling requirement whilst the 3-fluro-4-iodo appears to be slightly too large to be the optimal substituent.

The benoxadiazole substituent (291) is tolerated, with a small decrease in potency from that of the nitro substituent. This demonstrates that this substituent can be effective as a replacement for the nitro group in both steric and electronic properties. The Fieldview modelling of the negative field of compound 220 and 291 shows a similar distribution between both compounds.

![Comparison of the negative field of 220 (left) and 291 (right)](image)

**Figure 68** Comparison of the negative field of 220 (left) and 291 (right)

### 5.4 Analogues of the Ethynyl Group

Due to the potency of 4-chloroisooindolinone (253) a series of analogues were synthesised to develop the structure-activity relationships around the ethynyl analogue. Previously synthesised analogues have shown that substitution at the 3-position is not tolerated, to confirm this is also true for the benzyl ethynyl analogues, the 3-ethynyl analogue was synthesised *via* 3-iodobenzyl precursor. The 3-ethynyl analogue can be synthesised following a similar synthetic route to 4-ethynyl analogue, which is shown below in Scheme 78. Synthesis of 310 was achieved in similar yields to the synthesis of 253 in an overall yield of 6%.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Scheme 78 Reagents and conditions; a) i. SOCl$_2$, DMF, THF, 4 h; ii. 3-iodobenzylamine hydrochloride, Hüning’s base, THF, overnight, 37%; b) Triisopropylsilylethynyl, Et$_3$N, CuI, Pd(PPh$_3$)$_2$Cl$_2$, THF, 16 h, 80%; c) i. SOCl$_2$, DMF, THF, 4 h ii. 1,1-bis(hydroxymethyl)cyclopropyl, K$_2$CO$_3$, THF, overnight, 37% v. 1 M TBAF in THF, THF, 61%

Based on previous models the ethynyl group was thought to sit close to the bottom of the pocket of MDM2, leaving little room for further substitution. To probe whether any further substitution on the ethynyl group is tolerated two compounds were synthesised i.e. 311, which adds a methyl group to the terminal end of the ethynyl group and compound 312, which adds a phenyl ring to the terminal end of the ethynyl.

Figure 69

The synthesis of compound 313 was achieved using a Sonogashira reaction between the 4-iodobenzyl substituted isoindolinone (252) and propyne. Unfortunately, unlike
triisopropylacetylene, propyne is not a liquid but a gas, and therefore requires an alternative set-up of the reaction. The reaction mixture was prepared and degassed and cooled to -78 °C before the addition of the condensed propyne from a cold finger and the reaction gradually warmed to room temperature. As propyne is a gas at room temperature, it was not possible to measure 1.3 equivalents as the propyne boils within the needle syringe, therefore a large excess (as measured by the change in solvent level) was added. The general reaction scheme for the synthesis of 311 and 312 is shown below (Scheme 79).

Scheme 79 Reagents and conditions; a) R = CH₃ Pd(PPh₃)₂Cl₂, CuI, Et₃N, propyne, THF, 18 h, 94%; R = C₆H₁₁ Pd(PPh₃)₂Cl₂, CuI, Et₃N, phenyl acetylene, THF, 17 h, 91%; iv. 1. SOCl₂, DMF, THF, 4 h 2. 1,1-bis(hydroxymethyl)cyclopropyl, K₂CO₃, THF, overnight, R = CH₃ 20%, R = C₆H₅ 36%

Using the optimised Sonogashira conditions compound 314 was synthesised in excellent yield (91%), the complete synthetic route to synthesise the final compound is shown above.
5.4.1. Biological Results for Ethynyl Analogues

**Table 22** Biological activity for the ethynyl analogues

<table>
<thead>
<tr>
<th>Number</th>
<th>Structure</th>
<th>Biological Activity IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>310</td>
<td>![Structure 310]</td>
<td>2.0 ± 0.75</td>
</tr>
<tr>
<td>311</td>
<td>![Structure 311]</td>
<td>0.7$^a$</td>
</tr>
<tr>
<td>312</td>
<td>![Structure 312]</td>
<td>2.9$^a$</td>
</tr>
</tbody>
</table>

$^a$ indicates results where n = 1

Compound 310 demonstrates the narrow structure activity relationships for the benzyl substituents. No tolerance is observed for substituents larger than a fluoro group in the 3-benzyl position, suggesting that the benzyl group sits in a narrow deep pocket, ideal for a 4-ethynyl group, but intolerant of a change in position. Both compounds 311 and 312 demonstrate that there is insufficient space for any further extension from the ethynyl group, suggesting that the terminal hydrogen of the ethynyl sits close to the bottom of the pocket. Interesting, compound 312 has a smaller loss of potency than expected, suggesting that compound 312 may adopt an alternative binding mode.
A number of compounds have now been synthesised with a variety of substituents on the benzyl ring. Several highly activity substituents have been identified; to increase activity attention has been be directed to alternative areas of the molecule.

5.5 Further SAR for the Isoindolinone Ether-Groups

Previous work with the Northern Institute for Cancer Research drug discovery laboratories identified the oxymethyl-cyclopropyl methanol substituent optimal for the C3 position of the isoindolinone e.g. NU8604 (220). The addition of the cyclopropyl group to the ether side-chain significantly increased potency over the n-propoxy derivative the improvement was rationalised by the ability of the cyclopropyl to restrict the freedom of rotation of the propyl chain thus removing an entropic penalty to binding. However, addition of the cyclopropyl ring increases the clogP which is unfavourable for solubility and can cause more metabolic liability and the ether side chain may be a site for metabolic attack of the molecule in vivo.

Table 23 Ether derivatives with IC50 values and clogP values

<table>
<thead>
<tr>
<th>Number</th>
<th>R</th>
<th>IC50 (µM)</th>
<th>clogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>-OH</td>
<td>0.52 ± 0.14</td>
<td>4.4</td>
</tr>
<tr>
<td>316</td>
<td>-OH</td>
<td>0.34 ± 0.26</td>
<td>5.2</td>
</tr>
<tr>
<td>185</td>
<td>-OH</td>
<td>0.23 ± 0.11</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The gem-dimethyl group also improved activity, whilst commonly used to block metabolism, also increases the lipophilicity of molecules which can in turn place molecules at higher risk of metabolism.

Wuitschik et al233-235 have replaced a gem-dimethyl group with an oxetane ring. The van der Waals volumes of the gem-dimethyl group and the oxetane ring are thought to be similar but the oxetane ring is more hydrophilic. Within simple model systems the replacement of a tert-
butyl group with a 3-methyl-oxetanyl group improved the solubility of the compounds and reduced the logD value by one unit. The oxetane ring was also shown to have good stability within human and mice microsomes, a potential indicator of metabolic stability.\textsuperscript{235}

As the isoindolinone molecules have poor solubility (with a clogP of 5.7 for \textit{253}), making aqueous formulation difficult and leaving the molecule prone to metabolism, the inclusion of an oxetane ring may be beneficial. Therefore, the synthesis of oxetane \textit{317} was proposed.

![Diagram of oxetane 317]

Oxetane \textit{317} required the synthesis of the 3,3-bis(hydroxymethyl)oxetane (\textit{318}). The synthesis of 3,3-bis(hydroxymethyl)oxetane from pentaerythritol (2-bis-hydroxymethyl-propane-1,3-diol) (\textit{319}) using diethyl carbonate and potassium carbonate in ethanol has been reported.\textsuperscript{236} The reaction employs a catalytic amount of potassium hydroxide, as the reaction generates ethoxide ions to act as a base for further reactions (Scheme 80). The reaction was repeated following literature procedure. However, no product was observed using this reaction.

![Scheme 80 Proposed mechanism for formation of oxetane ring using diethyl carbonate]

An alternative route, used by Issidorides \textit{et al}\textsuperscript{237} proved more successful, this route uses 2-(bromomethyl)-2-(hydroxymethyl) propan-1,3-diol (\textit{320}) with potassium hydroxide in ethanol. \textit{320} was stirred at room temperature for 2 h, then heated to reflux. The length of
time the reaction was at reflux was gradually increased to optimise the yield of the reaction, the time and yield is shown below (Scheme 81 and Table 24).

Scheme 81 Reagents and conditions; a) KOH, Ethanol, RT-reflux

Table 24 Comparison of reaction time with yield

<table>
<thead>
<tr>
<th>Length of time at reflux (min)</th>
<th>Yield (%) of 318</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>15</td>
<td>78</td>
</tr>
<tr>
<td>20</td>
<td>62</td>
</tr>
<tr>
<td>1.5 h</td>
<td>62</td>
</tr>
</tbody>
</table>

Scheme 82 Proposed reagents and conditions; a) i. SOCl₂, DMF, THF, 4 h ii. 4-nitrobenzylamine hydrochloride, Hünig’s base, THF, overnight, 28%; c) i. SOCl₂, DMF, THF, 4 h ii. 3,3-bis(hydroxymethyl)oxetane, K₂CO₃, THF, overnight or BF₃·OEt₂, 3,3-bis(hydroxymethyl)oxetane, DCM 0 °C-RT

Formation of the ether linkage was then attempted using 3,3-bis(hydroxymethyl) oxetane in the standard synthetic procedure, with thionyl chloride and DMF. However, the synthesis of the desired product did not proceed as cleanly as previously seen when using bis(hydroxymethyl) cyclopropane and two minor spots were observed by TLC. Therefore the
reaction time was extended by 24 h, but no significant change was observed. On workup and purification of the reaction both compounds were isolated, but, only small amounts of each compound were isolated and the NMR spectrum of indicated poor purity. Both spectrum contained characteristic isoindolinone resonances. Full assignment was not possible.

The alternative BF$_3$.OEt$_2$ route to ether formation was then attempted. 3,3-Bis(hydroxymethyl)oxetane is not soluble in DCM so the solvent was switched to THF. However, rather than forming a yellow solution on addition of the BF$_3$.OEt$_2$ and a colourless solution on addition of the alcohol as is usually observed, a precipitate was instead observed, and no reaction occurred. The reaction was repeated, DCM was used as the main solvent, with 3,3-bis(hydroxymethyl)oxetane dissolved in the minimum of THF and added to the reaction, still no reaction was observed.

2-(Bromomethyl)-2-(hydroxymethyl)-propan-1,3-diol (320), which is used as the precursor to 3,3-bis(hydroxymethyl)oxetane is a white solid which is soluble in THF. It was decided to try and form the ether with 309 and then form the oxetane ring in situ. The cyclisation to form the oxetane ring should occur as previously seen, with compound 322 behaving as a monoprotected bromomethyl analogue. Formation of the ether was achieved in good yield under Vilsmeier conditions (42%) and cyclisation was achieved by stirring for 2 h at room temperature in the presence of potassium hydroxide in ethanol followed by 1 h at reflux in 68% yield (see Scheme 83). The 4-ethynyl derivative was prepared by the same route in an overall 13% yield (Scheme 84).

**Scheme 83** Reagents and conditions; a) i. SOCl$_2$, DMF, THF, 4 h; ii. 2-(bromomethyl)-2-(hydroxymethyl)-propan-1,3-diol, K$_2$CO$_3$, THF, overnight, 42%; b) KOH, EtOH, 3 h, RT-reflux, 68%
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

Scheme 84 Reagent and conditions a) i. SOCl₂, DMF, THF, 4 h; ii. 2-(bromomethyl)-2-(hydroxymethyl)-propan-1,3-diol, K₂CO₃, THF, overnight; 31% b) KOH, EtOH, 3 h, RT-reflux, 63%; c) 1 M TBAF in THF, THF, 1 h, 89%

Both compounds separated by chiral HPLC. Compound 317 was separated using a Daicel Chiralpak AD-H using hexane:ethanol 85:15 to give two compounds, both a foamy solid. NCL-00018566 (peak 1, 326) with [α] = -10.7° and NCL-00018565 (peak 2, 327) with [α] = + 30.3°. Compound 325 was separated using tert-butyl methyl ether:isopropanol 95:5. Peak 1 (NCL-00018710, 328), was a fluffy solid with optical rotation of with [α] = -7.74°, whilst peak 2 (NCL-00018711, 329) was a colourless oil which gradually solidified to a white solid. 329 had an optical rotation of [α] = +4.65°. Assignment of the absolute stereochemistry has not been possible as the compounds fail to give suitable crystals. The faster eluting peaks are expected to have an (R)-configuration by analogy with NU8354. A chiral HPLC trace is shown below (Figure 70) demonstrating the clear separation between compound 326 and 327 on the chiral column.

Figure 70
5.5.1. Biological Results for Isoindolinone Ether Groups

![Isoindolinone Ether Group](image)

**Table 25** Biological results for ether derivatives * indicates compounds synthesised by other chemist

<table>
<thead>
<tr>
<th>Number</th>
<th>Rotation</th>
<th>X</th>
<th>R</th>
<th>Biological Activity IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>clogP</th>
<th>Measured Solubility (midpoint)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220*</td>
<td>(+/-)</td>
<td>NO₂</td>
<td>OH</td>
<td>0.095 ± 0.0096</td>
<td>5.6</td>
<td>6.5 µM</td>
</tr>
<tr>
<td>317</td>
<td>(+/-)</td>
<td>NO₂</td>
<td>OH</td>
<td>0.048 ± 0.0091</td>
<td>4.7</td>
<td>18.75 µM</td>
</tr>
<tr>
<td>326</td>
<td>(-)</td>
<td>NO₂</td>
<td>OH</td>
<td>0.025 ± 0.0061</td>
<td>4.7</td>
<td>-</td>
</tr>
<tr>
<td>327</td>
<td>(+)</td>
<td>NO₂</td>
<td>OH</td>
<td>0.83 ± 0.12</td>
<td>4.7</td>
<td>-</td>
</tr>
<tr>
<td>253</td>
<td>(+/-)</td>
<td>CCH</td>
<td>OH</td>
<td>0.15 ± 0.084</td>
<td>6.1</td>
<td>3.75 µM</td>
</tr>
<tr>
<td>325</td>
<td>(+/-)</td>
<td>CCH</td>
<td>OH</td>
<td>0.090 ± 0.032</td>
<td>5.2</td>
<td>11.5 µM</td>
</tr>
<tr>
<td>328</td>
<td>(-)</td>
<td>CCH</td>
<td>OH</td>
<td>0.12 ± 0.096</td>
<td>5.2</td>
<td>-</td>
</tr>
<tr>
<td>329</td>
<td>(+)</td>
<td>CCH</td>
<td>OH</td>
<td>3.5 ± 1.7</td>
<td>5.2</td>
<td>-</td>
</tr>
</tbody>
</table>

The replacement of the cyclopropyl ring with an oxetane is calculated to give an improvement in the physical properties of the molecule, reducing the clogP by one unit. In the case of the 220 to 317 an increase in potency is also observed. Wuitschik *et al* describes 3-disubstituted oxetanes as possessing significant influence over the conformation of the carbon chain to which it is joined. With a gem-dimethyl substituted chain a staggered...
conformation is adapted, however, with a oxetane the chain form a gauche conformation.\textsuperscript{233} The introduction of the oxetane ring may therefore be holding the propyl chain in the ideal conformation, accounting for the increase in potency.

![Gem-dimethyl conformation](image)

**Figure 71**

When changing from the cyclopropyl ring to the oxetane ring with compound 253 to 325, the two compounds are essentially equipotent suggests that the isoindolinone adopts a somewhat different binding mode in MDM2 when substituted with an ethynyl group in comparison to the nitro analogues. When separating compound 317 into the two enantiomers 326 and 327, the (-)-enantiomer (326) improves activity beyond the parent compound. However, with compound 328, the (-)-enantiomer of compound 325, the activity is lower than the parent compound. It should be noted that this is the result of initial testing, and has a large error within the results, so further testing may result in improved activity.

The oxetane confers good properties without compromising the potency of the analogues. Consequently, it should be included in subsequent series of a future isoindolinones.

### 5.6 tert-Butyl ‘A’-Ring Analogues

The isoindolinone series has poor aqueous solubility, well below the target solubility for an orally available drug of 50 µM. Measurements of the aqueous solubility of a number of compounds by Cyprotex, a company which specialises in the measuring of physical properties of molecules developed by academia and pharmaceutical industry, observed that a significant difference could be seen within molecules of the isoindolinone series. Table 26 shows some of the results of a number of compounds below. The solubility was measured...
using a turbidimetric solubility assay, which measures kinetic solubility by diluting DMSO solution of the drug with an aqueous buffer at a pH of 7.4 and a temperature of 37 °C. Turbidity was measured at 620 nm. Results are quoted as start point, midpoint and endpoint of precipitation. The solubility midpoint was obtained by measuring the first concentration at which precipitation is observed and the concentration where complete precipitation is observed. From this, a midpoint is calculated.

**Table 26 Structure and related midpoint of solubility**

<table>
<thead>
<tr>
<th>Number</th>
<th>X</th>
<th>Ar</th>
<th>Lower Solubility point (µM)</th>
<th>Upper Solubility point (µM)</th>
<th>Solubility Midpoint (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>253</td>
<td>4-Cl</td>
<td><img src="image" alt="Structure" /></td>
<td>1</td>
<td>6.5</td>
<td>3.75</td>
</tr>
<tr>
<td>185</td>
<td>-</td>
<td><img src="image" alt="Structure" /></td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>220</td>
<td>4-Cl</td>
<td><img src="image" alt="Structure" /></td>
<td>3</td>
<td>10</td>
<td>6.5</td>
</tr>
<tr>
<td>224</td>
<td>6'-Bu</td>
<td><img src="image" alt="Structure" /></td>
<td>10</td>
<td>30</td>
<td>20</td>
</tr>
</tbody>
</table>
From Table 26 compound 253 can be seen to have particularly poor solubility. Interestingly, the change from compound 220 to compound 224 has a 3-fold change in solubility, which is a large difference for simply changing the ‘A’-ring 4-chloro to a 6-Bu. A tert-butyl group would be expected to decrease the aqueous solubility, as it increases lipophilicity. In terms of dissolution from a solid into a solution tert-butyl groups are known to have an interesting affect on solubility. Law describes tert-butyl as increasing organic solubility within a series of compounds. The tert-butyl increases steric hindrance, which reduces the interaction between molecules thus increasing solubility, a principle which can also be applied to aqueous solubility.

Compound 224, whilst having essentially equipotent biological activity (IC$_{50}$ = 194 nM) as compound 220 has significantly improved aqueous solubility. Compound 330, which maintains the favourable ethynyl substitution of the benzyl ring, and incorporates the solubilising tert-butyl group on the isoindolinone ‘A’-ring was therefore synthesised.

![Chemical Structure](image)

Compound 224 was synthesised starting from 4-tert-butyphthalic anhydride, forming the benzoyle benzoic acid via a Friedel-Crafts acylation analogous to 4-chloro analogues. Therefore, we hoped to utilise this synthetic route to access compound 330. The proposed synthetic route is shown below.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

**Scheme 85** Proposed reagents and conditions; a) AlCl₃, PhCl, 2 h, 90 °C; b) i. SOCl₂, DMF, THF, 4 h ii. 4-iodobenzylamine hydrochloride, Hünig’s base, THF, overnight; c) Triisopropylsilyl acetylene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, THF; d) i. SOCl₂, DMF, THF, 4 h ii. 1,1-bis(hydroxymethyl)cyclopropyl, K₂CO₃, THF, overnight e) 1 M TBAF in THF, THF, 1 h

Previous work undertaken within the laboratory identified that the Friedel-Crafts reaction between 3-tert-butyl phthalic anhydride and chlorophenyl would give approximately 1:1 mixture of the 5- and 6- isomers of the substituted benzoyl benzoic acid.²⁴⁰ The preparation was repeated as described and unfortunately, it proved to be very erratic in yield, and also very difficult to dry the product to a suitable level for the following reaction in the scheme. Therefore, an alternative method for formation of the benzoyl benzoic acid was sought.

An alternative synthetic route to the benzoyl benzoic acid had recently been developed in-house.²⁴¹ It utilises a lead tetraacetate mediated rearrangement to form a benzoyl benzaldehyde from 2-hydroxyhydrazide.²⁴² To utilise this new synthetic method the correctly substituted 2-hydroxyhydrazine was required. Jacq et al synthesised 2-hydroxyhydrazine from 2-hydroxybenzaldehydes and hydrazides,²⁴² a protocol which should provide access to the desired intermediates for synthesis of the desired final compound.
Synthesis of the 6-tert-butyldisubstituted isoindolinones following this synthetic scheme requires use of 5-tert-buty1-2-hydroxybenzaldehyde (340), which can be synthesised in one step from 4-tert-buty1 phenol (339). The Duff reaction is a method of installing aromatic formyl groups via electrophilic aromatic substitution. The reaction uses hexamethylenetetramine (HMTA) as a formaldehyde equivalent, which, when catalysed by trifluoroacetic acid will formylate an aromatic ring. The mechanism of is shown below. Acid catalysis results in formation of an iminium species, which is then attacked by the aromatic ring, to form the Wheland intermediate, a further iminium species then extracts a hydride from the benzylic position, forming a further iminium species, which on aqueous work-up will result in formation of the desired aldehyde.

Lindoy et al have synthesised 5-tert-buty1-2-hydroxybenzaldehyde (340) from HMTA and 4-tert-buty1phenol in refluxing TFA for in 29%. In-house development of this reaction suggested microwave heating was appropriate for this reaction. A solution of 4-tert-buty1phenol and HMTA in TFA was heated at 120 °C for 30 min in a yield of 46%. LCMS monitoring of this reaction suggested no starting material remained, but the lower yield suggests a large number of minor impurities are formed.
Benzaldehyde 340 was converted to hydrazide 341 on treatment with 4-chlorobenzhydrazide in acetic acid in excellent yield (94%) following the procedure described by Jacq et al. The correct substituents for the rearrangement reaction to give the benzoyl benzaldehyde were now in place. The synthetic scheme to give key intermediate benzoyl benzoic acid is shown below.

Scheme 88

Reagents and conditions; a) HMTA, TFA, 120 °C, 30 min, 46%; b) 4-chlorobenzhydrazide, AcOH, 15 min, 94%; c) Pb(OAc)$_4$, THF, 2 h, 0 °C, 73%; d) NaClO$_2$, H$_2$NSO$_3$H, MeCN, 1.5 h, 85%

The mechanism of the lead tetraacetate induced rearrangement has been extensively explored by Katritzky et al. Treatment of monosubstituted hydrazones (e.g. 344) leads to formation of acetoxyazo compounds like 345 (where R” = H). However, when R” = COAr e.g. 346 a cyclisation can occur to give 1,3,4-oxadiazolines such as 338.

Scheme 89

Scheme 90
When R’ = 2-phenol further rearrangement can occur, with the hydroxyl group replaced with the acyl substituent. This rearrangement leads to the formation of the desired benzoyl benzaldehydes. Katritzky et al demonstrated that the benzoyl benzaldehyde is formed via an intramolecular reaction, and proved that Pb(OAc)$_4$ does not act as an acid catalyst via minor contamination with acetic acid.$^{244}$ Instead, the proposed mechanism is shown below (Scheme 91). Co-ordination to lead (IV) acetate is followed by attack of an acetate ion on the imine, with displacement of lead (II) acetate. This can result in either a cis or trans double bond, with the cis required for the following mechanism to proceed. However this step of the mechanism may be reversible, allow reformation of the imine, preventing the loss of the trans product. Attack of the carbonyl with an acetate ion breaks the double bond, with the electrons displacing the first acetate ion and forming a 1,3,4-oxadiazolines. The second acetate ion is then displaced by the lone pair of electron of the phenol oxygen, this species then rearranges to extrude nitrogen and rearomatises to the benzoyl benzaldehyde. Labelling studies undertaken by Katritzky et al demonstrated that the acyl group oxygen eventually becomes the aldehyde oxygen, rather the hydroxyl oxygen atom via formation of 1,3,4-oxadiazolines as was seen previously.

Scheme 91 Mechanism of Pb(OAc)$_4$ induced rearrangement of 2-hydroxyacyl hydrazones to benzoyl benzaldehyde$^{244}$

The benzoyl benzoic aldehyde (342) was synthesised following the procedure described by Jacq et al.$^{242}$ The yield of this reaction was observed to be somewhat inconsistent, ranging from 54-73% and producing numerous minor side-products. A number of analogues with a range of substituents on the ‘A’-ring have been synthesised and a general trend has been observed, electron-donating substituents on the phenolic ring lowers the yield of the reaction.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

(Table 27). The few examples of electron-donating phenolic substituents reported also have a lower yield (e.g. 46% for a methoxy analogue). Benzoyl benzaldehyde (342) was oxidised to benzoyl benzoic acid (343) following the procedure of Lampe et al which, after recrystallisation gave 343 as a fluffy white solid.

**Table 27** Yields for lead (IV) acetate mediated rearrangement with a variety of phenolic substituents, * indicates results obtained by other chemists

<table>
<thead>
<tr>
<th>Phenolic ring substituents</th>
<th>Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>91*</td>
</tr>
<tr>
<td></td>
<td>94*</td>
</tr>
<tr>
<td></td>
<td>56*</td>
</tr>
<tr>
<td></td>
<td>30*</td>
</tr>
<tr>
<td></td>
<td>74*</td>
</tr>
</tbody>
</table>

Compound 343, which had previously been synthesised in one step from a Friedel-Crafts reaction (in 92% yield, mixture of isomers) has now been synthesised in 4-step route an overall yield of 26%. Although this scheme adds further synthetic steps to the synthesis of benzoyl benzoic acid (343), the regioselectivity removes the need for the difficult separation of isomers, as used in the Friedel-Crafts route. The desired final compound 330 can now be synthesised (Scheme 92).

Formation of the isoindolinone, using SOCl2/DMF, is achieved in significantly higher yield when using 5-tert-butyl-2-(4-chlorobenzoyl)benzoic acid (323) (86%) rather than 36% for 4-chloro 241, (even allowing for the mixture of isomers). The 4-chloro atoms appears to hinder
formation of the isoindolinone, either sterically, perhaps hindering formation of the Ψ acid chloride or electronically, due to the electron withdrawing nature of the chloro substituent.

The acetylene moiety was incorporated using the optimised Sonogashira conditions in excellent yield (91%). Formation of the ether linkage via the Vilsmeier chemistry was achieved in moderately higher yield for compound 336 in comparison to the 4-Cl analogue (238) (50% versus 24%), influenced by the change in steric and electronic factors. Deprotection of acetylene groups with TBAF gave compound 330 in 80% yield.

![Scheme 92](image)

**Scheme 92** Reagents and conditions; a) i. SOCl₂, DMF, THF, 4 h; ii. 4-iodobenzylamine hydrochloride, Hünig’s base, THF, overnight, 86%; b) Pd(PPh₃)₂Cl₂, CuI, Et₃N, triisopropylsilylacetylene, THF, 16 h, 91%; c) i. SOCl₂, DMF, THF, 4 h ii. 1,1-bis(hydroxymethyl)cyclopropyl, K₂CO₃, THF, overnight, 50%; d) 1 M TBAF in THF, THF, 1 h, 80%

Previous results showed that compound 224 displayed similar biological activity to the 4-Cl analogue, the replacement of the (hydroxymethyl)cyclopropylmethoxide side chain, with the alternative (hydroxymethyl)oxetanemethoxide side chain, with the aim of improving physical properties, was desired. The synthetic route is shown below and utilises the methodology of incorporating the bromomethyl species and forming the oxetane *in situ*.

It was decided to use the BF₃·OEt₂ reaction to incorporate the 2-(bromomethyl)-2-(hydroxymethyl)-propan-1,3-diol as it lacked the steric hindrance of the 4-Cl analogue.
Formation of the ether 349 was achieved in 4.5 h and as previously observed, conversion to the ether using BF$_3$.OEt$_2$ is high yielding, forming 349 in 87%. The cyclisation to form the oxetane ring was achieved in 78% yield on treatment with KOH in ethanol, and deprotection of the acetylene group gave the desired final compound (351).

Scheme 93 Reagents and conditions; a) 2-(bromomethyl)-2-(hydroxymethyl)propan-1,3-diol, BF$_3$.OEt$_2$, THF, 4.5 h 0°C-RT, 87%; b) KOH, EtOH, 3 h, RT-reflux, 78%; c) 1 M TBAF in THF, THF, 1 h, 59%

Both compounds 351 and 330 were selected for separation of the two enantiomers for chiral HPLC and are currently awaiting separation.
5.6.1. Biological Results for tert-Butyl Analogues

**Table 28** Biological activity for tert-butyl analogues * indicates compounds synthesised by other chemists

<table>
<thead>
<tr>
<th>Number</th>
<th>R</th>
<th>X</th>
<th>Ar</th>
<th>Rotation</th>
<th>Biological Activity IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220*</td>
<td>4-Cl</td>
<td>(+/-)</td>
<td>0.095 ± 0.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>224*</td>
<td>6-tBu</td>
<td>(+/-)</td>
<td>0.19 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>253</td>
<td>4-Cl</td>
<td>(+/-)</td>
<td>0.15 ± 0.083</td>
<td></td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>6-tBu</td>
<td>(+/-)</td>
<td>0.078(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>325</td>
<td>4-Cl</td>
<td>(+/-)</td>
<td>0.090 ± 0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>351</td>
<td>6-tBu</td>
<td>(+/-)</td>
<td>0.028(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>352*</td>
<td>4-Cl, 6-tBu</td>
<td>(+/-)</td>
<td>0.14 ± 0.055</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

<table>
<thead>
<tr>
<th>353*</th>
<th>( \text{\textsuperscript{353}O} \text{O} \text{O} \text{O} )</th>
<th>4-Cl, 6-\textsuperscript{t}Bu</th>
<th>(+/−)</th>
<th>0.27 ± 0.015</th>
</tr>
</thead>
</table>

* indicates n = 1

A change in ‘A’-ring substituent from the 4-chloro 253 to 6-\textsuperscript{t}Bu 330 gives equipotent compounds. However, when the oxetane side chain is also incorporated, an increase in potency is observed for the \textsuperscript{t}Bu 351 suggesting that the 4-chloro substituted isoindolinone and the 6-\textsuperscript{t}Bu substituted isoindolinone are slightly shifted in position within MDM2 pocket, allowing the oxetane to adopt a more favourable position. Interestingly, when the 4-Cl, 6-\textsuperscript{t}Bu A-ring substituents, which are active when combined with the cyclopropyl-substituted ether side-chain, is not when combined with the oxetane-substituted ether side-chain. This supports the evidence for a slightly different binding mode for the \textsuperscript{t}Bu-substituted isoindolinone, which adopts the optimal position for the oxetane substituted, whilst the chloro substituted isoindolinone does not.

5.7 Protein Crystallography of Isoindolinone-based Inhibitors of p53/MDM2 Protein-protein Interaction

Protein crystallography is an important aid for the drug discovery process. Although a number of MDM2/p53 inhibitors have been crystallised bound to the protein, no crystal structure of the isoindolinone has previously been available. Collaborators at the University of Oxford Biochemistry Department have previously identified the binding mode of isoindolinones through NMR studies,\textsuperscript{191} and suggested proposed which would lead to further increases in potency,\textsuperscript{192} a crystal structure would give further insights into the interactions between the isoindolinones and the protein.

Protein crystallography is traditionally used in lead optimisation to guide chemical modification.\textsuperscript{96} For X-ray crystallography to successfully obtain a structure a well-ordered crystal must first be grown. Protein crystals are less ordered that small molecule crystals, and frequently contain channels of disordered solvent.\textsuperscript{246} Protein crystallisation requires the synthesis of sufficient volumes of purified protein to screen a variety of crystallisation conditions. The development of molecular biology has aided that production of larger volumes of protein. Bacteria, such as E-coli, can be used to express the desired protein in
large quantities, through the introduction of plasmids containing the gene of the target protein. Within crystal structure of MDM2 the protein is stabilised by other molecules of MDM2 resulting in a number of molecules of MDM2 per unit cell. The crystal structure of Nutlin-2a (PDB code 1RV1) contains three molecules of MDM2 (Figure 72).

**Figure 72**

To gain understanding of the processes behind protein crystallography a period of four months was spent working alongside colleagues at the University of Oxford working on protein purification and protein crystallography. Both mutants and wild type samples of the MDM2 binding site were purified, of either residues 17-109 or 17-125 of MDM2. Removal of residues 110-125 removes an unstructured loop which may prevent crystallisation. A number of mutants were developed which replace either one, two or three residues with alanine residues. Crystals develop when good contacts are formed between protein molecules, and two residues, lysine and glutamic acid, have been shown to be prone to preventing the formation of good contacts between protein molecules. Therefore, Lys and Glu residues which commonly lie on the surface of proteins can be replaced by Ala to aid crystallography.247

A number of wild type and mutant MDM2 protein samples were then purified using the methodology shown below, all included a Glutathione S-Transferase tag to aid purification. E coli bacteria were employed to grow the protein in 1 L cultures, centrifuged and the supernatant removed. The remaining pellet was resuspended in PBS buffer (40 mL) and flash
frozen by co-workers in Oxford before my work began. For methodology for protein purification see appendix one.

A number of MDM2 samples were prepared and are summarised below.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>K94A</td>
<td>(17-109)</td>
</tr>
<tr>
<td>K94E95A</td>
<td>(17-109)</td>
</tr>
<tr>
<td>MDM2</td>
<td>(17-109)</td>
</tr>
<tr>
<td>E69K70A</td>
<td>(17-109)</td>
</tr>
<tr>
<td>E69A</td>
<td>(17-109)</td>
</tr>
<tr>
<td>E69K70E94A</td>
<td>(17-125)</td>
</tr>
<tr>
<td>E69A</td>
<td>(17-125)</td>
</tr>
</tbody>
</table>

All mutants were expressed in sufficient volumes for crystallography with the exception of K94E95A, and E69K70E94A which only allowed a small volume of crystallography tray to be set up. After purification the purified protein was concentrated to approximately 2 mg/mL and incubated overnight with a series of inhibitors – Nutlin-3a, NU8354a, NCL00013774, NCL00013775, NCL00018225 and NCL00018226, which includes the most active compound synthesised at the NICR, the less active opposite enantiomers, and Nutlin-3a, the classical example of an MDM2/p53 inhibitor. 1.5 equivalents of the inhibitor in DMSO was incubated at 4 °C overnight, followed by washing with Hepes buffer and concentrating to a concentration of approximately 12 mg/mL.
The protein and inhibitor mix was then set up into crystallisation trays. These were either purchased trays, to give a range of conditions to identify which conditions give crystals or custom trays, with a narrower range of conditions, with the aim of identifying optimal conditions for crystallisation. Purchased trays were dispensed in a 96-well plate using liquid handling robots and had a range of protein concentrations within each well. Trays were then sealed and stored in a fridge with automated image collection at 4 °C to allow monitoring of each well. Custom trays were alternatively set up in 24 well plates and the crystallisation conditions were prepared by hand to give a much smaller range of conditions, all based around conditions which had previously been identified from purchased screens as leading to crystals. Previous work undertaken at Oxford at identified polyethylene glycol (PEG) containing conditions as optimal for MDM2 crystallisation, therefore all custom trays were based either around PEG4000, PEG5000 or PEG8000. Each well contained a 500 µL reservoir and the raised well for the protein. Each reservoir was made up to 500 µL with water (volume determined by percentage of PEG used). Both ammonium sulphate and sodium acetate (from a 1 M stock of each) were used as additives to the solution, with the pH of the sodium acetate solution adjusted in some cases to pH 4.6. To the raised well was added 1 µL of the protein and inhibitor solution and 1 µL of the reservoir solution. These were also sealed and stored at 4 °C for crystallisation. All custom trays were prepared in this manner.
with the exception of the E69K70A mutant. Previous work had resulted in the growth of small spike-like crystals which were too small to be useful for X-ray structure determination. Therefore, a custom tray was prepared with only NCL-00018225 and NCL-00013775 as only these inhibitors had formed crystals, stored to two days at 4 °C before being seeded with fragments of these small needle-like crystals. Seeding provides a surface for crystallisation to occur and can result in the formation of larger crystals. A summary of crystallisation conditions is shown in appendix one. An illustrative example of the set up of a 24 well plate is also shown in appendix one.

From the crystallography tray seeded with the E69K70A mutant, a needle-like crystal was isolated. X-ray crystallography undertaken at the diamond synchrotron in Oxford with a 3 GeV electron beam, with a micro focus beamline under the supervision of Dr Ed Lowe and obtained a diffraction pattern of sufficiently high resolution to give a crystal structure which is shown below. The crystal structure was obtained with a resolution of 2.9 Å and the structural insights gained by this crystal structure will be discussed in more detail below.

To validate the use of mutants as a more crystallisable form of MDM2, we must firstly confirm that the isoindolinone bind to a similar manner to both the mutants and wild-type MDM2. For this confirmation two assay types were selected, a Thermofluor assay and a fluorescence anisotropy assay. Assays were initially run on the wild-type MDM2 and the E69K70A mutant, the only mutant at this point to crystallise in the presence of an isoindolinone ligand.

Thermofluor was a technique originally developed as high-throughput manner of identifying ligands for drug discovery targets. It requires a hydrophobic fluorescent probe or dye to distinguish between folded and unfolded proteins. When the protein is folded the probe is exposed to the aqueous environment, which quenches fluorescence. On heating, the protein will begin to unfold, exposing its hydrophobic core, to which the probe can then bind, and the fluorescence will no longer be quenched. This allows a measurement of unfolding as a function of temperature. This process can be undertaken using a real-time PCR machine in a 96-well plate. By introducing ligands to the wells, those which bind to the protein will stabilise it, and increase the thermal stability, increasing the melting temperature. This can be used to measure affinity of the ligand for the protein, a greater affinity will lead to a greater
increase in temperature. Results obtained should be consistent with results gained from other assays such as enzymatic assays.\textsuperscript{251}

The Thermofluor assay was used to compare the melting of wild-type and mutant MDM2 in the presence of ligands. The hydrophobic probe used was sypro orange, a fluorescent dye. Firstly the optimal conditions for fluorescence were identified using a range of protein concentration and dye concentrations. Sypro orange is sold at a concentration of 2000 x, so was therefore diluted with Hepes buffer to create a range of concentration 5 x, 10 x, 15 x and 20 x. The set up of the plate is shown is appendix one.

From this experiment, optimal conditions for monitoring of the protein melting was determined as 10 x dye concentration and after further optimisation the protein concentration was maintained at 9 µM. The inhibitor was added in a 2:1 inhibitor to protein ratio, with the inhibitor concentration maintained at 18 µM. To measure the inhibitor’s affinity, each plate contained 7.5 µL of 10 x dye, 1.76 µL protein at 77 µM and 0.54 µL inhibitor (from 500 µM DMSO stocks) and each well was made up to 15 µM with Hepes buffer. As a control, another well was run with identical conditions, with 0.54 µL of DMSO, and as a second control a well contained just dye and protein. A second row acted as a control for each inhibitor - each well contained 7.5 µL of 10 x dye and 0.54 µL inhibitor (or DMSO). For comparison a p53 peptide (sequence SQETFSDLWKLLPEN) was also included in these studies.

For the wild-type protein optimal concentrations were identified as 20 x concentration of sypro dye, 5 µM concentration of dye and 10 µM concentration of dye. With plates made up of 10 µL of 20x sypro dye, 0.56 µL protein (178 µM), 0.4 µL of inhibitor (500 µM) and were made up to 20 µL with Hepes buffer.

For both proteins fluorescence was measured over a temperature range of 25-89 °C, with three readings taken at each temperature, with each temperature maintained for one minute. The observed melting points are shown below for each protein; average change in melting point was calculated using DMSO as the blank for all inhibitors except the p53 peptide. As the peptide was an aqueous solution the buffer solution was used as the blank for this well.
### Table 29 Melting point for E69K70A protein with inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Melting point repeat 1 (°C)</th>
<th>Melting point repeat 2 (°C)</th>
<th>Average change in melting point from control (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td>59</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>DMSO</td>
<td>58</td>
<td>54</td>
<td>-</td>
</tr>
<tr>
<td>NU8354a</td>
<td>70</td>
<td>69</td>
<td>13</td>
</tr>
<tr>
<td>NCL-00013774</td>
<td>75</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>NCL-00013775</td>
<td>71</td>
<td>68</td>
<td>13</td>
</tr>
<tr>
<td>NCL-00018225</td>
<td>77</td>
<td>77</td>
<td>21</td>
</tr>
<tr>
<td>NCL-00018226</td>
<td>72</td>
<td>74</td>
<td>17</td>
</tr>
<tr>
<td>Nutlin-3a</td>
<td>79</td>
<td>77</td>
<td>22</td>
</tr>
<tr>
<td>p53 peptide</td>
<td>63</td>
<td>59</td>
<td>3.7</td>
</tr>
</tbody>
</table>

### Table 30 Melting point for wt MDM2 17-109 protein with inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Melting point repeat 1 (°C)</th>
<th>Melting point repeat 2 (°C)</th>
<th>Melting point repeat 3 (°C)</th>
<th>Average change in melting point from control (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>62</td>
<td>64</td>
<td>63</td>
<td>-</td>
</tr>
<tr>
<td>DMSO</td>
<td>68</td>
<td>64</td>
<td>67</td>
<td>-</td>
</tr>
<tr>
<td>NU8354a</td>
<td>72</td>
<td>75</td>
<td>76</td>
<td>8.0</td>
</tr>
<tr>
<td>NCL-00013774</td>
<td>75</td>
<td>79</td>
<td>76</td>
<td>12</td>
</tr>
<tr>
<td>NCL-00013775</td>
<td>64</td>
<td>71</td>
<td>70</td>
<td>2.2</td>
</tr>
<tr>
<td>NCL-00018225</td>
<td>76</td>
<td>70</td>
<td>77</td>
<td>7.6</td>
</tr>
<tr>
<td>NCL-00018226</td>
<td>65</td>
<td>68</td>
<td>68</td>
<td>1.0</td>
</tr>
<tr>
<td>Nutlin-3a</td>
<td>90</td>
<td>82</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Peptide</td>
<td>67</td>
<td>70</td>
<td>68</td>
<td>5.1</td>
</tr>
</tbody>
</table>

A comparison of the change in melting point between the E69K70A and wild-type protein is shown below, along with the ELISA assay IC\(_{50}\) values for each isoindolinone.
Table 31 Comparison of change in melting point between each protein and ELISA assay values for the wild-type protein

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>E69K70A change in melting point</th>
<th>Wt MDM2 change in melting point</th>
<th>ELISA assay IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NU8354a</td>
<td>13</td>
<td>8.0</td>
<td>0.17</td>
</tr>
<tr>
<td>NCL-00013774</td>
<td>14</td>
<td>12</td>
<td>0.044</td>
</tr>
<tr>
<td>NCL-00013775</td>
<td>13</td>
<td>2.2</td>
<td>0.73</td>
</tr>
<tr>
<td>NCL-00018225</td>
<td>21</td>
<td>7.6</td>
<td>0.041</td>
</tr>
<tr>
<td>NCL-00018226</td>
<td>17</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Nutlin-3a</td>
<td>22</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>peptide</td>
<td>3.7</td>
<td>5.1</td>
<td>-</td>
</tr>
</tbody>
</table>

By drawing a graph comparing the change in melting point against the IC$_{50}$ value it can be observed that for wild-type MDM2 some correlation can be observed between the thermofluor values and IC$_{50}$ values. However, when looking at the graph for the E69K70A mutant little correlation is observed between the change in melting points and the IC$_{50}$. For the (+)-isomers NCL-00013775 and NCL-00018226, a significant change in melting point is observed, unlike the wild-type protein.
The fluorescence anisotropy assay was then undertaken to give a further comparison between the mutant and wild-type protein. Fluorescence anisotropy uses a fluorescently labelled p53 peptide and monitors the displacement from MDM2 on titration of increasing concentration of inhibitor.

A fluorescent molecule, if excited by polarized light, will emit polarized light. However, in solution, the polarized light will be randomized, due to the rotation of the molecule in solution. Fluorescence anisotropy is based on the observation that the size of molecules can affect the polarization of fluorescence. A larger molecule results in less scattering of the polarized light than a smaller molecule. Therefore, when the p53 fluorescently labelled peptide is in complex with MDM2, the fluorophore behaves as a large molecule, but on titration of increasing concentrations of inhibitors, the p53 will be displaced from the p53/MDM2 complex and will behave as a smaller fluorophore. By monitoring the degree of scattering, the increasing levels of displacement can be observed. By plotting concentration of inhibitor against levels of polarization, a sigmoidal curve should be observed, of the type shown below, measured by Zhang et al.
Firstly the optimised concentrations without inhibitor were identified using a range of MDM2 and fluorescently labelled p53 concentrations (Table 35 Appendix one). Each well was made up to a final concentration of 20 µL with Hepes buffer.

For each concentration of MDM2 an increase in intensity of fluorescence was observed with increasing concentration of fluorescently labelled peptide. A MDM2 concentration of 10 nM was identified as the optimal concentration to use for the titration of inhibitor and a fluorescently labelled peptide concentration of 2 nM was used. NU8354a (345) was used as the inhibitor, and due to the extensive pipetting involved in the preparation of each experiment; conditions were optimised using a single inhibitor first. The concentration of inhibitor ranged from 2-57 nM from a DMSO solution, and to maintain a constant DMSO concentration each well was made up to a DMSO volume of 1.2 µL along with a blank well containing just DMSO. The volume for each well was maintained at 20 µL using Hepes buffer. However, on measuring the polarization no curve was observed.

After discussion with co-workers it was decided to alter the range of inhibition concentration, to give a range of 1-256 nM to give a range of values either side of the IC₅₀ value. On measuring the polarization, no curve was obtained. It was proposed that a greater
concentration of fluorescently labelled peptide was required. At low concentration of inhibitor, there are sufficient binding sites on MDM2 for both peptide and inhibitor; therefore the displacement of the peptide may not occur. By increasing the concentration of the peptide, the peptide should be displaced by the inhibitor of the peptide at a lower concentration, which should be observed by a change in fluorescence polarization.

Therefore, whilst both the MDM2 concentration and the range of inhibitor concentrations were maintained, the peptide concentration was increased to 5 nM. However, no curve was again observed. The experiment was repeated with Nutlin-3a to verify that the isoindolinone was not affecting the experiment, but this gave identical results. Work on this assay was therefore stopped. However, colleagues at Oxford, who continued to optimise this assay after my placement was completed, suggested that by saturating the MDM2 binding site with peptide, a curve can be observed on addition of the inhibitor.

The inhibitor may induce a change of behaviour of the MDM2 protein when in solution. To monitor this an analytical size S75 exclusion column was used. Size exclusion columns separate proteins according to size, with larger proteins coming off the column quicker. A sample of protein in Hepes buffer was run through the column, and the trace showed a peak at 12 min. The protein was then incubated with NU8354a (356) for 1 h in a 1:1 ratio of protein to inhibitor and the size exclusion column was then repeated. The trace showed two peaks, one at 12 min which corresponds to the protein in solution, and another peak at 4.2 min. This suggests that in the presence of the inhibitor the MDM2 protein forms a complex of a number of units of the protein. To identify the size of the complex a calibration column of proteins of known mass must be used. Two proteins; bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) were mixed together and ran through the size exclusion column. The largest of these two proteins appeared on the trace at 4.2 min, which agrees with the time of the MDM2 complex, suggesting a mass of 66 kDa. The wt MDM2 (17-109) has a mass of approximately 11 kDa, therefore six molecules of MDM2 form the complex.

The unusual behaviour of the MDM2 protein in the presence of inhibitors may influence the results of the fluorescence anisotropy and Thermofluor assay leading to the unusual results. Coworkers at Oxford are continuing work on assay optimisation.
5.7.1. Crystal Structure of NCL00013774

**Figure 75** X-ray crystallography of NCL-00017734 modelled using CCP4MG, the electron density map of the isoindolinone is shown

**Figure 76** Image of NCL-00013774 in MDM2 binding site modelled in PyMOL and (right) with surrounding residues displayed
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

A crystal structure of an isoindolinone was achieved using E69K70A mutant of MDM2 17-109 with a resolution of 2.9Å. From the crystal structure it can be observed that the chlorophenyl ring lies within the Trp23 pocket, the nitrobenzyl ring sits within the Leu26 pocket and the isoindolinone moiety lies over the Phe19 pocket.

**Figure 77** Modelling of crystal structure of NCL00013774 (top) and Nutlin-2a (bottom left) and p53 (bottom right), with the MDM2 protein represented as ribbons

**Figure 78** Halogen bond between 4-Cl and Gly58 of MDM2
The ‘A’-ring 4-Cl is observed to a halogen bond with a backbone carbonyl group of a glycine residue, with a length of 3.3 Å. The electron cloud surrounding the chloro atom interacts with the lone pair of the carbonyl. Chlorine, bromine and iodine atoms attached to either aryl or electron withdrawing alkyl groups can form bonds to electrophiles, nucleophiles and other halogens, \(^{254}\) dependent on direction of the interaction. \(^{255}\) A \(\sigma\)-hole is formed when halogen atoms are attached to either aryl or electron withdrawing alkyl groups. A \(\sigma\)-hole is a positive area within electron density, which lies opposite the carbon-halogen bond and is formed by the three pairs of unpaired electrons on the halogen, forming a ring of electron density surrounding the centre of the halogen atom, while any remaining electrons are involved in bonding to the carbon. \(^{254}\) A representation of the different interactions is shown below. Halogen bonds lie approximately 180 ° to the C-X bond, though this angle can be as little as 155 °. \(^{256}\) Halogen bonds are weaker than H-bonds but the larger the halogen atom the stronger the halogen bond. \(^{254}\) They can be increased in strength by increasing the electron-withdrawing nature of the substituents on the aromatic ring. The halogen bond acts to pull the isoindolinone in closer to the protein into a slightly different binding mode to the unsubstituted isoindolinone ‘A’-ring. This may account for differing degrees of activity between unsubstituted and substituted analogues.

![Representation of halogen and hydrogen bonds](Taken from reference 254)

**Figure 79** Representation of halogen and hydrogen bonds formed by a halogen atom (X) and hydrogen atom (H) (Taken from reference 254)

An edge-face stacking interaction is observed between the protons at the 5- and 6-position of the ‘A’-ring and Tyr67 within MDM2, an interaction which would not observed when the 6-
tert-butyl group is in place. The 7-position of the isoindolinone points out into solvent and should prove an interesting area to incorporate solubilising groups.

![Crystal Structure](image)

**Figure 80** The crystal structure demonstrates that the cyclopropyl substituted ether group lies along the surface of the protein

The cyclopropyl group of the ether side-chain was initially incorporated to restrict rotational freedom of the propyl ring. However, the protein crystallography (Figure 80) suggests that the cyclopropyl ring nestles up to the protein, contributing to the binding energy. The hydroxyl group itself points out into solution and does not form an interaction with the protein.

From the crystal structure it is unclear what role the nitro group is fulfilling. A direct interaction is not observed between the protein and the nitro group. Similarly, a clear role for the ethynyl functionality cannot be proposed, although His and Tyr residues are present at the bottom of the pocket, so this may be the ideal environment for the ethynyl group. The preference for the ethynyl over the nitro functionality in 253 and 220 may be a result of the halogen bond. By pulling the isoindolinone ‘A’ ring closer to the protein, the benzyl ring is pulled out of the Leu26 pocket. However, with the longer ethynyl substituent this may be counteracted.
5. Isoindolinone-Based Inhibitors of the MDM2 p53 Protein–protein Interaction

Figure 81 X-ray crystallography of NCL00013774 (left) and Nutlin-3a modelled using CCP4MG

From Figure 81 we can see that the benzyl aromatic ring of 354 does not protrude as deeply into the Leu26 pocket as Nutlin-3a. The ‘lid’ component, which partially covers the aromatic ring of Nutlin-3a does not cover NCL00013774, an area which could potentially be utilised by isoindolinone to give a further increase in potency.

The Tyr23 pocket of MDM2 is filled with a chlorophenyl ring on MDM2 binding. An overlay of a number of inhibitors of the MDM2/p53 interaction suggests that this ring is always filled with a chlorophenyl ring, suggesting the optimal substituent for this position has been identified. The crystal structure of 354 bound to MDM2 will guide the direction of synthetic efforts and a number of potential compounds designed as a result of this crystal structure will be discussed in chapter 5.8.

5.8 Future Work for MDM2/p53

The crystal structure of NCL-00013774 identified a number of areas to address to attempt to improve potency. A series of compounds designed to exploit these areas should result in an improvement in potency. NCL-00013774 was shown to -

- Form a halogen bond with the protein
- Form an edge-face interaction with a tyrosine residue
- Cα position of the isoindolinone points into solution
Halogen bonds and edge-face interactions can be enhanced by an electrodeficient aromatic ring.\textsuperscript{254} As the C\textsuperscript{7} position points out into solution, replacement of the isoindolinone phenyl ring with a pyridine ring may be tolerated, and should strengthen the halogen bond. The inclusion of a pyridine ring will also reduce the clogP from 6.6 for 253 to 5.6 for 357. Compound 357 may prove a useful compound to synthesise.

Figure 82

An ‘isoindolinone’ type molecule in which the pyridine ring replaced the phenyl ring has previously been synthesised from the furo[3,4-b]pyridine-5,7-dione, however no chloro substituted analogue is available. Spaeth \textit{et al} forms the chloro substituted anhydride from 4-chloroquinoline \textit{via} the diacid.\textsuperscript{257} However, formation of the benzoyl benzoic acid from the anhydride allows no control over which isomer is formed. Alternatively, the lead (IV) acetate mediated chemistry could be employed to synthesise the desired isomer of the benzoyl benzoic acid (359) (Scheme 93).

The retrosynthesis of this molecule is shown below. To utilise this synthetic route 4-chloro-2-formyl-3-hydroxypyridine must first be synthesised. Belley \textit{et al} synthesised 4-chloro-2-formyl-3-methoxypyridine from (4-chloro-3-methoxypyrid-2-yl)methanol oxidising the hydroxymethyl group to an aldehyde using Dess-Martin periodinane.\textsuperscript{258} Masaaki \textit{et al} synthesised (4-chloro-3-methoxypyrid-2-yl)methanol (364) from 4-chloro-3-methoxy-2-methylpyridine (366) \textit{via} the N-oxide (365). Treatment of the pyridine N-oxide (354) with acetic anhydride followed by potassium hydroxide can incorporate the hydroxymethyl functionality. Formation of the N-oxide acidifies the alkyl protons, allowing deprotonation with potassium hydroxide. To access 4-chloro-3-methoxy-2-methylpyridine (366), 3-methoxy-2-methylpyridin-4(1\textit{H})-one (367) can be chlorinated using POCl\textsubscript{3} as described by Kohl \textit{et al}.\textsuperscript{259} To synthesise the pyridone ring methyl maltol can be treated with a methylating
agent, followed by heating with ammonia as described by Ma et al will access compound 367.

The proposed synthetic route should allow access to compound 361 from which the lead (IV) acetate mediated chemistry can be utilised to form the desired isomer of the 3-(4-chlorobenzoyl)picinaldehyde. Electron-withdrawing groups have shown to improve the yield of the Pb(OAc)$_4$ mediated rearrangement, suggesting that the electron-deficient nature of the pyridine ring will not hinder rearrangement.

The crystal structure of NCL-00013774 also demonstrates that the isoindolinones fail to exploit the depth of the Leu26 pocket as fully as the Nutlin-type molecules. It was proposed that by replacing the benzyl moiety with phenylethyl moiety, the aromatic ring would sit deeper within the Leu26 pocket, mimicking the Nutlin series. Previously compound 370, failed to improved potency, compound 371 has an IC$_{50}$ of 0.36 µM whilst compound 370 with the addition CH$_2$ unit has an IC$_{50}$ of 3.3 µM. However, with the ‘A’-ring 4-chloro substituent forming a halogen bond, the molecules are expected to have a slightly different binding mode which may result in tolerance for the addition CH$_2$ moiety.
Both compounds 378 and 379 can be synthesised as analogues of the most potent non-nitro compounds. 4-Chloro substituted phenylethyl compound 372 can be purchased and via Buchwald-type Sonogashira reaction can be used to access 375 using chemistry optimised in-house.\textsuperscript{260} This utilises the chemistry developed by Gelman \textit{et al} to couple acetylene-derivative to aromatic chloro species.\textsuperscript{261}

However, the different vector of the substituent due to the addition CH\textsubscript{2} may lead to the aromatic ring not taking the optimal position within the pocket – resulting in a loss of potency. The addition flexibility of the chain may overcome this. The loss of potency that occurs in changing from the ethylene to the propylene substituent on the aromatic ring also suggests that the addition of the CH\textsubscript{2} to the N-benzyl substituent may not result in an increase in potency. However, by synthesising these two compounds 378 and 379 the SAR around the N-benzyl substituent will be developed further.

\textbf{Scheme 94} Proposed reagents and conditions; a) Boc\textsubscript{2}O, Amberlyst, 3 min; b) PdCl\textsubscript{2}(CH\textsubscript{3}CN)\textsubscript{2}, XPhos, CsCO\textsubscript{3}, MeCN, 110 °C, overnight; c) TFA, DCM, 1 h
The isoindolinone compounds 375 and 376 can be synthesised using the previously discussed synthetic route.

\[
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\text{Cl} \\
\text{HO} \\
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\[376 \ X = \text{Cl} \]
\[377 \ X = \text{CCH} \]
\[378 \ X = \text{Cl} \]
\[379 \ X = \text{CCH} \]

**Scheme 95** Proposed reagents and conditions; a) i. SOCl$_2$, DMF, THF, 4 h ii. $X = \text{Cl}$, 369, Hünig’s base, THF, overnight, $X = \text{CCH}$, 375, Hünig’s base, THF, overnight; b) i. SOCl$_2$, DMF, THF, 4 h ii. 1,1-bis(hydroxymethyl)cyclopropane, $K_2\text{CO}_3$, THF, overnight

An overlay of the crystal structures of NCL-00013774 and a number of published inhibitors identified that the spirooxindole series occupies a unique area of the binding pocket, in which a number of water-solubilising groups are located. This area lies between the isoindolinone phenyl ring and the benzyl groups as shown below. However, the published crystal structure of the spirooxindole series is not the same diastereoisomer as the preclinical candidates $2'R,3R,4'S,5'R$ for the crystallised form rather than $2'R,3S,4'R,5'R$. In the preclinical candidates the water-solubilising groups lies in a similar orientation as the Nutlin-3a water solubilising group. However, there are only slight differences in biological activity between the two diastereoisomers.\[^{146}\]

**Figure 84** Crystal structure of an analogue of MI-63
This area of the pocket could be accessed by substituents from the ortho-position of the benzyl ring. To confirm that substituents at this position are tolerated compound 377 could be synthesised. If compound 380 retains potency the alcohol group could provide synthetic access to a range of substituents at this position.

Nelson et al have synthesised (2-(aminomethyl)-5-chlorophenyl)methanol (384) from methyl 5-chloro-2-cyanobenzoate (383) by reducing the ester and nitrile group simultaneously with Zn(BH$_4$)$_2$. Copper cyanide was used to form methyl 5-chloro-2-cyanobenzoate from methyl 2-bromo-5-chlorobenzoate, and the analogous carboxylic acid is commercially available (Scheme 96). Following this synthetic scheme will allow formation of the required benzylamine, from which isoindolinone 380 can be synthesised.
Scheme 96 Proposed reagents and conditions; a) H$_2$SO$_4$, MeOH; b) CuCN, DMF; c) 1 M BH$_3$.THF in THF

Compounds 357, 378, 379 and 380 can act as a series of probes investigated areas suggested by the crystal structure of NCL-00013774. This will develop the SAR further for the isoindolinones, and potentially lead to a further increase in potency and improve physical and chemical properties.

5.9 p53/MDM2 Assay Details

Biological testing of compounds was undertaken by bioscientists at the NICR using an ELISA assay.$^{190}$ A 96-well plate was incubated overnight at 96 °C with streptavidin in a coating buffer. Plates were then rinsed 5 times with dissociation enhanced lanthanide immunoassay buffer, then incubated for 3 h at room temperature with a saturation buffer to block non-specific protein binding. After removal, plates were dried in sterile fumehood at room temperature before incubating at 4 °C for 1 h with biotinylated IP3 peptide$^{263}$ in 0.05% DMSO in PBS buffer, then plates were washed three times with PBS.

For initial testing compounds and controls were plated out in triplicate to give final concentrations of 500 µM, 100 µM and 20 µM. Controls were 5% DMSO as a negative control and 100 nM active peptide as a positive control.$^{264}$ Compounds and controls that were aliquoted in 96-well plate were preincubated with for 20 min at 20 °C with 190 µL aliquots of concentrated of in vitro translated MDM2, then transferred to the streptavidin coated plates and incubated at 4 °C for 90 min. Plates were then washed three times with PBS buffer, incubated for 1 h at 20 °C with TBS-Tween buffered solution of mouse monoclonal anti-MDM2 antibody, then washed three times with TBS-Tween solution, then incubated for 45 min at 20 °C with goat-antimouse house radish peroxidise conjugate secondary antibody. Unbound secondary antibody was removed by washing three times with TBS-Tween...
solution. HRP activity measured using oxidation of diacylhydrazide substrate luminal. The IC\textsubscript{50} were calculated using plot of % MDM2 inhibition versus concentration.\textsuperscript{190}

5.10 Conclusion

The p53/MDM2 protein-protein interaction is an important anti-cancer target. Isoindolinones have proved to be a valuable scaffold for the development of small molecules targeting the p53/MDM2 interaction, allowing elaboration of the scaffold in a number of positions. Modifications to the isoindolinone have led to a significant increase in potency from inhibitors with activity in the micromolar range to low nanomolar range.

The lead compound NCL-00008406 contains an undesirable nitro substituent on the benzyl group, however, synthetic efforts have identified appropriate replacements for the nitro group, which either improve or maintain activity against MDM2. The SAR around the benzyl group has been shown to be very narrow, substitution at the 3-position of the benzyl ring is only tolerated with small substituents, such as a fluoro group. Substituents at the 4-position of the benzyl ring have also demonstrated that the benzyl ring sits close to the bottom of the pocket. An ethylene group is tolerated, but addition of a terminal methyl group to the ethylene group is not tolerated, suggesting that this substituent is too large. The SARs around the isoindolinone A-ring have been developed and demonstrate that a number of substituents around the A-ring confer activity. Further investigation is required. Synthesis of an alternative ether chain, incorporating an oxetane ring has also shown to be tolerated.

An alternative regioselective synthesis of the important benzoic acid intermediate has also been developed which is proving more versatile in the synthesis of new molecules. Although adding a number of steps to the synthetic route, the new synthetic route results in only the desired isomer and the additional steps are mostly trivial and high yielding.

The crystal structure of an isoindolinone bound to MDM2 has been solved recently. This will guide the synthesis of more potent molecules, as well as allowing the incorporation of groups to improve the properties to the molecules, without disrupting the interaction with MDM2.

Several potent inhibitors of the MDM2/p53 interaction have been published, and the biological activity of lead isoindolinone compounds is comparable to those in the public
Isoindolinone-Based Inhibitors of the MDM2 p53 Protein-protein Interaction

domain. Interesting, biological studies have suggested that both Nutlin-3a and MI-63 are both substrates for PGP transporters, whereas isoindolinones series are not. It was demonstrated that isoindolinones induced p21 in a cell-line in which PGP is upregulated, unlike Nutlin-3a and MI-63 which failed to induce p21.\textsuperscript{265} Zhang \textit{et al} have also observed that Nutlin-3a is an inhibitor of the breast cancer resistance protein efflux pump, by inhibition of the ATPase activity of BCRP.\textsuperscript{266} Nutlin-3a was shown to inhibit the efflux of the anti-cancer agent Mitoxantrone. The inhibition of efflux-pumps may impact on the pharmacokinetic, pharmacodynamic and safety profile of any anti-cancer agents which are substrates for efflux pumps. As isoindolinones do not appear to be either a target of efflux pumps or an inhibitor of efflux pumps, these safety fears can be negated.

Isoindolinones have proved a versatile scaffold for the development of inhibitors of the MDM2/p53 protein-protein interaction. Synthetic efforts have successfully identified a replacement for the toxic nitro group of the lead compound NCL-00008406. The SAR has also been developed further around the isoindolinone ‘A’-ring and an alternative ether chain has been identified. The recent solving of the crystal structure of NCL-00013774 will guide further synthetic effort and the incorporation of groups to improve solubility. Isoindolinones are not substrates for cellular transporters which is unique amongst potent inhibitors of the p53/MDM2 interaction.
6. Overall Conclusion

Anti-cancer drug discovery has moved away from the development of broad spectrum cytotoxic agents towards the development of targeted agents, inhibiting a number of targets including receptors, kinases and protein-protein interactions. The drug discovery process entails a number of steps, and medicinal chemists are involved in a number of these steps, including hit and lead identification, hit validation and lead optimisation. The importance of the compound series has been demonstrated by the synthetic efforts towards the development of small molecule inhibitors of both mTOR and MDM2.

The pyrimidine-based series was shown to inhibit mTOR after a small screen of compounds. The molecules were originally developed as CDK2 inhibitors, so had modest activity against both mTOR and CDK2, and the pyrimidine heterocycle was already substituted at all available position around the pyrimidine. Design of a potent and selective inhibitor of mTOR required both the optimisation of mTOR activity and reduction of CDK2 activity around a ring which was already optimally substituted.

Alternatively, the isoindolinone series of inhibitors were identified as having cellular inhibitory activity and were then identified as inhibitors of the MDM2/p53 interaction, ensuring from the outset that compounds had cellular activity. As the isoindolinone scaffold was largely unsubstituted wide-ranging SAR studies have been undertaken to identify optimal substituents and position. As the isoindolinone compound were not part of a compound series directed at an alternative biological target, efforts were solely directed towards identification of active compounds.

The importance of hit compounds within the drug discovery process has lead to the development of a wide-range of techniques for the identification of compounds with activity against a particular biological target. Early techniques such as the use of natural compounds, screens of existing compounds and a me-too approach are being joined by more innovative techniques such as fragment-based drug discovery and computer-based techniques, making the drug discovery process increasingly efficient.
7. Experimental

Commercially available starting materials were purchased from fine chemical vendors and used as purchased unless otherwise stated. \(N,N,N,N\)-tetramethylethylenediamine was distilled over potassium hydroxide before use. Anhydrous THF, DMF, methanol, ethanol, DCM, acetonitrile and pyridine were obtained from Aldrich in SureSeal™ bottles or Acros in Acroseal™. Petrol refers to petroleum ether with the boiling range of 40-60 °C. All reactions, unless otherwise stated were carried out under an inert atmosphere of nitrogen or argon. Palladium and rhodium catalysed reactions were degassed by bubbling \(N_2\) through the reaction for the specified length of time.

All microwave assisted synthesis was performed using an Initiator Sixty Biotage apparatus. Hold temperature mode was used in all experiments.

LC-MS spectra were recorded either using a Micromass Platform LC in combination with a Waters 996 Photodiode Array Detector, a Waters 600 Controller and a Waters 2700 Sample Manager. Separation was achieved on a Waters Symmetry C18 column (4.6 x 20 nm) or Waters Atlantis C18 column (4.6 x 50 nm) using gradient elution with (A) 0.1% aqueous formic acid and (B) acetonitrile. The gradient used was A: B 95:5 to 5:95 over 3.5 min for the Symmetry column and 4.0 min for the Atlantis column. The Symmetry column had an overall time of 5.00 min and is referred to as (1) within experimental procedures. The Atlantis column has an overall time of 12.00 min and is referred to as (2) within experimental procedures. Alternatively, samples were run using a Waters Acquity UPLC system with PDA and ELSD. Separation was achieved using an Acquity UPLC BEH C18, 1.7 µM, 2.1 50 mm column. With a mobile phase of (A) 0.1% v/v aqueous formic acid and (B) 0.1% formic acid in acetonitrile A:B 95:5 to 5:95 over 1.5 min, overall times were either 2.00 min (3), 2.50 min (4) or 3.00 min (5) achieved with a range of hold times.

Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F\textsubscript{254} or NH\textsubscript{2}F\textsubscript{254s} plates (amino silica) with aluminium backing and was visualised with ultraviolet (UV) light or potassium permanganate. Column chromatography was performed either on Davisil silica gel 40-63 µm or on automated medium pressure chromatography apparatus, a Biotage SP4 system running a graduated system as
specified with either Biotage flash prepacket column KP-Sil, KP-NH (amino silica) and KP-C18 or Agilent Si 50 or 35 and NH (amino silica). Chromatography refers to medium pressure column chromatography or Biotage SP4 under the given conditions unless otherwise stated.

Chiral semi-preparative HPLC was performed using a Varian Prostar instrument equipped with a Daicel Chiralpak AD-H250 x 10mm column eluting with solvent (as specified), at a flow rate of 4.0 mL/min, monitoring by UV at λ = 254 nm. Analytical HPLC was performed using Waters X Terra RP185 μm (4.6 x 150 mm) column at 1 mL/min, with either (A) 0.1% aqueous ammonia and acetonitrile (B) 0.1% aqueous formic acid and acetonitrile (B), with a gradient of 5-100% acetonitrile over 25 min with the lowest purity quoted.

NMR spectra were recorded on a Bruker Spectrospin AC 300E spectrometer (1H at 300 MHz, 13C at 75 MHz) or using a Bruker Ultrashield 500 plus (1H at 500 MHz, 13C at 125 MHz) with CDCl3, d6-DMSO or d3-MeCN as the solvent. Chemical shifts (δH) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad); or combinations thereof. Coupling constants (J) were measured in Hertz (Hz).

Melting points were measured on either Stuart Scientific melting point apparatus (SMP3) or Stuart automatic melting point apparatus (SMP40) and are uncorrected.

IR spectra were recorded on a Bio-Rad FTS 3000MX diamond ATR as a neat sample. UV spectra were recorded on a Hitachi U-2800A spectrophotometer and were performed in ethanol.

High resolution mass spectra were performed by the EPSRC National mass spectrometry service, University of Wales, Swansea, Singleton Park, Swansea, SAZ 8PP. Elemental analyses were performed by The School of Pharmacy, Analytical Facility, University of London, WC1N 1AX.
7.1 mTOR Experimental Procedures

4-(Cyclohexylmethoxy)pyrimidine-2,6-diamine (55)

Sodium (0.40 g, 17 mmol, 1.3 eq) was added, in portions, to cyclohexylmethanol (20.0 mL, 189 mmol, 14 eq) and the mixture was heated to 160 °C until the sodium had dissolved. 4-Chloro-2,6-diaminopyrimidine (2.00 g, 13.8 mmol, 1 eq) was added to the solution and the temperature was increased to 180 °C. After 90 min the reaction mixture was cooled to room temperature and neutralised with acetic acid. Methanol (10 mL) was added and the solution was allowed to cool to 4 °C and then left overnight. The resulting white solid was filtered and recrystallised (methanol).

Additional precipitation of crude product was brought about by the addition of methanol to the remaining crude oil to give 55 as a white powder (0.55 g, 20%). R_f = 0.38 (100% EtOAc); mp = 137.5-138.8 °C (lit. 142 °C)\textsuperscript{147}; UV \lambda_{max} (EtOH): 266.5 nm; IR \upsilon_{max} 3344 (NH), 2920, 2850, 1589 cm\textsuperscript{-1}; \delta_H (500 MHz d<sub>6</sub>-DMSO): 0.93-1.00 (2H, m, C<sub>6</sub>H<sub>11</sub>), 1.34-1.23 (3H, m, C<sub>6</sub>H<sub>11</sub>), 1.63-1.73 (6H, m, C<sub>6</sub>H<sub>11</sub>), 3.88 (2H, d, J = 6.6 Hz, CH<sub>2</sub>), 5.04 (1H, s, H<sub>6</sub>), 5.83 (2H, s, N<sub>1</sub>H<sub>2</sub>), 5.99 (2H, s, N<sub>2</sub>H<sub>2</sub>); \delta_C (125 MHz, d<sub>6</sub>-DMSO): 25.2 (C<sub>6</sub>H<sub>11</sub>), 26.0 (C<sub>6</sub>H<sub>11</sub>), 29.3 (C<sub>6</sub>H<sub>11</sub>), 36.9 (CCH<sub>2</sub>O), 69.7 (OCH<sub>2</sub>), 76.0 (C<sup>5</sup>), 162.9 (C<sup>2</sup>), 165.9 (C<sup>6</sup>), 170.3 (CO); LCMS R<sub>t</sub> = 2.31 min, (1); MS (ESI+) m/z 223.15 [M+H]<sup>+</sup>

4-Cyclohexylmethoxy-5-nitrosopyrimidine-2,6-diamine (56)

4-(Cyclohexylmethoxy)pyrimidine-2,6-diamine (100 mg, 0.45 mmol, 1 eq) was dissolved in acetic acid (30% in water, 5 mL) at 80 °C. Sodium nitrite solution (40 mg in 0.8 mL of water, 0.58 mmol, 1.3 eq) was added dropwise to the solution, resulting in a purple solution being formed instantly which was stirred at 80 °C for 40 min and then cooled to room temperature. The resulting purple precipitate was filtered and recrystallised in ethanol to give a purple powder (97 mg, 86%). R<sub>f</sub> = 0.41 (15:85
7. mTOR Experimental Procedures

MeOH:DCM); mp = 247.8-248.5 °C (lit. 254 °C)\textsuperscript{147}; UV $\lambda_{\text{max}}$ (EtOH): 334.5, 235.0 nm; IR $\nu_{\text{max}}$ 3499 (NH), 3161, 1570, 1508 (NO), 1262 cm\textsuperscript{-1}; $\delta_{\text{H}}$ (300 MHz $d_6$-DMSO): 0.99-1.33 (5H, m, C$_6$H$_{11}$), 1.64-1.86 (6H, m, C$_6$H$_{11}$), 4.29 (2H, d, $J = 6.3$ Hz, CH$_2$) 7.77 (1H, s, N$_1$H$_2$), 7.82 (1H, s, N$_1$H$_2$), 8.00 (1H, s, N$_2$H/$H$), 10.09 (1H, s, N$_2$H/$H$); $\delta_{\text{C}}$ (75 MHz $d_6$-DMSO): 25.5 (C$_6$H$_{11}$), 26.3 (C$_6$H$_{11}$), 29.5 (C$_6$H$_{11}$), 37.2 (CCH$_2$O), 71.9 (CH$_2$O), 140.1 (C$^6$), 164.0; LCMS $R_t = 2.94$ min, (1); MS (ESI+) m/z 252.20 [M+H]$^+$

5-Bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-diamine (66)

![5-Bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-diamine](image)

To a solution of 4-(cyclohexylmethoxy)-pyrimidine-2,6-diamine (300 mg, 1.35 mmol, 1eq) in acetic acid (5.0 mL, 87 mmol, 64 eq) was added N-bromosuccinimide (240 mg, 1.35 mmol, 1 eq) and the mixture heated to 60 °C for 1 h then and diluted with water (10 mL) giving a white suspension and neutralised with 2.5M aqueous NaOH solution. The resulting white precipitate was filtered and redissolved in DCM (20 mL). The solution was dried over Na$_2$SO$_4$ and evaporated in vacuo. Chromatography (silica, 15% MeOH, DCM) gave 66 as a white solid (326 mg, 80 %). $R_t = 0.58$ (100% EtOAc); mp = 193.7 – 194.5 °C (193-194 °C)\textsuperscript{148}; UV $\lambda_{\text{max}}$ (EtOH): 338.5, 327.5, 273.5, 233.5 nm; IR $\nu_{\text{max}}$ 3497 (NH), 3472, 1053 cm\textsuperscript{-1}; $\delta_{\text{H}}$ (300 MHz $d_6$-DMSO): 0.93-1.29 (5H, m, C$_6$H$_{11}$), 1.62-1.72 (6H, m, C$_6$H$_{11}$), 3.98 (2H, d, $J = 6.3$ Hz, CH$_2$), 6.09 (2H, s, N$_1$H$_2$), 6.25 (2H, s, N$_1$H$_2$); $\delta_{\text{C}}$ (75 MHz $d_6$-DMSO) 25.6 (C$_6$H$_{11}$), 26.4 (C$_6$H$_{11}$), 29.5 (C$_6$H$_{11}$), 37.3 (CCH$_2$O), 71.1 (CH$_2$O) 161.5 (CNH$_2$), 162.2 (CNH$_2$), 165.4 (CO); LCMS $R_t = 2.99$ min, (1); MS (ESI+) m/z 301.23 [M+H]$^+$

$N,N'-$-(5-Bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-diyl)diacetamide (67)

![5-Bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-diamine](image)

5-Bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-diamine (200 mg, 0.66 mmol, 1 eq) was added to a mixture of acetic acid (2.5 mL, 44 mmol, 66 eq) and acetic anhydride (2.5 mL, 26 mmol, 40 eq) under nitrogen. The solution was heated to reflux overnight
resulting in a colour change from yellow to pale brown, then cooled to room
temperature and poured into water (10 mL), resulting in a white precipitate,
neutralised with concentrated ammonia and extracted into ethyl acetate (3 x 20 mL).
The combined organic layers were then washed with NaHCO₃ (3 x 30 mL) and then
water (3 x 30 mL) dried over Na₂SO₄, filtered and evaporated in vacuo. Recrystallised
(acetonitrile) gave 67 as a white solid (135 mg, 53%). Rᶠ = 0.5 (100% EtOAc); mp =
178.4-178.8 °C (lit. 179-182)¹⁴⁸, UV λ max (EtOH): 388.0, 228.0, 205.5 nm; IR ν max
3389 (NH), 1671 (C=O), 1258, 1111, 1067, 1036 cm⁻¹; δ H (300 MHz, d₆-DMSO):
0.98-1.31 (5H, m, C₆H₁₁), 1.69-1.79 (6H, m, C₆H₁₁), 2.17 (3H, s, COCH₃), 2.20 (3H,
s, COCH₃), 4.20 (2H, d, J = 6.3 Hz, CH₂), 9.98 (1H, s, NH), 10.48 (1H, s, NH); δ C (75
MHz d₆-DMSO): 24.2 (CH₃), 25.1 (CH₃), 25.5 (C₆H₁₁), 26.3 (C₆H₁₁), 29.4 (C₆H₁₁),
37.1 (CCH₂O), 73.0 (CH₂O), 157.5 (C-N), 166.9 (CO), 169.5 (C=O), 169.6 (C=O);
LCMS Rᵗ = 3.21 min, (1); MS (ESI+) m/z 385.35 [M+H]+

2,6-Diamo-4-(cyclohexylmethoxy)pyrimidine-5-carbonitrile (59)

To a solution of N,N’-(5-bromo-4-(cyclohexylmethoxy)pyrimidine-2,6-
diyldiacetamide (100 mg, 0.26 mmol, 1 eq) in DMF (5 mL) CuCN (28 mg, 0.31
mmol, 1.2 eq) was added, the mixture was heated to reflux for 5 h, then cooled to
room temperature, ethylenediamine (1.4 mL, 20 mmol, 77 eq) was added and stirred
overnight at room temperature. The mixture was filtered through Celite and washed
with DMF (2 x 5 mL), water (10 mL) was added to the filtrate and the product
extracted into ethyl acetate (3 x 20 mL). The combined organic layers were washed
with brine (50 mL), dried over Na₂SO₄ and evaporated under vacuum.
Recrystallisation (ethyl acetate / petrol) gave 59 as cream crystals (45 mg, 70%). Rᶠ =
0.64 (100 % EtOAc); mp = 187.5 – 188.3 °C (lit. 186-188 °C)¹⁴⁸; UV λ max (EtOH):
367.5, 267.0, 251.5, 234.5 nm; IR ν max 3512 (NH), 3381, 2210 (CN), 1549, 1226 cm⁻¹;
δ H (300 MHz d₆-DMSO): 0.96-1.26 (5H, m, C₆H₁₁), 1.63-1.75 (6H, m, C₆H₁₁), 4.06
(2H, d, J = 6.3 Hz, CH₂), 6.90 (2H, d, J = 15.3 Hz, N₁H₂) 7.95 (2H, s, N₂H₂); δ C (75
MHz d₆-DMSO): 25.5 (C₆H₁₁), 26.3 (C₆H₁₁), 29.4 (C₆H₁₁), 35.2 (CCH₂O), 71.1
(CH₂O), 163.8 (CNH₂), 166.3 (CNH₂), 177.9 (CO)
4-Ethoxypyrimidine-2,6-diamine (385)

\[
\begin{align*}
\text{O} & \\
\text{H}_2\text{N} & \\
\text{N} & \\
\text{N} & \\
\text{NH}_2 & \\
\end{align*}
\]

Ethanol (15 mL, 260 mmol, 19 eq) was added slowly to sodium (0.50 g, 22 mmol, 1.5 eq). Once the gas evolution had stopped the solution was heated to 80 °C until all the sodium had dissolved. 4-Chloro-2,6-diaminopyrimidine (2.00 g, 13.8 mmol, 1 eq) was added and the temperature increased to 98 °C for 5 h before cooling to room temperature, neutralising with acetic acid and evaporated *in vacuo*. The residue was suspended in water and extracted into ethyl acetate (3 x 20 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered and evaporated *in vacuo* resulting in cream crystals. Recrystallised (ethyl acetate /petrol) gave 385 as a white solid (1.25g, 58%). \(R_f = 0.44\) (1:9 MeOH:EtOAc); mp = 134.7 – 136.1 °C, (lit. 167-169 °C)\(^{148}\); UV \(\lambda_{\text{max}}\) (EtOH): 366.5, 284.5, 279.5, 267.0, 204.0 nm; IR \(\nu_{\text{max}}\) 3426 (NH), 1344, 1208, 1040 cm\(^{-1}\); \(\delta_{\text{H}}\) (300 MHz, \(d_6\)-DMSO): 1.21 (3H, t, \(J = 7.0\) Hz, CH$_3$), 4.12 (2H, q, \(J = 7.0\) Hz, CH$_2$), 5.01 (1H, s, H$^5$), 5.85 (2H, s, N$^1$H$_2$), 5.99 (2H, s, N$^2$H$_2$); \(\delta_{\text{C}}\) (75 MHz \(d_6\)-DMSO): 16.0 (CH$_3$), 61.5 (CH$_2$), 77.8 (C$^5$), 167.4 (CNH$_2$), 171.5 (CNH$_2$), 173.1 (CNH$_2$); LCMS \(R_t = 2.16\) min, (2); MS (ESI+) \(m/z\) 155.20 [M+H]$^+$

5-Bromo-6-ethoxypyrimidine-2,6-diamine (386)

\[
\begin{align*}
\text{O} & \\
\text{H}_2\text{N} & \\
\text{N} & \\
\text{N} & \\
\text{NH}_2 & \\
\text{Br} & \\
\end{align*}
\]

To a solution of 4-ethoxypyrimidine-2,6-diamine (1.00 g, 6.49 mmol, 1eq) in acetic acid (8.0 mL, 140 mmol, 22 eq) was added N-bromosuccinimide (1.17 g, 6.57 mmol, 1 eq) and the solution heated to 60 °C for 90 min, cooled to room temperature, diluted with water (45 mL) and neutralised with 2.5 M aqueous NaOH solution. Solution then extracted with ethyl acetate (3 x 50 mL) and combined organic layers were then dried over Na$_2$SO$_4$, filtered and evaporated *in vacuo*. Recrystallisation (methanol) to give 386 as a pale yellow powder which was then dried over P$_2$O$_5$ before further use (0.724 g, 48%). \(R_f = 0.35\) (100% EtOAc); mp = 179.7-180.5 °C; UV \(\lambda_{\text{max}}\) (EtOH): 353.5, 275.5, 235.5 nm; IR \(\nu_{\text{max}}\) 3426 (NH), 3344, 1206, 1047 cm\(^{-1}\); \(\delta_{\text{H}}\) (300 MHz, \(d_6\)-DMSO): 1.25 (3H, t, \(J = 7.1\) Hz, CH$_3$), 4.23 (2H, q, \(J = 7.1\) Hz, CH$_2$) 6.09 (2H, s,
N,N’-(5-Bromo-4-ethoxypyrimidine-2,6-diyldiacetamide (387)

A solution of 5-bromo-6-ethoxypyrimidine-2,6-diamine (500 mg, 2.15 mmol, 1 eq) in acetic acid (5.5 mL, 96 mmol, 45 eq) and acetic anhydride (7.5 mL, 68 mmol, 32 eq) was heated to reflux for 48 h, then cooled to room temperature, diluted with water (20 mL) and neutralised with concentrated ammonia, and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were washed with saturated NaHCO₃ (3 x 50 mL) followed by water (3 x 50 mL) and dried (Na₂SO₄). Recrystallised (methanol) gave 387 as off-white solid (0.19 g, 27%). Rᵣ = 0.48 (100% EtOAc); mp = 181.4 – 182.8 °C; UV λₓₜₙₐₓ (EtOH): 353.0, 256.0, 227.5, 207.0 nm; IR νₜₐₓₐₘₑₐₓ (300 MHz, d₆-DMSO): 1.35 (3H, t, J = 7.1, Hz, CH₂CH₃), 2.17 (3H, s, COCH₃), 2.21 (3H, s, COCH₃), 4.44 (2H, q, J =7.0 Hz, CH₂CH₃), 9.96 (1H, s, N¹H), 10.49 (1H, s, N²H); δC (75 MHz d₆-DMSO): 14.5 (CH₂CH₃), 24.1 (CH₃), 25.1 (CH₃), 64.3 (CH₂), 155.2 (CNH₂), 157.5 (CNH₂), 166.7 (CO), 169.4 (C=O), 169.6 (C=O); LCMS Rᵣ = 6.55 min, (2); MS (ESI+) m/z 247.07 [M+H]+

2,6-Diamino-4-ethoxypyrimidine-5-carbonitrile (81)

To a solution of N,N’-(5-bromo-4-ethoxypyrimidine-2,6-diyldiacetamide (110 mg, 0.35 mmol, 1 eq) in DMF (5 mL) was CuCN (48 mg, 0.54 mmol, 1.5 eq). The resulting green solution was heated to reflux for 3.5 h the solution before cooling to room temperature and ethylene diamine (2.5 mL, 37 mmol, 106 eq) added. The dark blue solution was stirred overnight, filtered through Celite and washed with further
DMF (8 mL). Water (25 mL) was added to the filtrate and the product was extracted into ethyl acetate (3 x 50 mL), dried over Na₂SO₄ and concentrated in vacuo. Chromatography (silica, 100% EtOAc) followed by recrystallisation (methanol) gave 81 as an off-white solid (0.047 g, 75%). Rf = 0.66 (100% EtOAc); mp = 226.7-227.1 °C; UV λmax (EtOH): 251.5, 216.5 nm; IR vmax 3433 (NH), 3372, 3093, 2199 (CN), 1668, 1615, 1539 cm⁻¹; δH (300 MHz, d₆-DMSO): 1.27 (3H, t, J=7.1 Hz, CH₃), 4.30 (2H, q, J=7.1 Hz, CH₂), 6.88 (2H, s, N¹H₂), 6.93 (2H, s, N²H₂); δC (75 MHz d₆-DMSO): 14.8 (CH₃), 62.2 (CH₂), 116.1 (CN), 163.4 (CNH₂), 166.3 (CNH₂), 171.2 (CO); LCMS Rf = 1.29 min, (1); MS (ESI+) m/z 180.12 [M+H]+; HRMS m/z: Calc. for C₇H₁₀N₅O: 180.0880 [M+H]+. Found 180.0880 [M+H]+. Analytical HPLC: 98.2% purity

4-Isopropoxypyrimidine-2,6-diamine (80)

To isopropanol (4.8 mL, 62 mmol, 18 eq) under constant flushing with nitrogen was gradually added sodium (0.13 g, 5.65 mmol, 1.6 eq). Once all gas evolution had stopped the solution was heated at 50 °C until all sodium had dissolved. 4-Chloro-2,6 diaminopyrimidine (0.50 g, 3.46 mmol, 1 eq) was added, which was sealed and irradiated for 20 min at 160 °C, then neutralised using acetic acid and evaporated. The resulting white solid was suspended in water (20 mL) and extracted with ethyl acetate (3 x 20 mL) and the resulting organic layers were dried over Na₂SO₄ and evaporated. Recrystallisation (ethyl acetate /petrol) gave 80 as a white solid (0.39 g, 68%). Rf = 0.31 (100% EtOAc); mp = 112.8-113.0 °C; UV λmax (EtOH): 379.0, 267.0 nm; IR vmax 3356 (NH), 1317, 1208 cm⁻¹; δH (300 MHz d₆-DMSO): 1.19 (6H, d, J = 6.2 Hz, 2 x CH₃), 4.97 (1H, s, H⁵), 5.10 (1H, septet, J = 6.2 Hz, CH) 5.81 (2H, s, N¹H₂), 5.94 (2H, s, N²H₂); δC (75 MHz d₆-DMSO): 22.4 (CH₃), 66.7 (OCH), 77.5 (C⁵), 163.4 (CNH₂), 166.4 (CNH₂), 172.0 (CO); LCMS Rf = 4.59 min, (2); MS (ESI+) m/z 169.21 [M+H]+
5-Bromo-4-isopropoxypyrimidine-2,6-diamine (388)

To a solution of 4-isopropoxypyrimidine-2,6-diamine (250 mg, 1.49 mmol, 1 eq) in acetic acid (5 mL, 87 mmol, 59 eq) was added N-bromosuccinimide (265 mg, 1.49 mmol, 1 eq), and the mixture was stirred at RT for 45 min, then diluted with water (10 mL) and neutralised using 2.5 M aqueous solution of NaOH. The resulting white precipitation was filtered, redissolved in methanol (15 mL), dried over Na₂SO₄, and evaporated. Recrystallisation (methanol) gave 388 as a white solid (21 mg, 58%). Rᵣ = 0.74 (100% EtOAc); mp = 160.9 – 161.2 °C; UV λₘₐₓ (EtOH): 379.0, 274.5, 232.0, 205.0 nm; IR νₘₐₓ 3426 (NH), 1335, 1206, 1047 cm⁻¹; δ_H (300 MHz d₆-DMSO): 1.24 (6H, d, J=6.2 Hz, CH₃), 5.19 (1H, septet, J = 6.3 Hz, CH) 6.05 (2H, s, N¹H₂), 6.23 (2H, s, N²H₂); δ_C (75 MHz d₆ DMSO) 29.9 (CH₃), 73.2 (OCH), 161.6 (CNH₂), 162.3 (CNH₂), 179.5; LCMS Rᵣ = 6.20 min, (2); MS (ESI+) m/z 247.10 [M+H]⁺; CHN Calculated C 34.03 H 4.49 N 22.67 Found C 34.65 H 4.18 N 21.78

N,N’-(5-Bromo-4-isopropoxypyrimidine-2,6-diyl)diacetamide (389)

To a solution of 5-bromo-4-isopropoxypyrimidine-2,6-diamine (150 mg, 0.61 mmol, 1 eq) in acetic acid (2.5 mL, 44 mmol, 72 eq) was added acetic anhydride (5 mL, 53 mmol, 86 mmol). The mixture heated to 160 °C for 16 h followed by 170°C for a further 8 h, diluted with water (10 mL) and neutralised with concentrated ammonia. The solution was then extracted into ethyl acetate (3 x 20 mL). The combined organic layers washed with saturated NaHCO₃ solution (1 x 50 mL) dried over Na₂SO₄, evaporated in vacuo. The residue was redissolved in petrol and evaporated to give 389 as a cream solid (184 mg, 91%). Rᵣ = 0.71 (100% EtOAc); mp = 174.4 – 174.9 °C; UV λₘₐₓ (EtOH): 367.0, 254.5, 227.5, 204.0 nm; IR νₘₐₓ 3375 (NH), 1669 (C=O), 1313, 1262, 1221, 1096 cm⁻¹; δ_H (300 MHz, d₆-DMSO): 2.69 (6H, d, J = 6.3 Hz,
7. mTOR Experimental Procedures

CH(CH₃)₂, 2.15 (3H, s, COCH₃), 2.20 (3H, s, COCH₃), 5.31 (1H, septet, J = 6.3 Hz, CH), 10.0 (1H, s, N₁H), 10.45 (1H, s, N²H); δC (75 MHz d₆-DMSO): 22.0 (COCH₃), 22.3 (COCH₃), 25.0 (CH(CH₃)₂), 71.7 (OCH), 99.6 (C⁵), 157.7 (C²), 166.8 (C⁶), 168.5 (CO), 169.5 (C=O), 169.9 (C=O); LCMS Rₜ = 3.01 min, (1), MS (ESI+) m/z 331.22 [M+H]⁺

2,6-Diamino-4-isopropoxypyrimidine-5-carbonitrile (82)

![Structure of 2,6-Diamino-4-isopropoxypyrimidine-5-carbonitrile (82)](image)

To a solution of N,N’-(5-bromo-4-isopropoxypyrimidine-2,6-diyl)diacetamide (200 mg, 0.60 mmol, 1 eq) in DMF (5 mL) was added CuCN (73 mg, 0.81 mmol, 1.4 eq). The reaction mixture was heated at 120 °C for 2 h, to room temperature and ethylenediamine (5 mL, 75 mmol, 125 eq) added and the mixture stirred overnight. The resulting blue solution was filtered through Celite, washed with DMF (10 mL) and diluted with water (50 mL). Solution was extracted with ethyl acetate (3 x 70 mL). The combined organic extracts were washed with brine (150 mL), dried (Na₂SO₄) and evaporated in vacuo. Chromatography (silica, 10% MeOH, EtOAc) followed by (SP4, silica, 15-100% EtOAc, PE). Final purification using HPLC (0.1% NH₃ aqueous solution: acetonitrile 75:25) to give 82 as a white solid (29 mg, 25%). Rₜ = 0.64 (1:9 MeOH:EtOAc); mp = 190.5-190.8 °C; UV λₘₐₓ (EtOH): 267.0, 252.0, 217.5 nm; IR νₘₐₓ 3381 (NH), 3129, 2208 (CN), 1669, 1614 cm⁻¹; δH (300 MHz, d₆-DMSO): 1.26 (6H, d, J = 5.1 Hz, CH₃), 5.27 (1H, septet, J = 6.3 Hz, CH), 6.84 (2H, s, N¹H₂), 6.89 (2H, s, N²H₂); δC (75 MHz, d₆-DMSO): 22.2 (CH(CH₃)₂), 69.2 (OCH(CH₃)₂), 116.2 (CN), 163.5 (C⁵), 166.4 (C⁶), 170.9 (CO); LCMS Rₜ = 2.59 min, (1); MS (ESI+) m/z 194.04 [M+H]⁺; HRMS m/z: Calc. for C₈H₁₂N₅O: 194.1036 [M+H]⁺. Found 194.1035 [M+H]⁺. Analytical HPLC: 99.7% purity

4-sec-Butoxypyrimidine-2,6-diamine (390)

![Structure of 4-sec-Butoxypyrimidine-2,6-diamine (390)](image)

226
To sodium (0.45 g, 19.6 mmol, 1.4 eq) under nitrogen was slowly added sec-butanol (18 mL, 196 mmol, 14 eq) and heated to 120 °C and until all sodium has dissolved. 4-Chloro-2,6-diaminopyrimidine (2.00 g, 13.8 mmol, 1 eq) was added and the mixture heated to reflux for 5.5 hours, then cooled to room temperature, neutralised with acetic acid and concentrated in vacuo. Crude was suspended in water (20 mL) and extracted into ethyl acetate (3 x 30 mL), combined organic layers were dried over Na$_2$SO$_4$, concentrated in vacuo resulting in a yellow solid. This crude product was recrystallised (ethyl acetate /petrol) to give 390 as a cream solid (1.20 g, 48%). $R_f = 0.44$ (100% EtOAc); mp = 92.7-93.1 °C; UV $\lambda_{\text{max}}$ (EtOH): 265.5, 234.5, 206.0 nm; IR $\nu_{\text{max}}$ 3356 (NH), 1315, 1208, 1098 cm$^{-1}$; $\delta_H$ (300 MHz $d_6$-DMSO): 0.86 (3H, t, $J = 7.4$, CH$_2$CH$_3$), 1.16 (3H, d, $J = 6.2$ Hz, CHCH$_3$), 1.47 -1.62 (2H, m, CH$_2$), 4.88- 4.98 (1H, m, OCH), 5.01 (1H, s, $H_5$), 6.00 (2H, s, NH$_2$), 6.12 (2H, s, NH$_2$); $\delta_C$ (75 MHz $d_6$-DMSO): 9.8 (CH$_3$CH$_2$), 18.9 (CH$_3$CH), 29.0 (CH$_3$CH$_2$), 71.3 (OCH$_2$), 77.4 (C$^5$), 163.4 (CNH$_2$), 166.5 (CNH$_2$), 170.5 (CO); LCMS $R_t$ = 5.39 min, (2), MS (ESI+) m/z 183.18 [M+H]$^+$

5-Bromo-4-sec-butoxypyrimidine-2,6-diamine (391)

To a solution of 4-sec-butoxypyrimidine-2,6-diamine (250 mg, 1.37 mmol, 1 eq) in acetic acid (5 mL, 87 mmol, 64 eq), was added N-bromosuccinimide (249 mg, 1.39 mmol, 1 eq). After 15 min of stirring at room temperature the yellow solution was diluted with water (10 mL) and neutralised with 2.5M aqueous NaOH solution. The product was extracted into ethyl acetate (3 x 30 mL), dried over Na$_2$SO$_4$, concentrated in vacuo giving 391 as a cream solid (257 mg, 72%) which was used without further purification. $R_t = 0.76$ (100% EtOAc); mp = 103.7 – 104.2 °C; UV $\lambda_{\text{max}}$ (EtOH): 344.0, 274.0, 234.5, 204.0 nm; IR $\nu_{\text{max}}$ 3451 (NH), 3411, 3357, 1043 cm$^{-1}$; $\delta_H$ (300 MHz $d_6$-DMSO): 0.88 (3H, t, $J = 7.4$ Hz, CH$_2$CH$_3$), 1.20 (3H, d, $J = 6.2$ Hz, OCHCH$_3$), 1.53-1.59 (2H, m, CH$_2$CH$_3$), 4.97-5.07 (1H, m, OCH), 6.06 (2H, s, NH$_2$), 6.26 (2H, s, NH$_2$); $\delta_C$ (75 MHz $d_6$-DMSO): 9.6 (CH$_3$CH$_2$), 19.8 (CH$_3$CH), 28.9 (CH$_3$CH$_2$), 73.1 (OCH), 161.5 (CNH$_2$), 162.3 (CNH$_2$), 165.1 (CO); HRMS m/z: Calc. for C$_8$H$_{14}$BrN$_4$O: 261.0346 [M+H]$^+$ Found 261.0343 [M+H]$^+$.
A solution of 391 (2.38 g, 9.65 mmol, 1 eq) in AcOH (23 mL, 405 mmol, 42 eq) and Ac₂O (58 mL, 1.01 mol, 105 eq) was heated to 170 °C for 40 h, cooled to room temperature, water (60 mL) and basified using conc. ammonia, the beige precipitate was collected by filtration, dissolved in DCM (30 mL) and dried over Na₂SO₄ and concentrated in vacuo. Chromatography (silica, 100% EtOAc) gave 392 as a white solid (0.86 g, 26%). \( R_f = 0.66 \) (100% EtOAc); mp = 139.3-142.0 °C; UV \( \lambda_{\text{max}} \) (EtOH): 253.5, 228.0 nm; IR \( \nu_{\text{max}} \) (C=O) 3215, 2974, 1677, 1571, 1311 cm⁻¹; \( \delta_H \) (500 MHz, \( d_6 \)-DMSO): 0.92 (3H, t, \( J = 7.4 \) Hz, \( CH_3CH_2 \)), 1.32 (3H, d, \( J = 6.1 \) Hz, \( CHCH_3 \)), 1.65-1.74 (2H, m, \( CH_2CH_3 \)), 2.18 (3H, s, COCH₃), 2.21 (3H, s, COCH₃), 5.15-5.19 (1H, m, OCHCH₃), 9.89 (1H, s, NH), 10.4 (1H, s, NH); \( \delta_C \) (125 MHz, \( d_6 \)-DMSO): 9.4 (\( CH_3CH_2 \)), 19.0 (\( CH_2CH \)), 23.7 (COCH₃), 24.7 (COCH₃), 28.2 (\( CH_2CH_2 \)), 75.9 (\( CH_3CHO \)), 90.3 (\( C^5 \)), 154.8 (\( C^2 \)), 157 (\( C^6 \)), 166.1 (C-O), 168.9 (C=O), 169.2 (C=O)

2,6-Diamino-4-sec-butoxypyrimidine-5-carbonitrile (83)

To a solution of \( N,N'-(5\text{-bromo-4-sec-butoxypyrimidine-2,6-diyl})\text{diacetamide (300 mg, 0.87 mmol, 1 eq)} \) in DMF (5 mL) was added CuCN (99 mg, 1.1 mmol, 1.3 eq) and the reaction mixture was heated to 120 °C for 6 h, then cooled to room temperature. Ethylenediamine (5 mL, 75 mmol, 86 eq) was added and the reaction mixture was stirred for 4 h, and the resulting blue solution was filtered through Celite, washed with DMF (8 mL) and diluted with water (30 mL). The filtrate was then extracted with ethyl acetate (3 x 50 mL) and the combined organic layers washed with brine (2 x 100 mL), dried over Na₂SO₄ and concentrated in vacuo. The residue was suspended in water (100 mL), extracted in to ethyl acetate (3 x 70 mL), dried over
Na$_2$SO$_4$ and concentrated in vacuo. Recrystallisation (methanol / petrol) gave 83 as a white solid (0.093 mg, 51%). $R_f = 0.71$ (7:3 EtOAc:PE); mp = 165.6-166.4 °C; UV $\lambda_{\text{max}}$ (EtOH): 265.5, 251.5, 218.0, 202.5 nm; IR $\nu_{\text{max}}$ 3380 (NH), 3125, 2199 (CN), 1660, 1628, 1572 cm$^{-1}$; $\delta_H$ (300 MHz, $d_6$-DMSO): 0.88 (3H, t, $J = 7.5$ Hz, CH$_2$CH$_3$), 1.23 (3H, d, $J = 6.0$ Hz, CHCH$_3$), 1.54-1.66 (2H, m, CH$_2$CH$_3$), 5.07-5.16 (1H, m, CHCH$_3$), 6.83 (2H, s, N$_1$H$_2$), 6.89 (2H, s, N$_2$H$_2$); $\delta_C$ (75 MHz, $d_6$-DMSO): 9.6 (CH$_3$CH$_2$), 19.6 (CH$_3$CH), 28.8 (CH$_3$CH$_2$), 73.6 (OCH), 116.1 (CN), 163.6 (CNH$_2$), 166.4 (CNH$_2$), 171.3 (CO); LCMS $R_t = 3.11$ min, (1), MS (ESI+) m/z 208.18 [M+H]$^+$; HRMS m/z: Calc. for C$_9$H$_{15}$N$_5$O: 208.1193 [M+H]$^+$. Found 208.1191 [M+H]$^+$.

Analytical HPLC: 96.8% purity

4-Isobutoxypyrimidine-2,6-diamine (393)

Sodium (0.45 g, 19.6 mmol, 1.4 eq) was added to (2-methyl-1-propanol (16 mL, 173 mmol, 13 eq) then heated to 80 °C until all the sodium had dissolved resulting in a yellow solution. 4-Chloro-2,6-diaminopyrimidine (2.00 g, 13.7 mmol, 1 eq) was added and the temperature increased to 120 °C for 1 h then 140 °C for 3 h, then cooled to room temperature, neutralised with acetic acid and evaporated. The resulting yellow solid was suspended in water (30 mL), and extracted in ethyl acetate (3 x 30 mL). The organic layers were dried over Na$_2$SO$_4$, evaporated resulting in a yellow oil that was triturated to form a cream solid. Recrystallisation (ethyl acetate /petrol) gave a sticky white solid which was dried over P$_2$O$_5$, then covered in diethyl ether (20 mL) which was allowed to slowly evaporate giving 393 as a white solid (1.31 g, 53%). $R_t = 0.39$ (100% EtOAc); mp = 81.8-82.8 °C; UV $\lambda_{\text{max}}$ (EtOH): 379.0, 266.5, 205.5 nm; IR $\nu_{\text{max}}$ 3439 (NH), 3323, 1213, 1027 cm$^{-1}$; $\delta_H$ (300 MHz $d_6$-DMSO): 0.90 (6H, d, 6.6 Hz), 1.87-1.96 (1H, m, CH), 3.84 (2H, d, $J = 6.6$ Hz, CH$_2$), 5.03 (1H, s, $H^5$), 5.88 (2H, s, N$_1$H$_2$), 6.01 (2H, s, N$_2$H$_2$); $\delta_C$ (75 MHz $d_6$-DMSO): 19.4 (2 x CH$_3$), 27.3 (CH), 71.2 (CH$_2$), 76.8 (C$^5$), 163.3 (CNH$_2$), 166.4 (CNH$_2$), 170.9 (CO); LCMS $R_t = 5.70$ min, (2); MS(ESI+) m/z 183.18 [M+H]$^+$.
To 4-isobutoxypyrimidine-2,6-diamine (250 mg, 1.37 mmol, 1 eq) in acetic acid (5 mL, 87 mmol, 64 eq) was added N-bromosuccinimide (255 mg, 1.43 mmol, 1 eq). After stirring at room temperature for 15 min the reaction was diluted with water (10 mL) and the neutralised with 2.5M aqueous NaOH solution, resulting in a white precipitate which was collected by filtration. The precipitation was redissolved in water (50 mL) and extracted into ethyl acetate (3 x 50 mL), dried over Na$_2$SO$_4$ and evaporated in vacuo giving 394 as a cream solid used without any further purification (309 mg, 86 %). $R_t = 0.77$ (100% EtOAc); mp = 136.3 – 136.7 °C; UV $\lambda_{\text{max}}$ (EtOH): 379.0, 274.5, 234.0, 204.0 nm; IR $\nu_{\text{max}}$ 3451 (NH), 3406, 1292, 1048 cm$^{-1}$; $\delta_H$ (300 MHz $d_6$-DMSO): 0.93 (6H, d, $J = 6.6$ Hz, CH$_3$), 1.89-2.02 (1H, m, CH), 3.95 (2H, d, $J = 6.6$ Hz, CH$_2$), 6.10 (2H, s, N$^1$H$_2$), 6.29 (2H, s, N$^2$H$_2$); $\delta_C$ (75 MHz $d_6$-DMSO): 19.2 (CH$_3$), 27.9 (CH), 72.2 (CH$_2$), 161.5 (CNH$_2$), 162.2 (CNH$_2$), 165.3 (CO); LCMS $R_t = 2.83$ min, (1); MS (ESI+) $m/z$ 261.10 [M]+; CHN Calculated C 36.80, H 5.02, N 21.45 Found C 36.81 H 4.81 N 21.49

$N,N'$-(5-Bromo-6-isobutoxypyrimidine-2,6-diyl)diacetamide (395)

A mixture of 5-bromo-4-isobutoxypyrimidine-2,6-diamine (150 mg, 0.57 mmol, 1 eq) in acetic acid (2.5 mL, 44 mmol, 76 eq) and acetic anhydride (5 mL, 52.9 mmol, 92 mmol) was heated to 150 °C for 28 h then to 160 °C for 11 h., then cooled to room temperature, diluted with water (10 mL) and neutralised with concentrated ammonia, then extracted into ethyl acetate (3 x 70 mL). The combined organic extracts were dried over Na$_2$SO$_4$, filtered and evaporated. Recrystallisation (methanol) gave 395 as a beige solid which was washed with petroleum ether (3 x 5 mL) (0.16 g, 79%). $R_t = 0.61$ (100% EtOAc); mp = 129.7-130.4 °C; UV $\lambda_{\text{max}}$ (EtOH): 254.5, 216.5 nm; IR $\nu_{\text{max}}$ 3395 (NH), 1670 (C=O), 1142, 1310, 1142, 1001 cm$^{-1}$; $\delta_H$ (300 MHZ $d_6$-DMSO):
7. mTOR Experimental Procedures

To a solution of N,N’-(5-bromo-4-isobutoxypyrimidine-2,6-diyl) (200 mg, 0.58 mmol, 1 eq) in DMF (5 mL) was added CuCN (70 mg, 0.78 mmol, 1.3 mmol) and the reaction heated to 120 °C for 7 h. The mixture was then cooled to room temperature, ethylenediamine (4 mL, 60 mmol, 103 eq) added and the reaction stirred for 17 h, filtered through Celite, washing with DMF (10 mL), the filtrate was diluted with water (30 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic layers were washed with brine (100 mL), dried over Na₂SO₄ and evaporated in vacuo resulting in a cream solid. Recrystallisation (MeOH/petrol). Chromatography (SP4, silica, 10-80% EtOAc, petrol) gave 84 as a white solid (36 mg, 30%). Rᵣ = 0.58 (100% EtOAc); mp = 197.4-198.2 °C; UV λₘₐₓ (EtOH): 266.0, 251.0, 217.5 nm; IR νₘₐₓ 3344 (NH), 3204, 2207 (CN), 1628 cm⁻¹; δₜ (300 MHz, d₆-DMSO): 0.93 (6H, d, J = 6.6 Hz, CH(CH₃)₂), 1.89-2.04 (1H, m, CH), 4.03 (2H, d, J = 6.6 Hz, OCH₂), 6.87 (2H, s, N¹H₂), 6.93 (2H, s, N²H₂); δ_C (75 MHz, d₆-DMSO): 19.1 (CH₃), 27.9 (CH), 72.3 (CH₂), 163.5 (CNH₂), 166.3 (CNH₂), 171.6 (CO); HRMS m/z: Calc. for C₉H₁₄N₅O: 208.1193 [M+H]+. Found 208.1193 [M+H]+. Analytical HPLC: 97.1% purity

4-Cyclopropylmethoxypyrimidine-2,6-diamine (396)

Sodium (0.55 g, 23.9 mmol, 1.7 eq) was added in portions to a flask containing cyclopropylmethanol (20 mL, 247 mmol, 18 eq) and heated to 60 °C until all the
sodium had dissolved. 4-Chloro-2,6-diaminopyrimidine (2.00g, 13.8 mmol, 1 eq) was added to the solution and the mixture was heated to 120 °C for 90 min, cooled to RT, neutralised with acetic acid, and evaporated. The resulting brown oil was suspended in water (50 mL) and extracted with EtOAc (3 x 70 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated in vacuo. Chromatography (silica, 100% Ethyl acetate) gave 396 as a yellow oil (1.90 g, 77%). $R_f = 0.33$ (100% EtOAc); UV $\lambda_{\text{max}}$ (EtOH): 265.0, 233.0, 206.0 nm; IR $\nu_{\text{max}}$ 3338 (NH), 3188, 1564 cm$^{-1}$; $\delta_H$ (300 MHz, $d_6$-DMSO): 0.22-0.27 (2H, m, CH$_2$), 0.47-0.53 (2H, m, CH$_2$), 1.15-1.20 (1H, m, CH), 3.91 (2H, d, $J = 6.9$ Hz, OCH$_2$), 5.01 (1H, s, H$^5$), 5.84 (2H, s, N$^1$H$_2$), 5.98 (2H, s, N$^2$H$_2$); $\delta_C$ (75 MHz, $d_6$-DMSO): 3.4 (cyclopropyl), 10.6 (cyclopropyl), 69.4 (OCH$_2$), 76.9 (C$^5$), 163.3 (CNH$_2$), 166.5 (CNH$_2$), 170.7 (CO); LCMS $R_t = 0.33$ min, (1); MS (ESI+) m/z 181.15 [M+H]$^+$

5-Bromo-4-cyclopropylmethoxyxypyrimidine-2,6-diamine (397)

A mixture 4-cyclopropylmethoxyxypyrimidine-2,6-diamine (0.72 g, 4.00 mmol, 1 eq) and N-bromosuccinimide (0.72 g, 4.06 mmol, 1.01 eq) was dissolved in acetic acid (8.0 mL, 130 mmol, 32 eq) was stirred at room temperature for 15 min neutralised using 2.5 M aqueous solution of NaOH resulting in a white precipitate which was filtered, redissolved in DCM (50 mL) and dried over Na$_2$SO$_4$ giving 397 as a white solid (1.03 g, 99%). $R_t = 0.8$ (100% EtOAc); mp = 147.6-147.8 °C; UV $\lambda_{\text{max}}$ (EtOH): 274.0, 236 nm; IR $\nu_{\text{max}}$ (NH): 3447 (NH), 3407, 3349, 3152, 1634, 1549 cm$^{-1}$; $\delta_H$ (300 MHz, $d_6$-DMSO): 0.27-0.32 (2H, m, CH$_2$), 0.49-0.54 (2H, m, CH$_2$), 1.15-1.25 (1H, m, CH), 4.03 (2H, d, $J = 7.2$ Hz, OCH$_2$), 6.09 (2H, s, N$^1$H$_2$), 6.28 (2H, s, N$^2$H$_2$); $\delta_C$ (75 MHz, $d_6$-DMSO): 3.3 (cyclopropyl), 10.5 (cyclopropyl), 70.4 (cyclopropyl), 161.4 (CNH$_2$), 162.2 (CNH$_2$), 165.3 (CO); LCMS $R_t = 2.34$ min, (1); MS (ESI+) m/z 259.91 [M+H]$^+$ HRMS m/z: Calc. for C$_8$H$_{12}$BrN$_4$O: 259.0192 [M+H]$^+$. Found 259.0192 [M+H]$^+$.

Analytical HPLC: 98.5% purity
N,N’-(5-Bromo-4-cyclopropylmethoxypyrimidine-2,6-diyl)diacetamide (398)

A mixture of 5-bromo-4-cyclopropylmethoxypyrimidine-2,6-diamine (1.03 g, 3.97 mmol, 1 eq) in acetic acid (12 mL, 207 mmol, 53 eq) and acetic anhydride (10 mL, 106 mmol, 27 eq) was heated to 80 °C for 17 h, cooled to room temperature and basified concentrated ammonia resulting in a white precipitate. The suspension was extracted into ethyl acetate (3 x 100 mL) and the combined organic layers washed with a saturated aqueous solution of NaHCO₃ (250 mL), dried over Na₂SO₄ and evaporated in vacuo. Chromatography (silica, EtOAc) gave 398 as a white solid (0.75g, 54%). R_f = 0.60 (100% EtOAc); mp = 191.3-192.5 °C; UV λ_max (EtOH): 256.0, 227.0 nm; IR ν_max (EtOH): 3207 (NH), 3140, 3003, 2925, 1674 (C=O), 1574, 1498 cm⁻¹; δ_H (300 MHz, d₆-DMSO): 0.35-0.41 (2H, m, CH₂), 0.52-0.60 (2H, m, CH₂), 1.24-1.37 (1H, m, CH), 2.17 (3H, s, COCH₃), 2.19 (3H, s, COCH₃), 4.25 (2H, d, J = 7.2 Hz, OCH₂), 9.96 (1H, s, N₁H), 10.49 (1H, s, N₂H); δ_C (75 MHz, d₆-DMSO) 3.4 (cyclopropyl), 10.1 (cyclopropyl), 24.3 (COCH₃), 25.1 (COCH₃), 72.7 (CH₂), 169.5,157.5; LCMS R_t = 3.07 min, (1); MS (ESI+) m/z 247.91 [M+H]+

2,6-Diamino-4-(cyclopropylmethoxy)pyrimidine-5-carbonitrile (85)

To a solution of 384 (80 mg, 0.23 mmol, 1 eq) in DMF (3 mL) was added CuCN (42 mg, 0.49 mmol, 2 eq) and the reaction heated to 120 °C for 4 h. After cooling to room temperature, ethylenediamine (1.20 mL, 18.0 mmol) was added and stirring continued for 20 h. The mixture was before diluted with water (10 mL), filtered through Celite and washed with further water (2 x 5 mL). The filtrate solution was extracted with EtOAc (3 x 30 mL), and the combined organic extracts washed with brine (40 mL), dried over Na₂SO₄ and concentrated in vacuo. Chromatography (SP4, silica, 18-100% EtOAc, PE) gave 85 as a white solid (39 mg, 82%). R_f = 0.55 (7:3 EtOAc:PE); mp = 216.2-217.7 °C; UV λ_max (EtOH): 265.5, 251.0, 216.5 nm; IR ν_max 3500 (NH), 3437,
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3379, 3096, 2202 (CN), 1666 cm$^{-1}$; δ$_H$ (500 MHz, $d_6$-DMSO): 0.05-0.08 (2H, m, cyclopropyl), 0.30 (2H, ddt, $J = 4.2$, 5.8 and 7.9 Hz, cyclopropyl CH$_2$), 0.94-1.00 (1H, m, cyclopropyl), 3.87 (2H, d, $J = 7.2$ Hz, OCH$_2$), 6.60 (2H, s, N$_1$H$_2$), 6.65 (2H, s, N$_2$H$_2$); δ$_C$ (125 MHz, $d_6$-DMSO): 3.09 (cyclopropyl), 9.90 (cyclopropyl), 62.7 (C$_5$), 70.4 (OCH$_2$), 116.1 (CN), 162.9 (C$^5$), 165.8 (C$^6$), 170.8 (C$^4$); LCMS $R_t = 1.01$ min (3); MS (ESI+) m/z = 206.3 [M+H]$^+$; HRMS m/z: Calc. for C$_9$H$_{11}$N$_5$O: 206.1036 [M+H]$^+$. Found 206.1034 [M+H]$^+$; Analytical HPLC: 98.6% purity

6-Amino-2-(4-methoxybenzylamino)pyrimidine-5-carbonitile (88)

6-Amino-2-chloropyrimidine-5-carbonitile (250 mg, 1.25 mmol, 1 eq) was dissolved in DMF (10 mL) and 4-methoxybenzylamine (0.5.4 mL, 3.88 mmol, 2.4 eq) was added. The reaction temperature was increased to 100 $^\circ$C. After 3 h the reaction mixture was cooled to room temperature and diluted with water (40 mL) resulting in a white precipitate which was collected by filtration to give 88 as a white solid used without further purification (375 mg, 91%). $R_t = 0.50$ (1:1 EtOAc:PE); mp = 227.6-228.2 $^\circ$C; UV $\lambda_{max}$ (EtOH): 220.0, 257.5; IR $\nu_{max}$ 3431 (NH), 3337 (NH), 3206, 2210 (CN), 1641, 1578, 1504 cm$^{-1}$; δ$_H$ (300 MHz $d_6$-DMSO): 3.72 (3H, s, OCH$_3$), 4.39 (2H, d, $J = 5.5$ Hz, NHCH$_2$), 6.86-6.88 (2H, m, Ar-H), 7.13 (1H, s, NH$_2$), 7.23-7.25 (2H, m, Ar-H), 7.82 (1H, s, NH$_2$), 8.00 (1H, s, NH), 8.17 (1H, s, H$^3$); δ$_C$ (125 MHz $d_6$-DMSO): 43.1 (CH$_2$), 55.0 (OCH$_3$), 77.7 (C$^5$), 113.7 (benzyl Ar-H), 117.5 (CN), 128.6 (benzyl Ar-H), 131.6 (CCH$_2$), 158.1 (COCH$_3$), 162.2 (CH), 162.2 (CNH), 163.2 (CNR$_2$); LCMS $R_t = 2.10$ min, (1); MS (ESI+) m/z 256.06 [M+H]$^+$

2,6-Diaminopyrimidine-5-carbonitile (86)

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6-Amino-2-(4-methoxybenzylamino)pyrimidine-5-carbonitrile (88) (334 mg, 1.31 mmol, 1 eq) was suspended in DCM (4.5 mL) was TFA (10.5 mL) added slowly and the mixture heated to reflux for 18 h, then cooled to room temperature, neutralised using saturated sodium bicarbonate, and extracted using DCM (4 x 100 mL). The combined organic layers were concentrated in vacuo. The resulting white solid was redissolved in THF (300 mL) and dried over Na₂SO₄ and evaporated. Chromatography (100% EtOAc) gave the title compound as a white solid (138 mg, 77%). Rf = 0.35 (100% EtOAc); mp > 300 °C (lit. 318 °C); UV λmax (EtOH): 284.5; IR νmax (NH): 3430, 3082, 2201 (CN), 1647, 1582, 1545 cm⁻¹; δH (500 MHz d6-DMSO): 6.93 (1H, s, NH₁), 7.03 (1H, s, NH₂), 7.15 (2H, s, NH₂), 8.20 (1H, s, H₄); δC (125 MHz d6-DMSO): 78.3 (C₅), 162.3 (CH), 163.2 (CNH₂), 163.6 (CNH₂); HRMS m/z: Calc. for C₅H₆N₅: 136.0618 [M+H]+. Found 136.0617 [M+H]+. Analytical HPLC: 98.9% purity.

4-Chloro-N-(6-methoxybenzyl)pyrimidin-4-amine (91)

A mixture of 4,6-dichloropyrimidine (103 mg, 0.69 mmol, 1 eq), 4-methoxybenzylamine (0.078 mL, 0.60 mmol, 0.86 eq) and DIPEA (0.23 mL, 1.34 mmol, 1.9 eq) in 2-propanol (2.5 mL) was heated by microwave for 20 min at 120 °C. After cooling to room temperature the solvent was evaporated in vacuo. Chromatography (silica, 30% EtOAc, PE) gave the title compound as a white solid (140 mg, 83%). Rf = 0.31 (3:7 EtOAc:PE); mp = 132.3-133.0 °C (lit. 118-120 °C); UV λmax (EtOH): 248.5 nm; IR νmax 3221 (NH), 3077, 1595, 1561, 1506 cm⁻¹; δH (300 MHz CDCl₃): 3.82 (3H, s, CH₃), 4.44 (2H, broad s, CH₂), 6.35 (1H, s, H₅), 6.90 (2H, d, J = 8.4 Hz, Ar-H), 7.25 (2H, d, J = 8.4 Hz, Ar-H), 8.23 (1H, s, H₄); δC (125 MHz CDCl₃): 45.2 (CH₂), 55.3 (CH₃), 114.3 (Benzyl CH), 128.9 (benzyl CH), 158.4 (C₅), 159.4 (CCl), 163.2 (CNH); LCMS Rf = 2.77 min, (1); MS (ESI+) m/z 250.0 [M+H]+.
4-(Cyclohexylmethoxy)-N-(4-methoxybenzyl) pyrimidin-6-amine (92)

Method A: using conventional heating

To cyclohexylmethanol (5.0 mL, 40 mmol, 20 eq) was added sodium (0.06 g, 2.6 mmol, 1.3 eq) in portions, the reaction mixture was heated at 160 °C until all sodium had dissolved, 4-chloro-N-(6-methoxybenzyl) pyrimidin-4-amine (91) (500 mg, 2.0 mmol, 1 eq) was added and the temperature increased to 180 ºC for 30 min, then cooled to room temperature and neutralised using acetic acid. Petrol (30 mL) was added and the solution cooled to 4 ºC overnight. The resulting precipitate was collected by filtration and the filtrate evaporated. Further petroleum ether (20 mL) was added to this filtrate and the solution cooled to 4 ºC. This was repeated until no further solid formed. Recrystallisation (methanol) gave 92 as a white solid (500 mg, 76%).

Method B: using microwave assisted heating

To a solution of cyclohexylmethanol (1.30 mL, 10.6 mmol, 5.3 eq) in THF (10 mL) was added NaH (140 mg, 5.83 mmol, 2.9 eq) and the mixture stirred at room temperature for 45 min. 4-Chloro-N-(6-methoxybenzyl)pyrimidin-4-amine (91) (500 mg, 2.00 mmol, 1 eq) was added and the reaction mixture was stirred for 20 min at room temperature. The vial was then heated at 120 ºC for 18 min then cooled to room temperature and neutralised using acetic acid. The solvent was evaporated to give a cream coloured oily solid. Petroleum ether (100 mL) was added to the residue and it was then cooled to 4 ºC. The resulting white precipitate was then filtered. The filtrate was then evaporated and further petrol (100 mL) was added and cooled to 4 ºC any further precipitate was then collected by filtration. The combined solid was recrystallised (methanol) to give 92 as a white solid (471 mg, 72%).
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Rf = 0.79 (8:2 EtOAc:PE); mp = 112.4-113.2 °C; UV λmax (EtOH): 245.5, 356.0 nm; IR νmax 3216 (NH), 2917, 2850, 1588, 1561, 1510 cm⁻¹; δH (300 MHz CDCl₃) 0.97-1.31 (5H, m, C₆H₁₁), 1.60-1.85 (6H, m, C₆H₁₁), 3.83 (3H, s, CH₃), 4.07 (2H, d, J = 6.3 Hz, OCH₂), 4.39 (2H, d, J = 5.4 Hz, NHCH₂), 5.15 (1H, s, NH), 5.66 (1H, s, H₅), 6.90 (2H, d, J = 8.4 Hz, 2 x Ar-H), 7.25-7.28 (m, 2 x Ar-H), 8.27 (1H, s, H₂); δC (125 MHz CDCl₃): 25.8 (C₆H₁₁), 26.5 (C₆H₁₁), 29.8 (C₆H₁₁), 37.4 (CHCH₂O), 45.3 (NCH₂), 55.3 (OCH₃), 85.1 (OCH₂), 114.2 (benzyl Ar-H), 128.7 (benzyl Ar-H), 129.7 (CCH₂NH), 157.8 (COCH₃), 159.1 (CH), 164.1 (CNH), 170.3 (CO); LCMS Rf = 3.17 min, (1); MS (ESI+) m/z 328.25 [M+H]^+

5-Bromo-4-(cyclohexylmethoxy)-N-(6-methoxybenzyl)pyrimidin-4-amine (93)

To a solution of 4-(cyclohexylmethoxy)-N-(4-methoxybenzyl)pyrimidin-6-amine (92) (100 mg, 0.31 mmol, 1 eq) in acetic acid (5 mL) was added N-bromosuccinimide (59 mg, 0.33 mmol, 1 eq) and the reaction stirred at room temperature for 1 h, then neutralised using 2.5 M aqueous solution of NaOH and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were dried over MgSO₄ and evaporated. Chromatography (silica, 30% EtOAc, PE) gave 93 as a white solid (87 mg, 69%). Rf = 0.86 (3:7 EtOAc:PE); mp = 62.4-63.7 °C; UV λmax (EtOH): 213.5, 251.0 nm; IR νmax 3417 (NH), 2922, 2843, 1578, 1503 cm⁻¹; δH (300 MHz CDCl₃): 1.02-1.37 (5H, m, C₆H₁₁), 1.68-1.88 (6H, m, C₆H₁₁), 3.28 (3H, s, CH₃), 4.19 (2H, d, J = 6 Hz, OCH₂), 4.65 (2H, d, J = 5.4 Hz, NHCH₂), 5.53 (1H, s, NH), 6.90 (2H, d, J = 8.7 Hz, 2 x Ar-H), 7.28 (2H, d, J = 8.7 Hz, 2 x Ar-H), 8.22 (1H, s, H₀); δC (75 MHz CDCl₃): 26.1 (C₆H₁₁), 26.9 (C₆H₁₁), 30.1 (C₆H₁₁), 37.9 (CHCH₂O), 45.4 (CH₂NH), 55.7 (COCH₃), 72.7 (OCH₂), 86.6 (C₀), 114.7 (benzyl Ar-H), 129.2 (benzyl Ar-H), 131.2 (CCH₂NH), 155.9 (COCH₃), 159.7 (CH), 160.3 (CNH), 165.3 (CO); LCMS Rf = 4.51 min, (1); MS (ESI+) m/z 406.26 [M+H]^+
4-(Cyclohexylmethoxy)-6-(4-methoxybenzylamino)pyrimidine-5-carbonitrile (94)

To a solution of 5-bromo-4-(cyclohexylmethoxy)-N-(6-methoxybenzyl)pyrimidin-4-amine (93) (280 mg, 0.69 mmol, 1 eq) in DMF (5 mL) was added CuCN (79 mg, 89 mmol, 1.3 eq) and the reaction heated to 120 °C. After 3 days the reaction was cooled to room temperature and ethylenediamine (5 mL, 75 mmol, 108 eq) added. After stirring at room temperature for 3 days the mixture was filtered through Celite, washed with further DMF (15 mL), diluted with water (30 mL) and extracted with EtOAc (3 x 100 mL). The combined organic layers were then dried (MgSO₄) and evaporated to give a brown oil. Chromatography (silica, 20% EtOAc, PE) gave the title compound as a white solid (68 mg, 28%). Rₚ = 0.59 (2:8 EtOAc:PE); mp = 119.8-120.4 °C; UV λₘₐₓ (EtOH): 228.0, 249.0, 296.5 nm; IR νₘₐₓ 3337 (NH), 2925, 2855, 2218 (CN), 1582, 1512 cm⁻¹; δₕ (500 MHz CDCl₃): 0.92-1.24 (5H, m, C₆H₁₁), 1.60-1.76 (6H, m, C₆H₁₁), 3.74 (3H, s, OCH₃), 4.14 (2H, d, J = 6.5 Hz, OCH₂), 4.59 (2H, d, J = 5.5 Hz, NHCH₂), 6.81 (2H, d, J = 8.5 Hz, 2 x Ar-H), 7.18 (2H, d, J = 8.5 Hz, 2 x Ar-H), 8.29 (1H, s, H²); δₒ (125 MHz CDCl₃): 25.7 (C₆H₁₁), 26.4 (C₆H₁₁), 29.5 (C₆H₁₁), 37.2 (CH₂CO), 44.8 (CH₂NH), 55.4 (OCH₃), 72.8 (OCH₂), 114.2 (benzyl Ar-H), 129.2 (benzyl Ar-H), 159.3 (COCH₃), 159.9 (CH), 163.5 (CNH), 170.5 (CO)

4-Amino-6-(cyclohexylmethoxy)pyrimidine-5-carbonitrile (95)

4-(Cyclohexylmethoxy)-6-(4-methoxybenzylamino)pyrimidine-5-carbonitrile (94) (63.9 mg, 0.18 mmol, 1 eq) was dissolved in DCM (3.5 mL) and TFA (1.5 mL) added dropwise. The reaction was heated to reflux for 18 h then neutralised using saturated sodium bicarbonate and extracted using DCM (3 x 40 mL). The combined organic layers were dried over Na₂SO₄ and concentrated in vacuo. Chromatography (silica, 50% EtOAc, PE) gave white solid, compound (95) (21 mg, 50%). Rₚ = 0.64 (1:1 EtOAc:PE); mp = 133.1-134.0 °C; UV λₘₐₓ (EtOH): 283.5, 343.0, 384.0 nm; IR νₘₐₓ
4-(Cyclohexylmethoxy)-N-(6-methoxybenzyl)-5-nitrosopyrimidin-4-amine (96)

To a solution of 4-(cyclohexylmethoxy)-N-(4-methoxybenzyl)pyrimidin-6-amine (96) (100 mg, 0.31 mmol, 1 eq) in 30% acetic acid in water (5 mL) at 80 °C. Sodium nitrite (30 mg, 0.43 mmol, 1.3 eq) in water (0.56 mL, resulting in a 0.7 M solution) was added dropwise and the mixture was stirred at 80 °C for 1 h, cooled to room temperature and neutralised using a 2.5 M aqueous solution of NaOH, then extracted with ethyl acetate (3x 50 mL). The combined organic layers were dried over MgSO$_4$ and concentrated in vacuo. Chromatography (silica, 50% EtOAc, PE) gave 96 as a yellow oil (87 mg, 79%). $R_f = 0.95$ (8:2 EtOAc:PE); UV $\lambda_{max}$ (EtOH): 224.5, 275.0 nm; IR $\nu_{max}$ (EtOH): 3007 (NH), 2933, 2917, 2849, 1585, 1562 (NO), 1510 cm$^{-1}$; $\delta^H$ (300 MHz CDCl$_3$): 1.04-1.32 (5H, m, C$_6$H$_{11}$), 1.75-1.86 (6H, m, C$_6$H$_{11}$), 3.76 (3H, s, CH$_3$), 4.21 (2H, d, $J = 6$ Hz, OCH$_2$), 5.31 (2H, s, NHCH$_2$), 6.79 (2H, d, $J = 8.7$ Hz, 2 x Ar-H), 7.25 (2H, d, $J = 8.4$ Hz, 2 x Ar-H), 7.32 (1H, s, NH), 8.71 (1H, s, $H^2$); $\delta^C$ (125 MHz CDCl$_3$): 25.7 (C$_6$H$_{11}$), 26.4 (C$_6$H$_{11}$), 29.7 (C$_6$H$_{11}$), 37.3 (CHCH$_2$O), 43.1 (CH$_2$NH), 55.2 (OCH$_3$), 72.5 (CH$_2$O), 93.5 ($C^5$), 113.8 (benzyl Ar-H), 127.0 (benzyl Ar-H), 130.0 (CCH$_2$NH), 157.9 (COCH$_3$), 159.0 ($C^5$), 161.6 (CNH), 171.0 (CO); LCMS $R_t = 4.22$ min, (1); MS (ESI+) $m/z$ 357.28 [M+H]$^+$.
4-(Cyclohexylmethoxy)pyrimidin-6-amine (99)

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\text{[Diagram of 4-(Cyclohexylmethoxy)pyrimidin-6-amine (99)]}
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4-(Cyclohexylmethoxy)-N-(4-methoxybenzyl) pyrimidin-6-amine (92) (500 mg, 1.53 mmol, 1 eq) was dissolved in DCM (13.5 mL) and TFA (6 mL) was added dropwise. The reaction was heated to reflux for 41 h, then neutralised using a saturated sodium bicarbonate, then extracted using DCM (3x 40 mL). The combined organic layer were dried over MgSO\(_4\) and evaporated. Chromatography (silica, 70% EtOAc, PE) gave compound (99) a white solid (245 mg, 77%). 

\[R_f = 0.33 (7:3 \text{EtOAc:PE})\; \text{mp} = 179.6-180.1 \, {^\circ}\text{C} ; \text{UV} \lambda_{\text{max}} \text{ (EtOH)}: 283.5, 343.0, 384.0 \, \text{nm} ; \text{IR} \nu_{\text{max}} 3378 \text{ (NH)}, 3336, 3211, 2919, 2847, 2220, 1666, 1574, 1551 \, \text{cm}^{-1} ; \delta_H \text{ (300 MHz \text{d}_6-\text{DMSO})}: 0.95-1.24 \, (5\text{H}, \text{ m}, \text{C}_6\text{H}_{11}), 1.67-1.74 \, (6\text{H}, \text{ m}, \text{C}_6\text{H}_{11}), 3.97 \, (2\text{H}, \text{ d}, \text{ J} = 6.3 \, \text{Hz}, \text{OCH}_2), 5.66 \, (1\text{H}, \text{ s}, \text{H}^5), 6.58 \, (2\text{H}, \text{ s}, \text{NH}_2), 8.06 \, (1\text{H}, \text{ s}, \text{H}^2) ; \delta_C \text{ (75 MHz \text{d}_6-\text{DMSO})}: 25.6 \, (\text{C}_6\text{H}_{11}), 26.4 \, (\text{C}_6\text{H}_{11}), 29.6 \, (\text{C}_6\text{H}_{11}), 37.3 \, (\text{CHCH}_2\text{O}), 70.7 \, (\text{CH}_2\text{O}), 86.0 \, (\text{C}^3), 158.0 \, (\text{C}^2), 165.9 \, (\text{CNH}_2), 169.7 \, (\text{CO}) ; \text{LCMS} R_t = 2.60 \, \text{min}, \text{ (1)} ; \text{MS (ESI+)} \, m/z 240.03 \, [\text{M+H}]^+\]

N-Allyl-4-chloropyrimidin-6-amine (100)

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\text{[Diagram of N-Allyl-4-chloropyrimidin-6-amine (100)]}
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To a solution of 4,6-dichloropyrimidine (500 mg, 3.36 mmol, 1 eq) in THF (10 mL) was added triethylamine (0.49 mmol, 3.52 mmol, 1.05 eq) and allylamine (264 µL, 3.52 mmol, 1.05 eq). The reaction was stirred at room temperature for 24 h then the solvent was evaporated in vacuo. The resulting solid was suspended in water (40 mL) and extracted using DCM (3 x 50 mL). The combined organic layers were dried (MgSO\(_4\)) and evaporated in vacuo. Chromatography (SP4, silica, 15-100% EtOAc, PE) gave 100 as a colourless oil which solidified to a white solid (560 mg, 98%). 

\[R_t = 0.52 \, (1:1 \text{EtOAc:PE}) \, \text{mp} = 87.4-88.1 \, {^\circ}\text{C} ; \text{UV} \lambda_{\text{max}} \text{ (EtOH)}: 340.5, 246.0 \, \text{nm} ; \text{IR} \nu_{\text{max}} 3233 \, (\text{NH}), 3054 \, (\text{alkene C-H}), 3015, 2965, 1598 \, (\text{alkene C-C}), 1568, 1531 \, \text{cm}^{-1} ; \delta_H \text{ (500 MHz \text{d}_6-\text{CDCl}_3)}: 3.89 \, (2\text{H}, \text{ s}, \text{NHCH}_2), 5.16-5.22 \, (2\text{H}, \text{ m}, \text{NHCH}_2\text{CHCH}_2), 5.79-7.5.86 \, (1\text{H}, \text{ m}, \text{NHCH}_2\text{CHCH}_2), 6.29 \, (1\text{H}, \text{ s}, \text{H}^5), 8.28 \, (1\text{H}, \text{ s}, \text{H}^2) ; \delta_C \text{ (125 MHz PE)}\]

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CDCl₃): 43.9 (NHCH₂), 117.3, (NHCH₂CHCH₂), 132.8 (NHCH₂CHCH₂), 158.2 (C²), 163.2 (C-Cl)

*N-Allyl-4-(cyclohexylmethoxy)pyrimidin-6-amine (101)*

Cyclohexylmethanol (1.01 mL, 8.84 mmol, 5 eq) was added to a microwave vial followed by THF (4mL) and NaH (0.13 mg, 3 eq). The reaction was stirred at rt for 30 min before *N*-allyl-4-chloropyrimidin-6-amine (300 mg, 177 mmol, 1 eq) was added, and the reaction irradiated at 120 °C for 20 min. The solvent was removed in vacuo. Chromatography (SP4, silica, 12-100 % EtOAc, PE) followed by SPE (C18 isoeolute, 20-80% MeOH with 0.1 % formic acid, 0.1 % formic acid (aq) gave 101 as a white solid (331 mg, 75%). Rᵣ = 0.78 (1:1 EtOAc:PE); mp = 62.4-63.5 °C; UV λₑₓₘₐₓ (EtOH): 340.5, 245.0, 211.0 nm; IR νₑₓₘₐₓ (NH): 3231 cm⁻¹; δH (500 MHz CDCl₃): 0.94-1.24 (5H, m, C₆H₁₁), 1.60-1.77 (6H, m, C₆H₁₁), 3.79-3.81 (2H, m, NHCH₂), 4.00, (2H, d, J = 6.6 Hz, OCH₂), 5.13 (1H, dd, J = 10.3 and 1.3 Hz, 1 x NHCH₂CHCH₂), 5.20 (1H, dd, J = 17.2 and 1.3, 1 x NHCH₂CHCH₂), 5.57 (1H, s, H₅), 5.85-5.78 (1H, m NHCH₂CH₂), 8.16 (1H, s, H²); δC (125 MHz CDCl₃): 25.7 (C₆H₁₁), 26.6 (C₆H₁₁), 29.8 (C₆H₁₁), 37.4 (CCH₂O), 44.1 (NHCH₂), 71.6 (OCH₂), 85.1 (C⁵), 116.8 (NHCH₂CHCH₂), 133.5 (NCH₂CHCH₂), 157.5 (C²), 163.8 (CNH), 170.4 (CO); LCMS Rᵣ = 2.82 min; (ESI+) m/z 248.13 [M+H]⁺

*N-Allyl-4-(cyclohexylmethoxy)-5-nitrosopyrimidin-6-amine (102)*

To a solution of *N*-allyl-4-(cyclohexylmethoxy)pyrimidin-6-amine (50 mg, 0.20 mmol, 1 eq) in acetic acid (1.5 mL) and water (3.5 mL) was added NaNO₂ (15 mg, 0.22 mmol, 1.1 eq) in water (1 mL) and the reaction heated to 80 °C for 75 min before then neutralised with a saturated NaHCO₃, and extracted with DCM (3 x 25 mL). The
combined organic layers dried (MgSO₄) and evaporated in vacuo. Chromatography (silica, 50% EtOAc, PE) gave 102 as a yellow oil (37 mg, 66%). Rᵡ = 0.94 (1:1 EtOAc:PE); UV λₘₐₓ (EtOH): 340.5 nm; IR νₘₐₓ 2922, 1656 (Alkene C=C), 1588 (NO) cm⁻¹; δₓ (500 MHz CDCl₃): 0.96-1.26 (5H, m, C₆H₁₁), 1.61-1.79 (6H, m, C₆H₁₁), 4.14 (2H, d, J = 6.3 Hz, OCH₂), 4.70-4.71 (2H, m, NHCH₂), 4.98-5.08 (1H, m, NHCH₂CHCH₂), 5.03-5.06 (1H, m, NHCH₂CHCH₂), 5.60-5.68 (1H, m, NHCH₂CHCH₂), 8.60 (1H, d, J = 0.9 Hz, H₂); δₓ (125 MHz CDCl₃): 25.5 (C₆H₁₁), 25.7 (C₆H₁₁), 26.4 (C₆H₁₁), 37.2 (CCH₂O), 42.6 (NHCH₂), 72.5 (OCH₂), 93.3 (C-NO), 118.1 (NHCH₂CHCH₂), 129.5 (NHCH₂CHCH₂), 158.0 (CNH), 161.4 (CO), 171.0 (C²); LCMS Rᵡ = 3.98 min (1); MS (ESI+) m/z 277.14 [M+H]⁺

6-(Benzyloxyamino)-4-chloropyrimidin-2-amine (109)

A mixture of 2-amino-4,6-dichloropyrimidine (100 mg, 0.61 mmol, 1 eq), DMSO (5 mL), DIPEA(0.35 mL, 2.01 mmol, 3.3 eq) and O-benzylhydroxylamine (0.11 mL, 0.91 mmol, 1.5 eq) was heated to 130 °C for 6 h then cooled to room temperature, diluted with water (30 mL) and extracted using ethyl acetate (3 x 40 mL). The organic layers were dried over Na₂SO₄ and evaporated. Chromatography (silica, 30-50% EtOAc, PE) gave 109 as a white solid (101 mg, 66%). Rᵡ = 0.14 (3:7 EtOAc:PE); mp = 172.3-172.9 °C; UV λₘₐₓ (EtOH): 208.5, 288.0, 341.5; IR νₘₐₓ 3470 (NH), 3298 (NH), 3173, 2918, 1632, 1593, 1546 cm⁻¹; δₓ (500 MHz d₆-DMSO): 4.84 (2H, s, OCH₂), 5.97 (1H, s, H₅), 6.73 (2H, s, NH₂), 7.44-7.46 (5H, m, Ar-H), 10.28 (1H, s, NH); δₓ (125 MHz d₆-DMSO): 77.0 (CH₂), 90.2 (C₅), 128.4 (Ar-H), 128.9 (Ar-H), 136.0 (Ar-H), 159.5 (CCl), 162.5 (CNH₂), 167.0 (CNH); LCMS Rᵡ = 2.66 min, (1); MS (ESI+) m/z 251.03 [M+H]⁺
4-Chloro-6-(methoxyamino)pyrimidin-2-amine (108)

A solution of methoxyamine hydrochloride (229 mg, 2.7 mmol, 4.5 eq) and DIPEA (0.90 mL, 7.3 mmol, 12 eq) in DMSO (5 mL) was stirred at room temperature for 30 min. 2-Amino-4,6-dichloropyrimidine (100 mg, 0.61 mmol, 1 eq) was added and the mixture heated to 130 °C for 16 h then diluted with water (30 mL) and extracted with EtOAc (3 x 70 mL). The combined organic layers were dried over Na₂SO₄ and evaporated. Chromatography (silica, 50% EtOAc, PE) gave the title compound as a white solid (55 mg, 52%). Rᶠ = 0.38 (1:1 EtOAc:PE); δ H (500 MHz d₆-DMSO): 3.61 (3H, s, CH₃), 5.93 (1H, s, H₅), 6.66 (2H, s, NH₂), 10.17 (1H, s, NH); LCMS Rₜ = 0.72 min, (1); MS (ESI+) m/z 174.93 [M+H]+

4-Chloro-6-(cyclohexylmethoxy)pyrimidin-2-amine (112)

To cyclohexylmethanol (15 mL, 122 mmol, 10 eq) was added sodium (0.25 g, 11.0 mmol, 0.9 eq) in portions, and the mixture heated to 100 °C until all sodium had dissolved. 2-Amino-4,6-dichloropyrimidine (2.00 g, 12.2 mmol, 1 eq) was added and the mixture stirred at 100 °C for 20 min, then cooled to room temperature, neutralised using acetic acid, diluted with petroleum ether (30 mL) and cooled to 4 °C. The precipitated solid was collected and purified using chromatography (silica, 30% EtOAc, PE). The filtrate was evaporated, diluted with petroleum ether and cooled to 4 °C. The collected solid was recrystallised from boiling methanol. The products were combined to give compound (112), a white solid (1.83 g 62%). Rᶠ = 0.61 (3:7 EtOAc:PE); mp = 108.6-109.3 °C; UV λmax (EtOH): 233.0, 278.0; IR νmax 3349 (NH), 3221, 2926, 2849, 1653, 1538 cm⁻¹; δ H (300 MHz d₆-DMSO): 0.92-1.30 (5H, m, C₆H₁₁), 1.63-1.74 (6H, m, C₆H₁₁), 4.03 (2H, d, J = 6.3 Hz, OCH₂), 6.08 (1H, s, H₅), 7.04 (2H, s, NH₂); δ C (75 MHz d₆-DMSO): 25.8 (C₆H₁₁), 26.5 (C₆H₁₁), 29.8 (C₆H₁₁), 243
7. mTOR Experimental Procedures

37.4 (CHCH₂O), 71.8 (CH₂O), 162.2 (Ar), 172.3 (CO); LCMS Rₜ = 3.36 min, (1); MS (ESI+) m/z 242.01 [M+H]⁺

4,6-Bis(cyclohexylmethoxy)pyrimidin-2-amine (113)

To cyclohexylmethanol (4.5 mL, 36 mmol, 60 eq) was added sodium (0.08, 3.49 mmol, 5.7 eq) in portions. The reaction mixture was heated to 120 °C and until all sodium had dissolved, 2-amino-4,6-dichloropyrimidine (100 mg, 0.61 mmol, 1 eq) was added and stirring continued at 120 °C followed by neutralisation using acetic acid. The mixture was diluted with petroleum ether (30 mL) cooled to 4 °C, and the white precipitate collected by filtration. Recrystallisation (methanol) gave the title compound as a white solid (65 mg, 33%). Rᵢ = 0.70 (3:7 EtOAc:PE); mp = 114.0-114.7 °C; UV λ_max (EtOH): 299.5, 260.5; IR ν_max 3350 (NH), 3235, 2920, 2820, 1644, 1566 cm⁻¹; δ_H (500 MHz d₆-DMSO): 0.88-1.18 (10H, m, CH₆H₁₁), 1.54-1.65 (12H, m, CH₆H₁₁), 3.88 (4H, d, J = 3.6 Hz, 2 x OCH₂), 5.21 (1H, s, H₅), 6.36 (2H, s, NH₂); δ_C (125 MHz CDCl₃): 25.8 (CH₆H₁₁), 26.5 (CH₆H₁₁), 29.8 (CH₆H₁₁), 37.4 (CHCH₂O), 71.8 (CH₂O), 79.6 (C₅), 162.2 (CNH₂), 172.4 (CO); LCMS Rᵢ = 3.86 min, (1); MS (ESI+) m/z 320.23 [M+H]⁺

2-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)acetonitrile (116)

To a solution of 2,6-diaminopyrimidone (250 mg, 1.98 mmol, 1 eq) and NaHCO₃ (366 mg, 4.36 mmol, 2.2 eq) in DMF (5 mL) was added bromoacetonitrile (0.30 mL, 4.36 mmol, 2.2 eq). The reaction was stirred at room temperature for 4 days, then concentration in vacuo, and water (40 mL) was added and the resulting precipitate was collected by filtration. Recrystallisation (ethanol) gave the title compound as beige needles (46 mg, 9%). Rᵢ = 0.63 (3:7 MeOH:EtOAc); mp = dec; UV λ_max (EtOH): 211.5, 269.5; IR ν_max 3437 (NH), 3363 (Amide NH), 2903, 2829, 2712, 2246
(weak, CN), 1630 (C=O), 1591 (Amide C-N) cm$^{-1}$; $\delta_H$ (300 MHz $d_6$-DMSO) 3.36 (2H, CH$_2$), 6.23 (4H, m, 2 x NH$_2$), 10.08 (1H, s, NH); $\delta_C$ (75 MHz $d_6$-DMSO): 11.9 (CH$_2$), 78.1 (C$_5$), 119.7 (CN), 154.4 (CNH$_2$), 162.2 (CNH$_2$), 162.4 (C=O); LCMS $R_t$ = 1.99 min, (2); MS (ESI+) $m/z$ 166.12 [M+H]$^+$

2-(2,4-Diamino-6-(cyclohexylmethoxy)pyrimidin-5-yl)acetonitrile (117)

A solution of 116 (244 mg, 1.48 mmol, 1 eq) and K$_2$CO$_3$ (230 mg, 1.63 mmol, 1.1 eq) in DMF (5 mL) was stirred at room temperature for 90 min, then (bromomethyl) cyclohexane (0.68 mL, 7.39 mmol, 5 eq) was added and the solution heated to 100 °C for 15h, cooled to room temperature, and poured into ice. The aqueous solution was extracted with EtOAc (4 x 70 mL), dried (Na$_2$SO$_4$) and concentrated in vacuo. Chromatography (silica, 100% EtOAc) and crystallisation gave 117 as a brown solid (84 mg, 22%). $R_t$ = 0.6 (100 % EtOAc); mp = 172.6-173.4 °C; UV $\lambda_{max}$ (EtOH): 267.0, 234.5; IR $\nu_{max}$ 3438 (NH), 3355, 3140, 2932, 2854, 2251 (weak, CN), 1624, 1569 cm$^{-1}$; $\delta_H$ (500 MHz $d_6$-DMSO): 1.04-1.35 (6H, m, C$_6$H$_{11}$), 1.70-1.85 (5H, m, C$_6$H$_{11}$), 3.54 (2H, s, CH$_2$CN), 4.05 (2H, d, $J = 6.0$ Hz, OCH$_2$), 6.05 (2H, s, N$^1$H$_2$), 6.32 (2H, s, N$^2$H$_2$); $\delta_C$ (500 MHz $d_6$-DMSO): 25.3 (C$_6$H$_{11}$), 26.0 (C$_6$H$_{11}$), 29.1 (C$_6$H$_{11}$), 36.9 ($CHCH_2$), 70.3 (CH$_2$O), 77.9 (C$_5$), 119.0 (CN), 161.7 (CNH$_2$), 162.9 (CNH$_2$), 167.2 (CO); LCMS $R_t$ = 2.29 min, (1); MS (ESI+) $m/z$ 262.12 [M+H]$^+$; HRMS $m/z$: Calc. for: C$_{13}$H$_{20}$N$_5$O 262.1662 [M+H]$^+$; Found 262.1666 [M+H]$^+$.; Analytical HPLC: 96.0% purity

4-Methoxypyridine 1-oxide (140)

To 4-methoxypyridine (0.50 mL, 4.92 mmol, 1 eq) in DCM (10 mL) was added m-CPBA (1.28 g, 7.41 mmol, 1.5 eq) in portions, and the mixture stirred for 20 h, then evaporated. Chromatography (silica, 30% MeOH, EtOAc) gave 140 as a cream solid
(0.48g, 79%). $R_f = 0.14$ (3:7 MeOH:EtOAc); mp = 101.7-102.3 °C (lit. 82-84 °C)$^{269}$; UV $\lambda_{\text{max}}$ (EtOH): 205.0, 268.0; IR $\nu_{\text{max}}$ 1561, 1286, 1207 cm$^{-1}$; $\delta_H$ (300 MHz, $d_6$-DMSO): 3.82 (3H, s, CH$_3$), 7.02 (2H, d, $J = 7.8$ Hz, Ar-H), 8.1 (2H, d, $J = 7.8$ Hz, Ar-H); $\delta_C$ (75 MHz $d_6$-DMSO): 56.6 (CH$_3$), 112.7 (Ar-H), 139.8 (CO), 156.7 (CHN)

$N$-Benzylacetamide (138)

$R_f = 0.14$ (3:7 MeOH:EtOAc); mp = 101.7-102.3 °C (lit. 82-84 °C)$^{269}$; UV $\lambda_{\text{max}}$ (EtOH): 205.0, 268.0; IR $\nu_{\text{max}}$ 1561, 1286, 1207 cm$^{-1}$; $\delta_H$ (300 MHz, $d_6$-DMSO): 3.82 (3H, s, CH$_3$), 7.02 (2H, d, $J = 7.8$ Hz, Ar-H), 8.1 (2H, d, $J = 7.8$ Hz, Ar-H); $\delta_C$ (75 MHz $d_6$-DMSO): 56.6 (CH$_3$), 112.7 (Ar-H), 139.8 (CO), 156.7 (CHN)

1-(Cyclohexylmethyl)pyridin-4(1H)-one (137)

4-Pyridone (100 mg, 1.05 mmol, 1 eq) was added to a microwave vial in the dark, then DMSO (2 mL) was added, followed by the potassium carbonate (218 mg, 1.58 mmol, 1.5 eq) and the mixture was stirred for 1h at room temperature. Cyclohexylmethanol (0.5 mL, 5.26 mmol, 5 eq) was added and the reaction then stirred at rt for 26 h. The mixture was added to ice water (50 mL) and extracted using DCM (3 x 50 mL). The combined organic layers were dried over MgSO$_4$ and evaporated. Chromatography (SP4, silica, 7-60% MeOH, EtOAc) gave the title compound as a colourless oil which solidified on standing (82 mg, 41%). $R_f = 0.5$ (3:7
MeOH:EtOAc); mp = 132.2-133.4 °C; UV $\lambda_{\text{max}}$ (EtOH): 263.5; IR $\nu_{\text{max}}$ 3427 (NH), 3366, 3427, 3366, 3289, 2918, 2849, 1638 (C=O), 1531 cm$^{-1}$; $\delta$H (300 MHz CDCl$_3$) 0.82-1.24 (5H, m, C$_6$H$_{11}$), 1.56-1.72 (6H, m, C$_6$H$_{11}$), 3.51 (2H, d, $J = 6.9$ Hz, NCH$_2$), 6.30 (2H, d, $J = 7.5$ Hz, CHCO), 7.17 (2H, d, $J = 7.5$ Hz, CHN; $\delta$C (75 MHz CDCl$_3$): 25.8 (C$_6$H$_{11}$), 26.4 (C$_6$H$_{11}$), 39.6 (C$_6$H$_{11}$), 41.5 (CHCH$_2$N), 63.5 (CH$_2$N), 118.9 (CH=O), 140.2 (CHN), 179.1 (C=O); LCMS $R_t = 1.76$ min, (1); MS (ESI+) m/z 192.09 [M+H]$^+$

4-(Cyclohexylmethoxy)pyridine (127)

To cyclohexylmethanol (30 mL, 244 mmol, 18 eq) was added to sodium (1.30 g, 56.5 mmol, 4.3 eq) in portions and the mixture heated to 140 °C until all the sodium had dissolved the solution. The solution was allowed to cool for 10 min then 4-chloropyridine hydrochloride (2.00 g, 13.3 mmol, 1 eq) was added and the reaction temperature increased to 200 °C with vigorous stirring for 3.5 h. The reaction mixture was cooled to room temperature and neutralised using acetic acid, diluted with petroleum ether (100 mL) and the precipitate filtered. The filtrate was concentrated in vacuo, diluted with DCM (100 mL) and extracted using 1 M HCl (6 x 70 mL). The combined acidic layers were basified using 2.5 M NaOH, and extracted using DCM (12 x 100 mL). The combined organic layers were dried over MgSO$_4$ and evaporated to give a yellow oil. Chromatography (silica, 80% EtOAc, PE) gave 127 as a pale yellow solid (1.02 g, 53%). $R_t = 0.47$ (8:2 EtOAc:PE); mp = 60.2-61.6 °C; UV $\lambda_{\text{max}}$ (EtOH): 218.0, 328.0; IR $\nu_{\text{max}}$ 2923, 2847, 1586, 1567 cm$^{-1}$; $\delta$H (300 MHz CDCl$_3$): 1.01-1.32 (5H, m, C$_6$H$_{11}$), 1.72 -1.90 (6H, m, C$_6$H$_{11}$), 3.81 (2H, d, $J = 5.4$ Hz, CH$_2$), 6.80 (2H, d, $J = 4.8$ Hz, 2 x Ar-H), 8.42 (2H, d, $J = 4.8$ Hz, 2 x Ar-H); $\delta$C (75 MHz CDCl$_3$): 26.1 (C$_6$H$_{11}$), 26.8 (C$_6$H$_{11}$), 30.2 (C$_6$H$_{11}$), 37.9 (CHCH$_2$O), 73.6 (CH$_2$O), 110.7 (CHCO), 151.4 (CHN), 165.7 (CO); LCMS $R_t = 1.24$ min, (1); MS (ESI+) m/z 192.08 [M+H]$^+$
4-(Cyclohexylmethoxy)pyridine-1-oxide (128)

![Chemical Structure](image)

To a solution of 4-cyclohexylmethoxypyridine (1.52 g, 7.93 mmol, 1 eq) in DCM (15 mL) was added mCPBA (2.29 g of 65% purity, 18.0 mmol, 1.5 eq) with stirring at room temperature for 2 days. The reaction mixture was then diluted with DCM (20 mL) and washed with a sat. NaHSO₃ (3 x 50 mL). The organic layer was then dried over MgSO₄ and evaporated. Chromatography (silica, 3-30% MeOH, EtOAc) to give the title compound as a white solid (1.59 g, 97%).

Rf = 0.17 (1:9 MeOH:EtOAc); UV λmax (EtOH): 202.0, 208.0, 261.0, 265.0, 268.5, 276.0; IR νmax 3105, 3028, 2924, 2850, 1611, 1553 cm⁻¹; δH (300 MHz CDCl₃): 0.99-1.33 (5H, m, C₆H₁₁), 1.75-1.85 (6H, m, C₆H₁₁), 3.79 (2H, d, J = 6 Hz CH₂), 6.80 (2H, d, J = 7.5 Hz, 2 x Ar-H), 8.13 (2H, d, J = 7.5 Hz, 2 x Ar-H); δC (75 MHz CDCl₃): 26.0 (C₆H₁₁), 26.7 (C₆H₁₁), 30.0 (C₆H₁₁), 37.8 (CHCH₂O), 75.0 (CH₂O), 112.5, 140.4; LCMS Rt = 9.40 min, (2); MS (ESI+) m/z 208.19 [M+H]+

2-Chloro-4-(cyclohexylmethoxy)pyridine (142)

![Chemical Structure](image)

A solution of 4-(cyclohexylmethoxy)pyridine 1-oxide (128) (0.646 g, 3.11 mmol, 1 eq) in acetonitrile (6 mL) was added phosphorus oxychloride (7.5 mL, 107 mmol, 34 eq) was added and the mixture was heated at 160 °C for 30 min in a microwave, then poured into ice (2 x 20 mL) and stirred overnight. The aqueous solution was basified using 1M aqueous KOH solution and extracted using DCM (9 x 100 mL). The combined organic layers were then dried over MgSO₄ and concentrated in vacuo to give a brown oil. Chromatography (silica, 100% EtOAc) gave the title compound as a brown oil (0.40 g, 56%).

Rf = 0.87 (100% EtOAc); mp = 49.2-49.8 °C; UV λmax (EtOH): 217.5; IR νmax 3073, 2922, 2846, 1730, 1584, 1551 cm⁻¹; δH (300 MHz CDCl₃) 0.78-1.25 (5H, m, C₆H₁₁), 1.62-1.78 (6H, m, C₆H₁₁), 3.72 (2H, d, J = 6.0 Hz, CH₂), 6.66 (1H, dd, J = 2.4 and 6.0 Hz, CHCO ), 6.74 (1H, d, J = 2.1 Hz, CHCl),
Diethyl 4-oxo-1,4-dihydropyridine-2,6-dicarboxylate (153)

To a solution of chelidamic acid monohydrate (500 mg, 2.73 mmol, 1 eq) in ethanol (15 mL) was added thionyl chloride (0.8 mL, 10.9 mmol, 4 eq) dropwise. The reaction heated to reflux for 2 h, then cooled to room temperature and the solvent evaporated. The mixture was neutralised using a saturated sodium bicarbonate solution, and the solvent evaporated. The residue was diluted with water (15 mL) and extracted using EtOAc (3 x 40 mL). The combined organic extracts were dried over MgSO₄ and concentrated in vacuo. Chromatography (SP4, silica, 12-100% EtOAc, PE) gave the title compound as a white solid (0.30 g, 46%). Rf = 0.38 (100% EtOAc); mp = 118.2-118.8 °C (lit 115-116 °C); UV λ max (EtOH): 214.5; IR ν max 2985, 1722 (Ester C=O), 1603 (Pyridone C=O), 1568 cm⁻¹; δH (500 MHz CDCl₃): 1.29 (6H, t, J = 7.5 Hz, CH₃), 4.34 (4H, q, J = 7.5 Hz, CH₂), 7.49 (2H, s, Ar-H); δC (125 MHz CDCl₃): 14.1 (CH₃), 63.0 (CH₂), 118.7 (Ar-H), 162.6 (COOEt); LCMS Rₘ = 2.34 min, (1); MS (ESI+) m/z 240.01 [M+H]⁺

Diethyl 4-(benzyloxy)pyridine-2,6-dicarboxylate (154)

To a solution of diethyl 4-oxo-1,4-dihydropyridine-2,6-dicarboxylate (153) (500 mg, 2.09 mmol, 1 eq) in DMF (5 mL) was added K₂CO₃ (318 mg, 2.30 mmol, 1.1 eq) and benzyl bromide (0.255 mL, 2.14 mmol, 1.03 eq). The reaction was heated to 80 °C for 30 min, then cooled to room temperature and water (20 mL) was added, and extracted using EtOAc (3 x 50 mL). The combined organic layers were dried over Na₂SO₄ and evaporated. Chromatography (silica, 40% EtOAc, PE) gave compound 154 a white
solid (659 mg, 96%). $R_t = 0.46$ (4:6 EtOAc:PE); mp = 74.6-75.2 °C; UV $\lambda_{\text{max}}$ (EtOH): 215.5; IR $\nu_{\text{max}}$ 3075, 2975, 1712 (Ester C=O), 1592, 1567 cm$^{-1}$; $\delta_H$ (300 MHz CDCl$_3$): 1.35 (6H, t, $J = 7.2$ Hz, CH$_3$), 4.37 (4H, q, $J = 7.2$ Hz, CH$_2$), 5.13 (2H, s, OCH$_2$), 7.26-7.34 (5H, m, C$_6$H$_5$), 7.78 (2H, s, Ar-H); $\delta_C$ (75 MHz CDCl$_3$): 13.2 (CH$_3$), 61.2 (CH$_2$CH$_3$), 69.9 (OCH$_2$), 113.6 (Ar-H), 126.7 (benzyl Ar-H), 127.7 benzyl (Ar-H), 127.9 (benzyl Ar-H), 134.1 (CCH$_2$), 149.7 (CCO$_2$Et), 163.7 (C-O), 165.7 (C=O); LCMS $R_t = 3.81$ min, (1); MS (ESI+) m/z 330.14 [M+H]$^+$

4-(Benzyloxy)pyridine-2,6-dicarboxamide (152)

A solution of diethyl 4-(benzyloxy)pyridine-2,6-dicarboxylate (150) (290 mg, 0.88 mmol, 1 eq) in 7 N methanolic ammonia in methanol (10 mL, 70 mmol, 80 eq) was stirred for 3.5 h. The white precipitate was collected by filtration and used without further purification. The filtrate was concentrated in vacuo. Chromatography (SP4, silica, 2-20% MeOH, EtOAc) gave a white solid compound (152) (212 mg, 88%) in total. $R_t = 0.26$ (100% EtOAc); mp = 271.3-272.1 °C (lit: > 220 °C)$^{180}$; UV $\lambda_{\text{max}}$ (EtOH): 214.0 nm; IR $\nu_{\text{max}}$ 3070 (NH), 1711, 1663 (Amide C=O), 1591, 1564 (NH) cm$^{-1}$; $\delta_H$ (500 MHz $d_6$-DMSO): 5.29 (2H, s, OCH$_2$), 7.28-7.42 (5H, m, Ar-H), 7.65 (2H, s, NH), 7.67 (2H, s, Ar-H), 8.77 (2H, s, NH); $\delta_C$ (125 MHz $d_6$-DMSO): 69.8 (CH$_2$), 110.5 (Ar-H), 127.7 (benzyl Ar-H), 128.1 (benzyl Ar-H), 128.8 (benzyl Ar-H), 135.8 (CCH$_2$O), 151.2 (CCONH$_2$), 165.1 (CO), 166.7 (C=O); LCMS $R_t = 3.04$ min, (1); MS (ESI+) m/z 272.00 [M+H]$^+$

4-Benzyl-2,6-diaminopyridine (153)

To an aqueous solution of 5M KOH (60 mL, 300 mmol, 55 eq) was added bromine (700 µL, 13.6 mmol, 2.5 eq) followed by 4-benzyloxy-3,2-dicarboxamide (1.48 g, 5.45 mmol, 1 eq). The mixture was heated to 90 °C for 2 h, cooled to room temperature, and extracted with DCM (6 x 100 mL), and THF (3 x 60 mL). The
combined organic layers were dried (MgSO₄). Chromatography (SP4, silica, 3-20% MeOH, EtOAc) gave **153** as a white solid (837 mg, 71%). *R*<sub>f</sub> = 0.2 (1:9 MeOH:EtOAc); mp. = 166.2 -167.0 °C (lit. 164-166 °C)<sup>180</sup>; UV *λ*<sub>max</sub> (EtOH): 289.0, 248.0 nm; IR *ʋ*<sub>max</sub> 3372 (NH), 3188, 2198, 1590, 1559 cm<sup>-1</sup>; δ<sub>H</sub> (500 MHz d<sub>6</sub>-DMSO): 5.02 (2H, s, OCH<sub>2</sub>), 5.39 (2H, s, Ar-H), 5.40 (4H, s, NH<sub>2</sub>), 7.38-7.45 (5H, m, benzyl); δ<sub>C</sub> (125 MHz d<sub>6</sub>-DMSO): 68.3 (OCH<sub>2</sub>), 82.3 (Ar-H), 127.4 (benzyl Ar-H), 127.7 (benzyl Ar-H), 128.4 (benzyl Ar-H), 137.1 (CCH₂O), 159.7 (CNH₂), 167.1 (CO); LCMS *R*<sub>t</sub> = 2.00 min (1); MS (ESI+) *m/z* 216.04 [M+H]<sup>+</sup>

*N,N’-*(4-Benzylxopyridine-2,6-diyl)diacetamide (**155**)

![Diacetamide Structure](image)

A mixture of 4-benzylxoy-2,6-diaminopyridine (193 mg, 0.90 mmol, 1 eq), acetic acid (3 mL) and acetic anhydride (3 mL) was stirred at room temperature for 3 days, then diluted with water (15 mL) and neutralised with ammonia. The resulting precipitate was collected by filtration, dissolved in DCM (20 mL), dried (MgSO₄) and the solvent removed *in vacuo*. Chromatography (silica, 10% MeOH, EtOAc) gave **155** as a white solid (247 mg, 92%). *R*<sub>f</sub> = 0.66 (1:9 MeOH:EtOAc) mp.= 116.6-117.3 °C; UV *λ*<sub>max</sub> (EtOH): 340.5, 283.0, 218.5 nm; IR *ʋ*<sub>max</sub> 3322 (NH), 1663 (C=O), 1618, 1580, 1521 cm<sup>-1</sup>; δ<sub>H</sub> (500 MHz CDCl₃): 2.08 (6H, s, 2 x CH₃), 5.05 (2H, s, OCH₂), 7.25-7.36 (5H, m, benzyl), 7.55 (2H, Ar-H), 7.70 (2H, s, NH); δ<sub>C</sub> (125 MHz CDCl₃): 23.7 (CH₃), 69.4 (OCH₂), 95.5 (Ar-H), 126.7 (benzyl Ar-H), 127.2 (benzyl Ar-H), 127.6 (benzyl Ar-H), 134.7 (CCH₂O), 149.5 (CNH), 167 (CO); LCMS *R*<sub>t</sub> = 2.49 min; MS (ESI+) *m/z* 300.16 [M+H]<sup>+</sup>

*N,N’-*(4-Benzylxoy-3-bromopyridine-2,6-diyl)diacetamide (**156**)

![Diacetamide Structure](image)
To a solution of \(N,N'-(4\text{-benzyloxy}p\text{yridine}-2,6\text{-diyl})\text{diacetamide}\) (117 mg, 0.39 mmol, 1 eq) and acetic acid (7 mL), \(N\text{-bromosuccinimide}\) (69 mg, 0.39 mmol, 1 eq) was added, then stirred at room temperature for 30 min and neutralised with NaOH (2.5 M). The resulting precipitate was collected by filtration, dissolved in DCM (30 mL), dried (MgSO\(_4\)) and evaporated in vacuo. Chromatography (silica, 10% MeOH, EtOAc) to give 156 as a white solid (0.115 mg, 77%). \(R_t = 0.7\) (1:9 MeOH:EtOAc)

mp. = 212.0 - 212.6 °C; UV \(\lambda_{\text{max}}\) (EtOH): 340.5, 257.5, 221.5 nm; IR \(\nu_{\text{max}}\) 3327 (NH), 1669, 1619, 1590, 1590, 1559, 1511 cm\(^{-1}\); \(\delta_H\) (500 MHz \text{d}_6-\text{DMSO}): 2.06 (3H, s, \(\text{CH}_3\)), 2.13 (3H, s, \(\text{CH}_3\)), 5.31 (2H, s, OCH\(_2\)), 7.41 - 7.756 (5H, m, benzyl), 7.95 (1H, s, Ar-\(H\)), 9.97 (1H, s, NH), 10.68 (1H, s, NH); \(\delta_C\) (125 MHz \text{d}_6-\text{DMSO}): 22.8 (\(\text{CH}_3\)), 24.0 (\(\text{CH}_3\)), 70.4 (OCH\(_2\)), 96.5 (Ar-\(H\)), 127.6 (benzyl Ar-\(H\)), 128.2 (benzyl Ar-\(H\)), 128.5 (benzyl Ar-\(H\)), 135.6 (CCH\(_2\)), 148.6 (CNH\(_2\)), 163.1 (carbonyl), 168.5 (carbonyl), 169.6 (CO); LCMS \(R_t = 2.80\) min (1); MS (ESI+) \text{m/z} 378.06 [M+H]\(^+\)

4-Cyclohexylmethoxypyridine-2,6-dicarboxylic acid diethyl ester (157)

A mixture of diethyl 5-oxocyclohexa-3,6-diene-1,3-dicarboxylate (1.00 g, 4.18 mmol, 1 eq), DMF (7 mL), \(\text{K}_2\text{CO}_3\) (0.64 g, 4.60 mmol, 1.1 eq) and (bromomethyl)
cyclohexane (0.70 mL, 5.02 mmol, 1.2 eq) was heated to 80 °C for 16 h, cooled to room temperature, diluted with saturated NaHCO\(_3\) (20 mL) and extracted using DCM (3 x 30 mL). The combined organic layers were dried (MgSO\(_4\)) and evaporated in vacuo. Chromatography (silica, 50% EtOAc, PE) gave 157 as a colourless oil (491 mg, 70%). \(R_t = 0.75\) (1:1 EtOAc:PE); UV \(\lambda_{\text{max}}\) (EtOH): 340.5, 213.5 nm; IR \(\nu_{\text{max}}\) 2926, 2853, 1717 (Ester \(\text{C}=\text{O}\)), 1593 cm\(^{-1}\); \(\delta_H\) (500 MHz \text{CDCl}_3): 1.00 (5H, m, C\(_6\)H\(_11\)), 1.38 (6H, t, 7.2 Hz, OCH\(_2\)CH\(_3\)), 1.62-1.81 (6H, m, C\(_6\)H\(_11\)), 3.85 (2H, d, \(J = 6.0\) Hz, OCH\(_2\)), 4.40 (4H, q, \(J = 7.2\) Hz, OCH\(_2\)CH\(_3\)), 7.70 (2H, s, Ar-\(H\)); \(\delta_C\) (125 MHz \text{CDCl}_3): 14.1 (CH\(_3\)), 25.6 (C\(_6\)H\(_11\)), 26.3 (C\(_6\)H\(_11\)), 29.6 (C\(_6\)H\(_11\)), 37.4 (CCH\(_2\)), 62.4 (OCH\(_2\)CH\(_3\)), 74.3 (OCH\(_2\)), 114.4 (Ar-\(H\)), 150.1 (CCO\(_2\)Et), 164.8 (CO), 167.2 (C=O)
4-Cyclohexylmethoxypyridine-2,6-dicarboxamide (158)

![Chemical Structure]

4-Cyclohexylmethoxypyridine-2,6-dicarboxylic acid diethyl ester (270 mg, 0.80 mmol, 1 eq) was dissolved in 7 M methanoic ammonia (7 mL, 49 mmol, 61 eq) and stirred at room temperature for 1h. The white precipitate was collected by filtration and recrystallised from boiling methanol to give the title compound as a white solid (105 mg, 47%). $R_f = 0.68$ (1:9 MeOH:EtOAc) mp = 269.0-269.9 °C; UV $\lambda_{max}$ (EtOH): 340.5, 212.0 nm; IR $\upsilon_{max}$ 3447 (NH), 3341, 3181, 2921, 2845, 1672 (C=O), 1588 (NH) cm$^{-1}$; $\delta_H$ (500 MHz $d_6$-DMSO) 1.08-1.35 (5H, m, $C_6H_{11}$), 1.69-1.86 (6H, m, $C_6H_{11}$), 4.05 (2H, d, $J = 5.9$ Hz, OCH$_2$), 7.68 (2H, s, Ar-H), 7.76 (2H, d, $J = 1.8$ Hz, NH), 8.89 (2H, d, $J = 1.75$ Hz, NH) $\delta_C$ (125 MHz $d_6$-DMSO): 25.1 ($C_6H_{11}$), 25.9 ($C_6H_{11}$), 28.9 ($C_6H_{11}$), 39.0 (CCH$_2$O), 73.3 (OCH$_2$), 110.0 (Ar-H), 151.2 ($C\text{CONH}_2$), 165.2 (CO), 167.2 (C=O); LCMS $R_t = 2.73$ min (1); ESI+ $m/z$ 278.12 [M+H]$^+$

4-Cyclohexylmethoxy-2,6-diaminopyridine (159)

![Chemical Structure]

To an aqueous solution of 5M KOH (3.96 mL, 19.8 mmol, 55 eq) was added bromine (46 µL, 0.90 mmol, 2.5 eq) followed by 4-cyclohexylmethoxypyridine-2,6-dicarboxylic acid diamine (100 mg, 0.36 mmol, 1 eq). The reaction mixture was heated to 90 °C for 2 h, cooled to room temperature, extracted with DCM (3 x 25 mL). The combined organic layers were dried (MgSO$_4$) and evaporated. Chromatography (SP4, silica, 3-20% MeOH, EtOAc) to give 4-cyclohexylmethoxy-2,6-diaminopyridine as a white solid (35 mg, 44%). $R_t = 0.18$ (1:9 MeOH:EtOAc); mp = 152.0-152.8 °C; UV $\lambda_{max}$ (EtOH): 289.5, 246.5 nm; IR $\upsilon_{max}$ 3435 (NH), 3363, 3333, 3187, 2926, 2854, 1626, 1578 cm$^{-1}$; $\delta_H$ (500 MHz $d_6$-DMSO): 0.97-1.25 (5H, m, $C_6H_{11}$), 1.64-1.76 (6H, m, $C_6H_{11}$), 3.65 (2H, d, $J = 6.5$ Hz, OCH$_2$), 5.27 (2H, s, Ar-H), 5.42 (4H, s, NH$_2$); $\delta_C$ (125 MHz $d_6$-DMSO): 25.2 ($C_6H_{11}$), 26.0 ($C_6H_{11}$), 29.2 ($C_6H_{11}$), 36.9 (CCH$_2$O), 71.9 (OCH$_2$), 82.1 (Ar-H), 159.2 (CNH$_2$), 167.6 (CO); LCMS $R_t = 2.33$ min (1); MS (ESI+) $m/z$ 222.16 [M+H]$^+$
4-Cyclohexylmethoxy-3-nitrosopyridine-2,6-diamine (160)

A solution of 4-cyclohexylmethoxy-2,6-diaminopyridine (50 mg, 0.23 mmol, 1 eq), in acetic acid (1.5 mL) and water (3.5 mL) was heated to 80 °C and NaNO₂ (17 mg, 0.25 mL, 1.1 eq) in water (1 mL) was added. The brown solution was stirred at 80 °C for 30 min, cooled to room temperature, neutralised using a saturated NaHCO₃ and extracted using DCM (4 x 25 mL). The combined organic layers were dried (Na₂SO₄), and evaporated in vacuo. Chromatography (silica, 10% MeOH, EtOAc), followed by recrystallisation (methanol) gave 160 as a purple solid (24 mg, 42%).

\[
R_f = 0.57 \quad \text{MeOH:EtOAc); \quad mp = 253.0-254.2 \degree C; \quad UV \lambda_{max} \quad \text{EtOH): 365.0, 323.5, 247.5, 204.0 nm; IR \nu_{max} \quad \text{cm}^{-1}; \quad \delta_H \quad (500 MHz, d_6-DMSO): 1.04-1.31 (5H, m, C₆H₁₁), 1.66-1.85 (6H, m, C₆H₁₁), 3.91 (2H, d, J = 6.05 Hz, OCH₂), 5.62 (1H, s, H³), 7.46 (1H, s, N¹H¹), 7.06 (1H, s, N¹H²), 7.66 (1H, d, J = 5.25 Hz, N²H¹), 11.14 (1H, d, J = 5.2 Hz, N²H²); \delta_C \quad (125 MHz, d_6-DMSO): 25.2 (C₆H₁₁), 25.9 (C₆H₁₁), 29.1 (C₆H₁₁), 37.8 (CCH₂O), 73.3 (OCH₂), 83.3 (Ar-H), 142.3 (Ar-NO), 148.8 (CNH₂), 165.0 (CNH₂), 166.0 (CO); \quad \text{LCMS} \quad R_t = 2.16 \text{ min (1); MS (ESI+) } m/z \quad 251.10 [M+H]^+ \quad \text{HRMS} \quad m/z : \quad \text{Calc. for } C_{12}H_{19}N₄O₂: 251.1503 [M+H]^+; \quad \text{Found: 251.1506 [M+H]^+; Analytical HPLC: 97.0% purity}
\]

4,6-Diamino-2-methoxynicotinitrile (165)

Sodium (0.72 g, 30.0 mmol, 3 eq) was added in portions to methanol (30 mL, 698 mmol, 70 eq), after all the sodium had dissolved 2-amino-1,1,3-tricyanopropene (1.32 g, 9.99 mmol, 1 eq) was added and the reaction heated to reflux for 48 h, then cooled to room temperature and the solvent removed in vacuo. The resulting oil was diluted with ice water (5 mL) and chilled to 4 °C for 2 days. The precipitate was collected by filtration and purified by chromatography (silica, 4:1 Et₂O:PE) to give the title compound as a white solid (0.33 g, 20%). \(R_t = 0.26 \quad (4:1 \text{ Et}_2\text{O:PE}); \quad \text{mp.} = 183.3-183.8 °C \quad \text{(lit. 176 °C)}^{186}; \quad \text{UV } \lambda_{max} \quad \text{(EtOH): 267.5, 220.5 nm; IR } \nu_{max} \quad 3511 \quad \text{(NH), 3398, 3337,}
3240, 2197 (CN), 1638, 1597, 1547 cm\(^{-1}\); \(\delta\)\(_H\) (500 MHz \(d_6\)-DMSO): 3.82 (3H, s, CH\(_3\)), 5.39 (1H, s, Ar-H), 6.26 (2H, s, NH\(_2\)), 6.32 (2H, s, NH\(_2\)); \(\delta\)\(_C\) (125 MHz \(d_6\)-DMSO): 53.0 (CH\(_3\)), 67.5 (C-CN), 82.5 (CCN), 116.7 (Ar-H), 158.4 (CNH\(_2\)), 160.2 (CNH\(_2\)), 165.7 (CO); LCMS \(R_t\) = 1.56 min (1); MS (ESI+) \(m/z\) 164.98 [M+H]\(^+\)

4,6-Diamino-2-methoxynicotinitrile (164)

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N}
\end{array}
\]

The title compound was synthesised as a by-product of the above reaction as a pale yellow solid (77 mg, 5%). \(R_t\) = 0.53 (4:1 Et\(_2\)O:PE); mp = 139.5-140.9 \(^0\)C; UV \(\lambda_{max}\) (EtOH): 341.0, 293.5, 225.5 nm; IR \(\nu_{max}\) 3357 (NH), 3232, 2195 (CN), 1626, 1569 cm\(^{-1}\); \(\delta\)\(_H\) (500 MHz CDCl\(_3\)): 3.74 (3H, s, CH\(_3\)), 4.52 (2H, s, NH\(_2\)), 4.87 (2H, s, NH\(_2\)), 5.36 (1H, s, Ar-H); \(\delta\)\(_C\) (125 MHz CDCl\(_3\)): 37.4 (CH\(_3\)), 4.52 (2H, s, NH\(_2\)), 4.87 (2H, s, NH\(_2\)), 5.36 (1H, s, Ar-H); \(\delta\)\(_C\) (125 MHz CDCl\(_3\)): 53.7 (CH\(_3\)), 70.4 (CCN), 83.8 (Ar-H), 116.5 (CN), 157.6 (CNH\(_2\)), 160.3 (CNH\(_2\)), 167.0 (CO); LCMS \(R_t\) = 2.45 min (1); MS (ESI+) \(m/z\) 165.06 [M+H]\(^+\)

4,6-Diamino-2-cyclohexylmethoxynicotinitrile (162)

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N}
\end{array}
\]

Sodium (0.73 g, 31.2 mmol, 3.2 eq) was added in portions to cyclohexylmethanol (30 mL, 244 mmol, 24 eq) and the reaction heated to 160 \(^0\)C until the sodium had dissolved, the reaction mixture was cooled to 120 \(^0\)C and 2-amino-1-propene-1,1,3-tricarbonitrile (1.32 g, 10.0 mmol, 1 eq) added. The temperature was maintained at 120 \(^0\)C for 48 h before cooling to room temperature. The solvent was removed \textit{in vacuo}. The resulting solid was dissolved in DCM (250 mL) and the precipitate was removed by filtration. The filtrate was purified using repeated rounds of medium pressure column chromatography (silica, 80% Et\(_2\)O, PE) followed by (silica, 40% EtOAc, PE). The crude product was then purified by semi-preparative HPLC (60:40 0.1% formic acid in MeCN:0.1 % aqueous formic acid) to give 162 as a off-white solid (0.11g, 4%). \(R_t\) = 0.8 (6:4 EtOAc:PE); mp = 85.2-86.0 \(^0\)C; UV \(\lambda_{max}\) (EtOH):
341.5, 268.0, 223.0 nm; IR $\nu_{\text{max}}$ 3442 (NH), 3327, 3215, 2922, 2849, 2203 (C=N), 1599, 1552 cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.93-1.21 (5H, m, C$_6$H$_{11}$), 1.59-1.77 (6H, m, C$_6$H$_{11}$), 3.98 (2H, d, $J = 6.6$ Hz, OCH$_2$), 4.45 (2H, s, NH$_2$), 4.54 (2H, s, NH$_2$), 5.28 (1H, s, Ar-H); $\delta_C$ (125 MHz CDCl$_3$): 25.8 (C$_6$H$_{11}$), 26.5 (C$_6$H$_{11}$), 29.7 (C$_6$H$_{11}$), 37.8 (CCH$_2$O), 71.2 (CCN), 71.7 (OCH$_2$), Ar-H (OCH$_2$), 116.8 (CN), 158.0 (CNH$_2$), 159.5 (CNH$_2$), 166.3 (CO); LCMS $R_t = 3.18$ min (1); MS (ESI+) $m/z$ 247.04 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{13}$H$_{19}$N$_4$O: 247.1553 [M+H]$^+$. Found 247.1554 [M+H]$^+$.; Analytical HPLC: 98.1% purity
7.2 MDM2 Experimental Procedures

**General Procedure A** – Freidel-Crafts Reaction

To the appropriate phthalic anhydride (1 eq) was added chlorobenzene (8 eq) and AlCl₃ (2.4 eq) under N₂. The reaction mixture was heated to 90 °C for 2 h before cooling to room temperature and quenching with ice. Concentrated HCl (1 mL per 3 mmol of anhydride) was added and the solution extracted with DCM (3 x 3 mL per mmol of anhydride). The combined organic layers were washed using 20 % Na₂CO₃ aqueous solution (3 mL per mmol of anhydride). On standing a precipitate formed which was collected by filtration, giving the desired 2-(4-chlorobenzooyl) benzoic acid as the sodium salt.

**General procedure B** – Formation of the 2-aryl-3-(4-chlorophenyl)-3-hydroxyisoindolin-1-one

To the appropriate 2-benzoyl benzoic acid (1 eq) in THF was added thionyl chloride (2 eq) followed by DMF (4 drops) under N₂. The reaction mixture was stirred at room temperature for 4 h before then concentrated in vacuo. The residue was dissolved in THF and DIPEA (2.2 eq) and the appropriate benzylamine or benzylamine hydrochloride salt (1.1 eq) was added. The reaction mixture was stirred for 16 h at room temperature, then the solvent removed in vacuo. The residue was partitioned between ethyl acetate (1 mL per 0.1 mmol of benzoyl benzoic acid) and water (1 mL per 0.1 mg). The aqueous layer was extracted with ethyl acetate (2 x 1 mL per 0.1 mmol of benzoyl benzoic acid). The combined organic layers were washed with brine (1 mL per 0.1 mmol of benzoyl benzoic acid), dried (MgSO₄), filtered and evaporated in vacuo. Chromatography (silica, with appropriate solvents) gave the desired 3-hydroxyisoindolinone.

**General procedure C** – Synthesis of the alkoxy isoindolinones via 3-chloride

To the appropriate 3-hydroxy isoindolinone (1 eq) in THF was added thionyl chloride (2 eq) and DMF (4 drops) under N₂ and stirred for 4 h at room temperature, then concentrated in vacuo. The residue was dissolved in THF and K₂CO₃ (2 eq) and the
appropriate diol (2 eq) were added and the reaction stirred at room temperature for 16 h then concentrated in vacuo and the residue partitioned between ethyl acetate (1 mL per 0.1 mmol of isoindolinone) and water (1 mL per 0.1 mmol of isoindolinone). The aqueous layer extracted using ethyl acetate (2 x 1 mL per 0.1 mmol of isoindolinone). The combined organic layers were washed with brine (1 mL per 0.1 mmol of isoindolinone), dried over Na$_2$SO$_4$ and evaporated in vacuo. Chromatography (silica, appropriate solvents) gave the desired compound.

**General procedure D** – Synthesis of alkoxy isoindolinone via Lewis acid catalysis

To a solution of the appropriate 3-hydroxyisoindolinone (1 eq) in either DCM (1.5 mL per 100 mg of isoindolinone) at 0 °C was added BF$_3$.OEt$_2$ (2.5 eq) under N$_2$. After stirring at 0 °C for 15 min, the appropriate diol (10 eq) in DCM (1 mL per 10 mmol of diol) was added dropwise to the reaction mixture. After stirring at 0 °C for 1.5 h the reaction was warmed to room temperature and stirred until the reaction is complete. Saturated NH$_4$Cl (10 mL per 1 mmol of isoindolinone) was then added and extracted using DCM (3 x 40 mL per mmol of isoindolinone). The combined organic layers were dried over Na$_2$SO$_4$ and evaporated in vacuo. Chromatography (silica, appropriate solvents) gave the desired compounds.

**General procedure E** – Formation of alkoxy isoindolinone succinic esters

To a solution of the required 2-(4-aryl)-3-(4-chlorophenyl)-3-((1-(hydroxymethyl)cyclopropyl)methoxy)isoindolin-1-one in THF (3 mL per mmol) was added DMAP (0.2 eq), pyridine (2 eq) and succinic anhydride (2 eq) and the mixture was heated to reflux for 14 h, cooled to room temperature and concentrated in vacuo. The residue was partitioned between ethyl acetate (5 mL per and 0.01 mmol) and water (10 mL per 0.01 mmol). The aqueous layer was extracted with ethyl acetate (2 x 5 mL per 0.01 mmol). The combined organic layers were washed with brine (10 mL per 0.01 mmol), dried over Na$_2$SO$_4$ evaporated in vacuo. Chromatography (silica, appropriate solvent) gave the title compound.
General procedure F - Sonogashira Reaction

To a solution of the required 3-(4-chlorophenyl)-3-hydroxy-2-(4-iodobenzyl)isoindolin-1-one (1 eq) in THF (4 mL per mmol) was added Pd(PPh₃)₂ (0.03 eq) and CuI (0.02 eq) and the solution degassed for 5 min. Et₃N (2.5 eq) and appropriate substituted acetylene (1.3 eq) were added and the solution degassed for a further 15 min, before stirring overnight in the dark, filtering through Celite and washing with MeOH (60 mL per mmol of isoindolinone). Crude product concentrated in vacuo and purified (Chromatography, silica, appropriate solvent).

General procedure G – Triisopropylsilyl group deprotection

To 3-(alkoxy)-3-(4-chlorophenyl)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindol-1-one (1 eq) in THF (mL per mmol) was added 1M tetrabutylammonium fluoride in THF (1.5 eq) and the reaction stirred at room temperature for 1 h. The solvent was removed in vacuo and the crude product purified using chromatography (silica, appropriate solvents).

General procedure H – Formation of the oxetane ring

To a solution of 3-(3-bromo-2,2-bis(hydroxymethyl)propoxyisoindolinone (1 eq) in ethanol (10 mL per mmol) was added powdered potassium hydroxide (1.5 eq) and the reaction mixture stirred at room temperature for 2 h, then heated to reflux for 1 h. The reaction mixture was then cooled to room temperature and neutralised using 1 M aqueous HCl and concentrated in vacuo. The residue was partitioned between water (30 mL per mmol) and EtOAc (100 mL per mmol). The aqueous solution was extracted further with EtOAc (2 x mL per mmol) and the combined organic layers dried over Na₂SO₄ and evaporated in vacuo. Chromatography (silica, appropriate solvents) gave the desired compound.
Sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (238)

**General procedure A**: 3-chlorophthalic anhydride (5.00 g, 27.00 mmol). White solid (7.85 g, 91%). Dried over P₂O₅ before use. \( R_t = 0.5 \) (100 % EtOAc); mp > 350 °C; UV \( \lambda_{max} \) (EtOH): 256 nm; IR \( \nu_{max} \) 2163, 1975, 1666 (Acid C=O), 1608 (Ketone C=O), 1583, 1556 cm⁻¹; \( \delta_H \) (500 MHz \( d_6 \)-DMSO) 7.40-7.49 (4H, m, 4 x Ar-H), 7.54-7.57 (2H, m, 2 x Ar-H), 7.87 (1H, dd, \( J = 1.3 \) and 7.4 Hz, \( CHCOOH \)); \( \delta_C \) (125 MHz \( d_6 \)-DMSO): 128.3, 128.4, 128.9, 129.4, 129.7, 129.8, 136.4, 137.0, 138.9, 141.7 (\( I^3 \)), 167.0 (acid carbonyl), 193.0 (ketone carbonyl); LCMS \( R_t = 3.18 \) min, (1); MS (ESI+) \( m/z \) 295.02 [M+H]⁺

4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-iodobenzyl)isoindolin-1-one (252)

**General procedure B**: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (846 mg, 2.87 mmol) and 4-iodobenzylamine hydrochloride (850 mg, 3.15 mmol). Chromatography (SP4, silica, 7-60% EtOAc, petrol) gave 252 white solid (523 mg, 36%). \( R_t = 0.35 \) (3:7 EtOAc:PE); mp = 233.7-234.3 °C; UV \( \lambda_{max} \) (EtOH): 340.5, 227.5 nm; IR \( \nu_{max} \) 3194, 2929, 1655 (C=O), 1587 (amide C-N) cm⁻¹; \( \delta_H \) (500 MHz \( d_6 \)-DMSO): 4.20 (1H, d, \( J = 15.7 \) Hz, NCH₂), 4.32 (1H, d, \( J = 15.7 \) Hz, NCH₂), 6.92 (2H, d, \( J = 8.3 \) Hz, Ar-H), 7.22-7.7.29 (3H, m, Ar-H), 7.43 (1H, s, OH), 7.50 (2H, d, \( J = 8.3 \) Hz, Ar-H), 7.61-7.62 (2H, m, Ar-H), 7.77 (1H, dd, \( J = 2.6 \) and 5.8 Hz, \( H^3 \)); \( \delta_C \) (125 MHz \( d_6 \)-DMSO): 41.5 (NCH₂), 89.8 (Ar-I), 92.4 (hemi-aminal carbon), 121.6, 128.0, 128.4, 128.6, 130.2, 131.8, 132.8, 133.2, 133.6, 136.5, 136.6, 137.5, 144.3 (CCCI), 165.3 (carbonyl); LCMS \( R_t = 3.78 \) min,(1); MS (ESI+) \( m/z \) 510.06 [M+H]⁺; HRMS \( m/z \): Calc. for C₂₁H₁₅³⁵Cl₂INO₂: 509.9519 [M+H]⁺. Found 509.9507 [M+H]⁺.
3-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (247)

**General procedure F:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-iodobenzyl)-isoindolin-1-one (500 mg, 0.98 mmol) and triisopropylsilylacetylene (286 µL, 1.27 mmol). Chromatography (silica, 30% EtOAc, PE) to give 247 as an off-white solid (488 mg, 88%). \( R_f = 0.54 \) (3:7 EtOAc:PE); mp = 192.6-193.4 °C; UV \( \lambda_{max} \) (EtOH): 266.5, 254.5, 232.0 nm; IR \( \nu_{max} \) 3231, 2942, 2865, 2155 (Ethynyl stretch), 1680 (C=O), 1587 (Amide C-N) cm\(^{-1}\); \( \delta_{H} \) (500 MHz CDCl\(_3\)): 1.03 (18H, s, CH(CH\(_3\))\(_2\)), 1.18 (3H, m, CH(CH\(_3\))\(_2\)), 3.35 (1H, s, OH), 3.98 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 4.43 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 6.97 (2H, d, \( J = 8.3 \) Hz, Ar-H), 7.14-7.16 (6H, m, Ar-H), 7.33-7.35 (2H, m, Ar-H), 7.57 (1H, dd, \( J = 3.1 \) and 5.35, \( H^7 \)); \( \delta_{C} \) (125 MHz CDCl\(_3\)): 11.3 (CH(CH\(_3\))\(_2\)), 18.7 (3 x C(CH\(_3\))\(_2\)), 42.6 (NCH\(_2\)), 90.7 (hemi-aminial carbon), 91.0 (ethynyl), 106.8 (ethynyl), 122.0 (Ar-ethynyl), 122.4, 128.3, 128.5, 128.6, 129.6, 131.5, 131.9, 132.9, 134.0, 134.7, 134.9, 137.7, 143.9 (CCCl), 166.3 (carbonyl); LCMS \( R_t = 4.74 \) min; MS (ESI+) \( m/z \) 564.36 [M+H]\(^+\); HRMS \( m/z \): Calc. for C\(_{32}\)H\(_{36}\)\(^{35}\)Cl\(_2\)NO\(_2\)Si: 564.1887 [M+H]\(^+\). Found: 564.1884 [M+H]\(^+\).

4-Chloro-3-(4-chlorophenyl)-3-(((1-hydroxymethyl)cyclopropyl)methoxy)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-2-one (248)
**General procedure C:** 3-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-((triisopropylsilyl)ethynyl)benzyl)-isoindolin-1-one (308 mg, 0.55 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.11 mL, 1.15 mmol). Chromatography (SP4, silica, gradient 7-60% EtOAc, petrol) gave 248 as a colourless oil (85 mg, 24%). R_f = 0.24 (4:6 EtOAc:PE); UV λ_{max} (EtOH): 340.5, 267.0, 254.5 nm; IR ʋ_{max} 3388, 2940, 2862, 2157 (Ethynyl stretch), 1682 (C=O), 1588 (Amide C-N) cm^{-1}; δ_H (500 MHz CDCl_3): 0.15-0.17 (1H, m, cyclopropyl), 0.29-0.31 (1H, m, cyclopropyl), 0.44-0.46 (2H, m, cyclopropyl), 1.12 (18H, s, CH_3), 2.83 (1H, d, J = 9.1 Hz, OCH_2), 2.91 (1H, d, J = 9.1 Hz, OCH_2), 3.44 (1H, d, J = 11.5 Hz, CH_2OH), 4.21 (1H, d, J = 15.0 Hz, NCH_2), 4.48 (1H, d, J = 15.0 Hz, NCH_2), 7.07 (2H, d, J = 8.25 Hz, Ar-H), 7.20-7.28 (6H, m, Ar-H), 7.46 (1H, dd, J = 1 and 7.9 Hz, \(H^7\)), 7.50-7.53 (2H, Ar-H), 7.81 (1H, dd, J = 1.0 and 7.4 Hz, \(H^7\)); δ_C (125 MHz CDCl_3): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 18.7 (CH(CH_3)_2), 22.1 (cyclopropyl), 42.7 (NCH_2), 67.9 (CH_2OH), 68.1 (OCH_2), 90.9 (ethynyl), 94.6 (ethynyl), 106.7 (hemi-aminol carbon), 122.3, 122.6, 128.3, 128.5, 128.9, 129.7, 131.8, 133.9, 134.2, 134.7, 135.0, 137.2, 140.8, 166.7 (carbonyl); LCMS R_t = 4.78 min, (1); HRMS m/z: Calc. for C_{37}H_{44}Cl_2NO_3Si: 648.2462 [M+H]^+. Found: 648.2447 [M+H]^+.

4-Chloro-3-(4-chlorophenyl)-2-(4-ethynylbenzyl)-3-((1-(hydroxymethyl)cyclopropyl) methoxy)isoindolin-1-one (253)

![Chemical structure](image)

To a solution of 4-chloro-3-(4-chlorophenyl)-3-(((1-hydroxymethyl)cyclopropyl) methoxy)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-2-one (99 mg, 0.15 mmol, 1 eq) in THF (2 mL) was added 1M TBAF in THF (0.23 mL, 0.23 mmol, 1.5 eq). The reaction was stirred at for 75 min, concentrated in vacuo. Chromatography (silica, EtOAc:PE), followed by semi-preparative HPLC (1:1 MeCN: 0.1% aqueous formic acid) gave 253 as a colourless oil (30 mg, 41%). R_t = 0.44 (1:1 EtOAc:PE);
UV $\lambda_{max}$ (EtOH): 229.5 nm; IR $\nu_{max}$ 3438 (OH), 3296, 2924, 2874, 1693 (C=O), 1588 (Amide C-N) cm$^{-1}$; $\delta_{H}$ (500 MHz, CDCl$_3$) 0.05-0.11 (1H, m, cyclopropyl), 0.24-0.30 (1H, m, cyclopropyl), 0.40-0.44 (2H, m, cyclopropyl), 1.67 (1H, s, OH), 2.79 (1H, d, $J = 9.1$ Hz, OCH$_2$), 2.90 (1H, d, $J = 9.1$ Hz, OCH$_2$) 3.04 (1H, s, CCH), 3.40 (1H, d, $J = 11.4$ Hz , CH$_2$OH) 3.50 (1H, d, $J = 11.4$ Hz, CH$_2$OH), 4.16 (2H, d, $J = 15.0$ Hz, NCH$_2$), 4.50 (1H, d, $J = 15.0$ Hz, NCH$_2$), 7.11-7.30 (8H, m, Ar-H) 7.44-7.52 (2H, m, Ar-H), 7.85 (1H, dd, $J = 0.65$ and 7.3 Hz, $R^7$); $\delta_{C}$ (125 MHz, CDCl$_3$): 8.5, (cyclopropyl) 8.5 (cyclopropyl), 22.1 (cyclopropyl), 42.7 (NCH$_2$), 67.9 (OCH$_2$), 68.1 (CH$_2$OH), 83.3 (ethynyl CH), 94.7 (hemi-aminal carbon),121.2 (C-ethynyl), 122.3, 128.3, 128.5, 129.0, 129.7, 131.9, 133.0, 134.0, 134.1, 134.7, 135.0, 137.9, 140.7, 166.7 (carbonyl carbon); LCMS $R_t = 3.71$ min, (1); MS (ESI+) m/z 492.24 [M+H]$^+$; HRMS m/z: Calc. for C$_{28}$H$_{24}$Cl$_2$NO$_3$: 492.1128 [M+H]$^+$ Found: 492.1124 [M+H]$^+$; Analytical HPLC: 99.9% purity

Chiral Separation: mobile phase hexane:ethanol 92.5:7.5

254 Peak 1: (33.8 mg, 42%); Specific rotation $[\alpha] = -9.16$° (at 21.0 °C, wavelength = 589 nm, tube length = 0.1 dm, concentration = 0.328 g per 100 mL);

255 Peak 2: (33.8 mg, 42%); Specific rotation $[\alpha] = +11.4$° (at 21.0 °C, wavelength = 589 nm, tube length = 0.1 dm, concentration = 0.336 g per 100 mL)

4-Chloro-3-((4-chlorophenyl)-3-(((1-methoxy(succinic ester))cyclopropyl)methoxy)-2-(((4-triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (256)

General procedure E: 4-chloro-3-((4-chlorophenyl)-3-(((1-hydroxymethyl) cyclopropyl)methoxy)-2-((4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-2-one (248)
(84 mg, 0.13 mmol). Chromatography (silica, 50-85% EtOAc, petrol) gave 256 as a pale yellow oil (92 mg, 94%). Rf = 0.15 (85:15 EtOAc:PE); UV λmax (EtOH): 266.5, 254.5 nm; IR νmax 2942, 2865, 2158 (Ethynyl stretch), 1708 (Carboxylic acid C=O), 1608 (Amide C=O), 1582 (Amide C-N), 1556 cm⁻¹; δH (500 MHz CDCl3) 0.18-0.20 (1H, m, cyclopropyl), 0.29-0.36 (1H, m, cyclopropyl), 0.47-0.52 (2H, m, cyclopropyl), 1.11 (18H, s, CH₃), 1.24-1.27 (3H, m, CH(CH₃)₂), 2.55-2.64 (4H, m, succinic ester CH₂), 2.71 (1H, d, J = 9.3 Hz, OCH₂), 2.86 (1H, d, J = 9.3 Hz, OCH₂), 3.93 (1H, d, J = 11.4 Hz, CH₂OH), 4.21 (1H, d, J = 11.4 Hz, CH₂OH), 4.29 (1H, d, J = 15.0 Hz, NCH₂), 4.35 (1H, d, J = 15.0 Hz, NCH₂), 4.70 (1H, d, J = 8.2 Hz, Ar-H), 7.15-7.23 (6H, m, Ar-H), 7.43 (1H, dd, J = 1.0 and 8.0 Hz, H°), 7.48-7.51 (1H, m, H°), 7.84 (1H, dd, J = 1.0 and 7.4 Hz, H°); δc (125 MHz CDCl3): 8.9 (cyclopropyl), 11.3 (cyclopropyl), 18.7 (CH(CH₃)₂), 19.4 (CH(CH₃)₂), 28.8 (CH₂CO₂H), 28.9 (COCH₂), 42.6 (NCH₂), 66.5 (OCH₂), 68.4 (CH₂OH), 90.8 (ethyl), 94.4 (ethyl), 106.8 (hemi-aminal), 122.2 (Ar-ethyl), 122.5, 128.3, 128.4, 128.9, 129.9, 131.7, 131.8, 133.8, 134.1, 134.6, 134.8, 137.1, 140.8, 167.0 (isoindolinone carbonyl), 172.1 (succinic ester carbonyl), 177.2 (succinic acid carbonyl)

4-Chloro-3-(4-chlorophenyl)-2-(4-ethynylbenzyl)-3-(((1-methoxy(succinic ester))cyclopropyl)methoxy)isoindolin-1-one (257)

To 4-chloro-3-(4-chlorophenyl)-3-(((1-methoxy(succinic ester))cyclopropyl)methoxy)-2-(((4-trisopropylsilyl)ethyl)benzyl)isoindolin-1-one (81 mg, 0.11 mol) in THF (2 mL) was added 1M TBAF in THF (162 µL, 0.16 mmol). The reaction was stirred for 165 min and concentrated in vacuo. Chromatography (SP4, silica, 3-20% MeOH, EtOAc) to give 257 as a colourless oil (53 mg, 81%). Rf = 0.56 (1:9 MeOH:EtOAc); UV λmax (EtOH): 228.5 nm; IR νmax 3295, 2927, 1706 (Carboxylic
acid C=O), 1588 (Amide C-N) cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 0.20-0.22 (1H, m, cyclopropyl), 0.36-0.39 (1H, m, cyclopropyl), 0.50-0.53 (2H, m, cyclopropyl), 2.57-2.64 (4H, m, succinic 2 x CH\(_2\)), 2.80 (1H, d, \(J = 9.3\) Hz, OCH\(_2\)), 2.84 (1H, d, \(J = 9.3\) Hz, OCH\(_3\)), 3.04 (1H, s, ethynyl), 4.00 (1H, d, \(J = 11.5\) Hz, CH\(_2\)OH), 4.16 (1H, d, \(J = 11.5\) Hz, CH\(_2\)OH), 4.32 (1H, d, \(J = 15.0\) Hz, NCH\(_2\)), 4.39 (1H, d, \(J = 15.0\) Hz, NCH\(_3\)), 7.06 (2H, d, \(J = 8.3\) Hz, Ar-H), 7.18-7.28 (6H, m, Ar-H), 7.45 (1H, dd, \(J = 1.0\) and 8.0 Hz, Ar-H), 7.49-7.52 (1H, m, Ar-H), 7.86 (1H, d, \(J = 1.0\) and 7.4 Hz, Ar-H); \(\delta_C\) (125 MHz CDCl\(_3\)): 8.9 (cyclopropyl), 8.9 (cyclopropyl), 19.4 (cyclopropyl), 28.8 (CH\(_2\)CO\(_2\)H), 29.0 (COCH\(_2\)), 42.6 (NCH\(_2\)), 66.5 (OCH\(_2\)), 68.4 (CH\(_2\)O), 83.3 (ethynyl), 94.5 (ethynyl), 121.2 (Ar-ethynyl), 122.2, 128.3, 128.4, 129.0, 131.0, 131.9, 133.8, 134.2, 134.7, 134.9, 137.7, 140.8, 167.0 (isoindolinone CO), 172.0 (succinic ester carbonyl), 176.0 (succinic acid carbonyl); LCMS \(R_t = 3.67\) min, (1); MS (ESI+) \(m/z\) 592.11 [M+H]\(^+\) HRMS \(m/z\): Calc. for C\(_{32}\)H\(_{28}\)Cl\(_2\)NO\(_6\): 592.1288 [M+H]\(^+\). Found: 592.1290 [M+H]\(^+\).; Analytical HPLC: 95.8% purity

4-((Trifluoromethyl)sulfonyl)benzylamine hydrochloride (265)

![Chemical structure](image)

To 4-((trifluoromethyl)sulfonyl)benzonitrile (400 mg, 1.70 mmol, 1 eq) in THF was added 1M BH\(_3\).THF complex in THF (13.6 mL, 13.60 mmol, 8 eq) and the reaction mixture stirred at room temperature for 1 h then heated to reflux for 4 days before cooling to room temperature. A 1:1 mixture of water (5 mL) and acetic acid (5 mL) was added slowly and stirred at room temperature for 15 min, then concentrated \(\text{in vacuo}\). The resulting white solid was suspended in water (10 mL) cooled in an ice-bath, the pH was adjusted to 8 with a saturated sodium bicarbonate solution then extracted with ethyl acetate (3 x 50 mL). The combined organic layers were dried (MgSO\(_4\)), and concentrated \(\text{in vacuo}\). Chromatography (SP4, silica, 3-20% MeOH, EtOAc). The resulting yellow oil was redissolved in the minimum amount of methanol and 1M HCl (5 mL) added, then evaporated \(\text{in vacuo}\) to give 265 as a white solid (240 mg, 51%) and dried (P\(_2\)O\(_5\)). \(R_t = 0.14\) (1:9 MeOH:EtOAc) mp. = 278.1-278.8 °C; UV \(\lambda_{max}\) (EtOH): 271.0 nm; IR \(\nu_{max}\) 2860, 2162, 1361 (Sulfone), 661 (C-F) cm\(^{-1}\); \(\delta_H\) (500 MHz \(d_6\)-DMSO): 4.26 (2H, s, CH\(_2\)), 7.97 (2H, d, \(J = 8.5\) Hz, Ar-H), 8.24 (2H, d, \(J = 8.5\) Hz, Ar-H), 8.68 (3H, s, NH\(_3\)); \(\delta_C\) (125 MHz \(d_6\)-DMSO): 41.2
(CH$_2$), 120.7, 129.2, 130.9, 131.0, 144.6; $\delta$F (470 MHz $d_6$-DMSO): -78.4; LCMS R$_t$ = 0.44 min, (1); MS (ESI+) $m/z$ 239.99 [M+H]$^+$

4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(((4-trifluoromethyl)sulfonyl)benzyl)isoindolin-1-one (263)

[Chemical structure image]

**General procedure B**: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (156 mg, 0.49 mmol) and 4-((trifluoromethyl)sulfonyl)benzylamine hydrochloride. Chromatography (SP4, silica, 12-100% EtOAc, petrol) to give 263 as an off-white solid (90 mg, 35%). $R_f$ = 0.7 (1:1 EtOAc:PE); mp = 228.3-229.1 °C; UV $\lambda_{\text{max}}$ (EtOH): 340.5 nm; IR $\nu_{\text{max}}$ 3269 (OH), 1682 (C=O), 1363 (sulfone) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 3.05 (1H, s, OH), 4.32 (1H, d, $J = 15.5$ Hz, NCH$_2$), 4.59 (1H, d, $J = 15.5$ Hz, NCH$_2$), 7.13-7.14 (4H, m, Ar-H), 7.39-7.45 (4H, m, Ar-H), 7.73-7.76 (3H, m, Ar-H); $\delta_C$ (125 MHz $d_6$-DMSO): 41.4 (NCH$_2$), 89.6 (hemiaminal) 121.8, 123.2, 127.5, 127.9, 128.4, 128.7, 129.7, 130.3, 131.9, 132.8, 133.0, 133.8, 136.4, 144.2, 148.6, 165.5 (C=O); $\delta_F$ (470 MHz $d_3$-MeCN): -78.4; LCMS R$_t$ = 3.74 min, (1); MS (ESI+) $m/z$ 516.04 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{22}$H$_{14}$Cl$_2$F$_3$N$_2$O$_4$: 514.9967 [M+H]$^+$; Found: 514.9962 [M+H]$^+$.

4-Chloro-3-(4-chlorophenyl)-3-(1-(hydroxymethyl)cyclopropyl)methoxy)-2-((4-((trifluoromethyl)sulfonyl)benzyl)isoindolin-one (246)

[Chemical structure image]
**General procedure C**: 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(((4-trifluoromethyl)sulfonyl)benzyl)isoindolin-1-one \( \text{252} \) (162 mg, 0.31 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.06 mL, mmol). Chromatography (SP4, silica, 12-100 % EtOAc, petrol) gave \( \text{246} \) as a colourless oil (55 mg, 29%). \( R_f = 0.34 \) (1:1 EtOAc:PE); UV \( \lambda_{\text{max}} \) (EtOH): 225.5 nm; IR \( \nu_{\text{max}} \) (OH): 3457 cm\(^{-1}\); \( \delta_H \) (500 MHz \( d_3\)-MeCN): 0.04-0.06 (1H, m, cyclopropyl), 0.13-0.16 (1H, m, cyclopropyl), 0.19-0.23 (2H, m, cyclopropyl), 2.36 (1H, t, \( J = 5.5 \) Hz, OH), 2.56 (1H, d, \( J = 9.1 \) Hz, OCH\(_2\)), 2.89 (1H, d, \( J = 9.1 \) Hz OCH\(_2\)), 3.23 (1H, dd, \( J = 5.0 \) and 11.2 Hz, CH\(_2\)O), 3.34 (1H, dd, \( J = 5.0, 11.2 \) Hz, CH\(_2\)OH), 4.14 (1H, d, \( J = 15.9 \) Hz, NCH\(_2\)), 4.57 (1H, d, \( J = 15.9 \) Hz, NCH\(_2\)), 6.86-6.97 (4H, m, Ar-\( H \)), 7.19 (2H, d, \( J = 8.0 \) Hz, Ar-H), 7.35 (1H, dd, \( J = 1.1 \) and 8.0 Hz, \( H^6 \)), 7.39-7.42 (1H, m, \( H^6 \)), 7.55 (2H, d, \( J = 8.5 \) Hz, Ar-H), 7.63 (1H, dd, \( J = 1.1 \) and 7.4 Hz, \( H^6 \)); \( \delta_C \) (125 MHz \( d_3\)-MeCN): 7.3 (cyclopropyl, 7.4 (cyclopropyl), 21.6 (cyclopropyl), 41.2 (NCH\(_2\)), 64.9 (CH\(_2\)OH) 66.1 (OCH\(_2\)), 93.2 (hemi-amin C), 120.7, 121.7, 127.6, 128.3, 128.4, 129.3, 129.8, 130.1, 132.0, 133.6, 133.9, 135.5, 140.3, 147.6 (CF\(_3\)), 166.1 (C=O); \( \delta_F \) (470 MHz \( d_3\)-MeCN) -93.9; LCMS \( R_f = 3.78 \) min, (1); HRMS \( m/z \): Calc. for \( C_{27}H_{25}^{35}Cl_2F_3N_2O_5S: 617.0886 \) [M+NH\(_4\)]\(^+\). Found: 617.0884 [M + NH\(_4\)]\(^+\); Analytical HPLC: 96.7% purity

3-(4-Chlorophenyl)-2-(4-((trifluoromethyl)sulfonyl)benzyl)isoindolin-1-one (\( \text{262} \))

**General procedure B**: 2-(4-chloro-benzoyl)benzoic acid (56 mg, 0.21 mmol) and 4-((trifluoromethyl)sulfonyl)benzylamine hydrochloride (65 mg, 0.24 mmol). Chromatography (SP4, silica, 15-100 % EtOAc, petrol) gave the title compound as a white solid (88 mg, 87%). \( R_f = 0.55 \) (1:1 EtOAc:PE); mp = 208.8-209.2 °C; UV \( \lambda_{\text{max}} \) (EtOH): 340.5 nm; IR \( \nu_{\text{max}} \) 2393, 1655 (C=O), 1598 (Amide C-N), 1365 (Sulfone) cm\(^{-1}\); \( \delta_H \) (500 MHz \( d_6\)-DMSO): 4.52 (1H, d, \( J = 16.4 \) Hz, NCH\(_2\)), 4.63 (1H, d, \( J = 16.4 \) Hz, NCH\(_2\)), 7.23-7.27 (4H, m, Ar-H), 7.31 (1H, d, \( J = 7.2 \) Hz, Ar-H), 7.41 (1H,
s, OH), 7.57-7.64 (4H, m, Ar-H), 7.81 (1H, dd, J = 0.7 and 6.8 Hz, Ar-H), 7.93 (2H, d, J = 8.4 Hz, H^2); δ_c (125 MHz d_6-DSMO): 41.8 (NCH2), 90.0 (semi-aminal carbon), 122.8, 122.9, 127.4, 128.0, 128.2, 129.6, 129.8, 129.9, 130.4, 132.8, 133.1, 138.5, 148.9, 149.0, 166.9 (carbonyl); δ_F (470 MHz, d_6-DSMO) -78.6; LCMS R_t = 3.64 min, (1); MS (ESI+) m/z 482.11 [M+H]^+; HRMS m/z: Calc. for C_{22}H_{16}ClF_3N_O_4S: 482.0435 [M+H]^+. Found: 482.0431 [M+H]^+.

3-((4-Chlorophenyl)-3-(1-((hydromethyl)cyclopropyl)methoxy)-2-(((trifluoromethyl)sulfonyl)benzyl)isoindolin-1-one (245)

**General procedure D:** 3-((4-chlorophenyl)-2-(4-((trifluoromethyl)sulfonyl)cyclopropyl)methoxy)benzyl)isoindolin-1-one (209 mg, 0.43 mmol) and 1,1-bis(hydroxymethyl)cyclopropyl (0.27 mL, 4.34 mmol). The reaction was quenched after 1.5 h at 0 °C and 1 h at room temperature. Chromatography (SP4, silica, 15-100% EtOAc, petrol) gave 245 as a colourless oil (151 mg, 62%). R_t = 0.43 (1:1 EtOAc:PE); UV λ_max (EtOH): 203.0 nm; IR ν_max 3408 (OH), 2159, 1698 (C=O), 1597 (Amide C-N), 1364 (Sulphone), 1216 (CF_3), 1191 cm^{-1}; δ_H (500 MHz d_3-MeCN): 0.15-0.18 (1H, m, cyclopropyl), 0.20-0.23 (1H, cyclopropyl), 0.36-0.38 (2H, m, cyclopropyl), 2.56 (1H, t, J = 5.7 Hz, OH), 2.82 (1H, d, J = 9.3 Hz, OCH_2), 2.87 (1H, d, J = 9.3 Hz, OCH_2), 3.41 (1H, dd, J = 6.2 and 11.2 Hz, CH_2OH), 3.47 (1H, dd, J = 6.2 Hz and 11.2 Hz, CH_2OH), 4.43 (1H, d, J = 15.8 Hz, NCH_2), 4.72 (1H, d, J = 15.8 Hz, NCH_2), 7.10 (2H, d, J = 8.9 Hz, Ar-H), 7.18-7.20 (3H, m, Ar-H), 7.46 (2H, d, J = 8.6 Hz, Ar-H), 7.59-7.61 (2H, m Ar-H), 7.79 (2H, d, J = 8.4 Hz, Ar-H), 7.86-7.88 (1H, m, H^3) δ_c (125 MHz d_3-MeCN): 7.3, 7.3 (cyclopropyl), 21.6 (cyclopropyl), 41.4 (NCH_2), 64.9 (CH_2OH), 65.7 (OCH_2), 93.5 (semi-aminal carbon), 122.9, 123.0, 127.8, 128.0, 128.5, 129.9, 130.1, 133.5, 137.3, 144.8, 148.0 (CCl), 167.5 (carbonyl); δ_F (470 MHz d_3-MeCN): -79.8;
LCMS $R_t = 3.69$ min, (1); HRMS $m/z$: Calc. for C$_{27}$H$_{27}$$^{35}$Cl$_2$F$_3$N$_2$O$_5$S: 583.1261 [M+NH$_4$]$^+$. Found: 583.1276 [M + NH$_4$]$^+$. Analytical HPLC: 99.6% purity.

4-Chloro-3-(4-chlorophenyl)-2-(3,4-difluorobenzyl)-3-hydroxyisoindolinone (268)

**General procedure B:** sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (750 mg, 2.37 mmol) and 3,4-difluorobenzylamine (618 µL, 5.22 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 268 as a white solid (390 mg, 39%). $R_t = 0.45$ (3:7 EtOAc:PE); mp = 147.3-149.1 °C; UV $\lambda_{max}$ (EtOH): 210.0 nm; IR $\nu_{max}$ 3244 (OH), 2162, 1686 (C=O), 1588 (amideC-N), 1278 (C-F), 815, 757 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 3.83 (1H, s, OH), 4.03 (1H, $d$, $J = 14.7$ Hz, NCH$_2$), 4.33 91H, $d$, $J = 14.7$ Hz, NCH$_2$), 6.71-6.74 (1H, m, benzyl Ar-H), 6.83 (1H, ap. dt, $J = 8.2$ and 10.2 Hz, benzyl Ar-H), 6.88 (1H, ddd, $J = 2.1$, 7.7 and 11.0 Hz, benzyl Ar-H), 7.15-7.19 (4H, m, Ar-H), 7.36-7.36 (2H, m, $H^5$ and $H^6$), 7.58-7.60 (1H, m, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 41.9 (NCH$_2$), 90.8 (hemi-aminal), 116.8 (d, $J = 17.1$ Hz, benzyl Ar-H), 117.7 (d, $J = 17.3$ Hz, benzyl Ar-H), 122.0, 124.8 (dd, $J = 3.6$ and 6.4 Hz, benzyl Ar-H), 128.2, 128.5, 129.7, 131.6, 134.1, 134.4 (dd, $J = 4.0$ and 5.3 Hz, benzyl Ar-H), 134.8, 134.9, 143.8, 148.7 (dd, $J = 12.6$ and 34.7 Hz, benzyl Ar-H), 150.7 (dd, $J = 12.6$ and 35.1 Hz, benzyl Ar-H), 166.3 (C=O); $\delta_F$ (470 MHz CDCl$_3$) -139.7 (d, $J = 20.7$ Hz), -137.7 (d, $J = 21.1$Hz); LCMS $R_t = 1.63$ min, (4); MS (ESI+) $m/z$ 420.2 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{21}$H$_{13}$$^{35}$Cl$_2$F$_2$NO$_2$: 420.0364 [M+H]$^+$. Found 420.0365 [M+H]$^+$. 

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General procedure C: 4-Chloro-3-(4-chlorophenyl)-2-(3,4-difluorobenzyl)-3-hydroxyisoindolinone (210 mg, 0.50 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.1 mL, 1.00 mmol). Chromatography (SP4, silica, 10-80% EtOAc, PE) gave 269 as a colourless oil (59 mg, 23%). $R_f = 0.24$ (4:6 EtOAc:PE); UV $\lambda_{\text{max}}$ (EtOH): 232.0, 258.0 nm; IR $\nu_{\text{max}}$ (500 MHz CDCl$_3$): 0.17-0.20 (1H, m, cyclopropyl), 0.32-0.35 (1H, m, cyclopropyl), 0.45-0.51 (2H, m, cyclopropyl), 1.78 (1H, s, OH), 2.86 (1H, d, $J = 9.2$ Hz, OCH$_2$), 2.92 (1H, d, $J = 9.2$ Hz, OCH$_2$), 3.49 (1H, d, $J = 11.4$ Hz, $CH_2$OH), 3.56 (1H, d, $J = 11.4$ Hz, $CH_2$OH), 6.79-6.82 (1H, m, Benzyl Ar-H), 6.91 (1H, ap. dt, $J = 8.3$ and 10.2 Hz, benzyl Ar-H), 6.97 (1H, ddd, $J = 2.1$, 7.6 and 10.7, benzyl Ar-H), 7.18-7.19 (4H, m, Ar-H), 7.45 (1H, dd, $J = 1.0$ and 8.0 Hz, $H^6$), 7.49-7.52 (1H, m, $H^6$), 7.84 (1H, d, $J = 1.0$ and 7.4, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 22.2 (cyclopropyl), 41.9 (NCH$_2$) 67.7 ($CH_2$OH), 68.0 (OCH$_2$), 94.4 (hemi-aminial), 116.8 (d, $J = 17.1$ Hz, benzyl Ar-H), 118.1 (d, $J = 17.3$, benzyl Ar-H), 122.3, 125.1 (dd, $J = 3.6$ and 6.2, benzyl Ar-H), 128.3, 128.4, 129.8, 131.9, 134.0, 134.8, 135.0, 140.7, 148.8 (dd, $J = 6.4$ and 21.0, benzyl Ar-F), 150.7 (dd, $J = 12.7$ and 21.3, benzyl Ar-H), 166.7 (C=O); $\delta_F$ (470 MHz CDCl$_3$) -138.88 (d, $J = 21.9$), -137.62 (d, $J = 21.9$); LCMS $R_t$=1.66 min, (3); HRMS m/z: Calc. for C$_{26}$H$_{22}$Cl$_2$F$_2$NO$_3$: 504.0939 [M+H]$^+$. Found: 504.0937 [M+H]$^+$; Analytical HPLC: 98.3% purity
4-Chloro-3-fluorobenzylamine (271)

To a solution of 4-chloro-3-fluorobenzonitrile (500 mg, 3.21 mmol, 1 eq) in THF (1 mL) was added 1M BH$_3$.THF in THF (13 mL, 12.9 mmol, 4 eq) and the mixture stirred at room temperature for 15 min then heated to reflux for 16 h, cooled, and diluted by dropwise addition of a 1:1 solution of AcOH (10 mL) and water (10 mL). Stirred at RT for 15 min, concentrated in vacuo. The residue was cooled in an ice-bath and basified to pH 9 by sat. NaHCO$_3$, then extracted using EtOAc (3 x 50 mL). Combined organic extracts were washed with brine (50 mL), dried over MgSO$_4$ and concentrated in vacuo. Chromatography (SP4, amino silica, 50-100% EtOAc, PE) gave 271 as a white solid (321 mg, 63%). $R_f$ = 0.71 (1:4 EtOAc:PE, amino silica); mp = 93.5-95.9 °C; UV $\lambda_{max}$ (EtOH): 253.5, 217.0 nm; IR $\nu_{max}$ 3295 (N-H), 2491, 2172, 1231 (C-F) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.48 (2H, s, NH$_2$), 3.86 (2H, s, CH$_2$), 7.04 (1H, d, $J$ = 8.0 Hz, CHCF), 7.14 (1H, d, $J$ = 10.0 Hz, CHCCl), 7.34 (1H, ap. t, $J$ = 8.0 Hz, Ar-H); $\delta_C$ (125 MHz CDCl$_3$): 45.2 (CH$_2$), 115.2 (d, $J$ = 20.9 Hz, CHCF), 119.0 (d, $J$ = 17.5 Hz, CCl), 123.3 (d, $J$ = 14.1 Hz, Ar-H), 130.5 (CHCCl), 144.1 (CCH$_2$), 158.2 (d, $J$ = 227.2 Hz, CF); $^1$F (470 MHz CDCl$_3$): -115.6; LCMS (R$_f$ = 0.81 min, (3); MS (ESI+) m/z 160.1 [M+H]$^+$; HRMS m/z: Calc. for C$_7$H$_8$F$_3$ClN: 160.0324 [M+H]$^+$. Found 160.0320 [M+H]$^+$.

4-Chloro-2-(4-chloro-3-fluorobenzyl)-3-(4-chlorophenyl)-3-hydroxyisoindolinone (273)

General procedure B: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (1.06 g, 3.34 mmol) and 4-chloro-3-fluorobenzylamine (587 mg, 3.63 mmol). Chromatography (SP4, silica, 7-60 % EtOAc, PE) gave 273 as a white solid (538 mg, 37%). $R_f$ = 0.39
(3:7 EtOAc:PE); mp = 184.7-187.0 °C; UV $\lambda_{\text{max}}$ (EtOH): 231.0 nm; IR $\nu_{\text{max}}$ 3217, 2158, 2028, 1683 (C=O), 1588 (amide C-N), 1093 (C-F), 814, 757 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 3.44 (1H, s, OH), 4.03 (1H, d, $J = 15.2$ Hz, NCH$_2$), 4.42 (1H, d, $J = 15.2$ Hz, NCH$_2$), 6.79 (1H, d, $J = 7.9$ Hz, CHCF), 6.88 (1H, d, $J = 9.8$ Hz, CHCCl), 7.09 (1H, ap. t, $J = 7.9$ Hz, benzyl Ar-H), 7.17-7.18 (4H, m, Ar-H), 7.37-7.40 (2H, m, H$_5$ and H$_6$), 7.64 (1H, dd, $J = 2.4$ and 5.8 Hz, H$_7$); $\delta_C$ (125 MHz CDCl$_3$): 42.0 (NCH$_2$), 90.8 (hemi-aminal), 116.9 (d, $J = 20.6$ Hz, CHCCl), 138.4 (d, $J = 25.8$ Hz, CCH$_2$), 143.7, 157.7 (d, $J = 247.4$ Hz, CF), 166.2 (C=O); $\delta_F$ (470 MHz CDCl$_3$): -115.3; LCMS $R_t = 1.68$ min, (4); MS (ESI+) m/z 436.2 [M+H]$^+$; HRMS m/z: Calc. for C$_{21}$H$_{14}$Cl$_3$FNO$_3$: 436.0069 [M+H]$^+$. Found 436.0068 [M+H]$^+$.  

4-Chloro-2-(4-chloro-3-fluorobenzyl)-3-(4-chlorophenyl)-3-((1-(hydroxymethyl)cyclopropyl)methoxy)isoindolin-1-one (274) 

**General procedure C**: 4-chloro-2-(4-chloro-3-fluorobenzyl)-3-(4-chlorophenyl)-3-hydroxyisoindolinone (501 mg, 1.15 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.22 mL, 2.29 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) followed by (SP4, C$_{60}$, 40-100% 0.1% HCOOH in MeCN, 0.1% HCOOH in water) to give 274 as a white oily solid (107 mg, 18%). $R_t = 0.25$ (3:7 EtOAc:PE); UV $\lambda_{\text{max}}$ (EtOH): 224.0 nm; IR $\nu_{\text{max}}$: 3375 (OH), 2925, 2361, 1694 (C=O), 1585 (Amide C-N), 1489, 1063 (C-F) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.17-0.20 (1H, m, cyclopropyl), 0.32-0.35 (1H, m, cyclopropyl), 0.45-0.51 (2H, m, cyclopropyl), 1.66 (1H, s, OH), 2.86 (1H, d, $J = 9.1$ Hz, OCH$_2$), 2.92 (1H, d, $J = 9.1$ Hz, OCH$_2$), 3.48 (1H, d, $J = 11.1$ Hz, CH$_2$OH), 3.56 (1H, d, $J = 11.1$ Hz, CH$_2$OH), 3.21 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.36 (1H, d, $J = 15.1$, NCH$_2$), 6.83 (1H, d, $J = 8.2$ Hz, Benzyl Ar-H), 6.93 (1H, dd, $J = 1.7$ and 9.8 Hz, benzyl Ar-H), 7.14-7.20 (5H, m, Ar-H), 7.47 (1H, d, $J = 7.9$ Hz, H$_7$), 7.50-7.53 (1H,
m, $H^6$, 7.85 (1H, d, $J = 7.4$ Hz, $H^7$); δC (125 MHz CDCl$_3$): 8.5 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 41.9 (NCH$_2$), 67.7 (CH$_2$OH), 67.9 (OCH$_2$), 94.4 (hemi-aminal), 117.3 (d, $J = 21.2$ Hz, CHCF), 120.0 (d, $J = 17.5$ Hz, CCl), 122.3, 125.4 (d, $J = 3.6$ Hz, benzyl Ar), 128.3, 128.5, 130.0, 130.2, 132.0, 134.0, 134.0, 134.9 (d, $J = 10.2$ Hz, benzyl Ar), 137.8 (d, $J = 6.4$ Hz, benzyl Ar), 140.6, 157.6 (d, $J = 247.9$ Hz, C-F), 166.7 C=O); δF (470 MHz CDCl$_3$): -115.2; LCMS $R_t$ = 1.74 min, (3); MS (ESI+) m/z 520.3 [M+H]$^+$; HRMS m/z: Calc. for C$_{26}$H$_{22}$Cl$_3$FNO$_3$: 520.0644 [M+H]$^+$. Found 520.0643 [M+H]$^+$.; Analytical HPLC: 97.7% purity

4-Bromo-3-fluorobenzylamine (278)

![Image of 4-Bromo-3-fluorobenzylamine](image)

To a solution of 4-bromo-3-benzonitrile (500 mg, 2.50 mmol, 1 eq), in THF (1 mL) was added 1M BH$_3$.THF in THF (10 mL, 10.0 mmol, 4 eq) and the reaction was stirred at RT for 20 min then heated to reflux for 14 h, cooled, and a 1:1 solution of AcOH(10 mL) and water (10 mL) was added dropwise with stirring for 15 min, concentrated in vacuo. The resulting solid was cooled in an ice-bath, suspended in water (10 mL) and basified to pH 9 with sat. NaHCO$_3$ solution, and extracted using EtOAc (3 x 70 mL). The combined organic extracts were washed with brine (50 mL), dried over MgSO$_4$ and concentrated in vacuo. Chromatography (SP4, amino silica, 60-100% EtOAc, PE) gave 278 as a white solid (432 mg, 84%). $R_t$ = 0.87 (7:3 EtOAc:PE, amino silica); mp = 90.8-93.0 °C; UV $\lambda_{max}$ (EtOH): 256.0 nm; IR $\nu_{max}$ 3297 (N-H), 2873, 1574, 1237 (C-F) cm$^{-1}$; δH (500 MHz CDCl$_3$): 1.48 (2H, s, NH$_2$), 3.85 (2H, s, CH$_2$), 6.99 (1H, d, $J = 8.1$ Hz, CHCF), 7.12 (1H, d, $J = 9.5$ Hz, Ar-H), 7.48 (1H, s, ap. t, $J = 7.6$ Hz, Ar-H); δC (125 MHz CDCl$_3$): 45.5 (CH$_2$), 115.1 (d, $J = 22.1$ Hz, CHCF), 123.8 (d, $J = 13.1$ Hz, CHCCH$_2$), 133.4 (CHCBr), 145.2 (CCH$_2$), 159.2 (d, $J = 248.7$, CF); LCMS $R_t$ = 0.31 min, (1) ; MS (ESI+) m/z 203.8 [M+H]$^+$; HRMS m/z: Calc. for C$_7$H$_8$FBrFN: 203.9819 [M+H]$^+$. Found: 203.9822 [M+H]$^+$. 
2-(4-Bromo-3-fluorobenzyl)-4-chloro-3-(4-chlorophenyl)-3-hydroxyisoindolinone (279)

General procedure B: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (989 mg, 3.12 mmol) and 4-bromo-3-fluorobenzylamine (700 mg, 3.43 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 279 as an off-white solid (765 mg, 51%). R_f = 0.43 (3:7 EtOAc:PE); mp = 192.4-195.1 °C; UV λ_max (EtOH): 230.0 nm; IR ʋ_max (O-H) 3236 cm⁻¹, 2157, 1587 (Amide C-N), 1400, 1093 (C-F), 818, 756 (C-Cl) cm⁻¹; δ_H (500 MHz CDCl₃): 3.08 (1H, s, OH), 4.03 (1H, d, J = 15.2 Hz, NCH₂), 4.46 (1H, d, J = 15.2 Hz, NCH₂), 6.77 (1H, dd, J = 1.8 and 4.1 Hz, benzyl Ar-H), 6.89 (1H, dd, J = 1.8 and 9.4 Hz, CHCF), 7.18-7.19 (4H, m, Ar-H), 7.26 (1H, dd, J = 7.3 and 8.1 Hz, CHCBr), 7.37-7.42 (2H, m, H⁻⁵ and H⁻⁶), 7.68 (1H, dd, J = 1.7 and 6.7 Hz, H⁻⁷); δ_C (125 MHz CDCl₃): 42.0 (NCH₂), 90.8 (hemi-aminal), 107.8 (d, J = 20.8 Hz, C-Br), 116.8 (d, J = 22.5 Hz, CHCF), 122.2, 125.5 (d, J = 3.5 Hz, benzyl Ar-H), 128.2, 128.6, 129.6, 131.8, 132.8, 133.2, 134.2, 134.9 (d, J = 26.7 Hz, CHCBr), 139.3 (d, J = 6.6 Hz, benzyl CH₂C), 143.6, 158.7 (d, 246.2 Hz, CF), 166.1; δ_F (470 MHz CDCl₃): -107.2; LCMS R_t = 1.70 min, (4); MS (ESI+) m/z 579.4 [M+H]^+; HRMS m/z: Calc. for C_{21}H_{13}Br^{35}ClFNO₂: 479.9564 [M+H]^+. Found 479.9551 [M+H]^+.

2-(4-Bromo-3-fluorobenzyl)-4-chloro-3-(4-chlorophenyl)-3-((1-(hydroxymethyl)cyclopropane)methoxy)isoindolin-1-one (281)

General procedure C: 2-(4-bromo-3-fluorobenzyl)-4-chloro-3-(4-chlorophenyl)-3-hydroxyisoindolinone (294 mg, 0.61 mmol) and 1,1-bis(hydroxymethyl)cyclopropane

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(0.12 mL, 1.22 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) followed by (SP4, silica, 20-100% EtOAc, PE) gave 281 as an oily white solid (65 mg, 19%). Rf = 0.33 (6:4 EtOAc:PE); UV λmax (EtOH): 260.0, 225.0, 205.0 nm; IR νmax 3458 (OH), 2925, 2872, 2159, 1694 (C=O), 1587 (Amide C-N), 1075 (C-F) cm⁻¹; δH (500 MHz CDCl3): 0.17-0.20 (1H, m, cyclopropyl), 0.32-0.35 (1H, m, cyclopropyl), 0.45-0.51 (2H, m, cyclopropyl), 1.67 (1H, s, OH), 2.86 (1H, d, J = 9.2 Hz, OCH2), 2.92 (1H, d, J = 9.2 Hz, OCH2), 3.48 (1H, d, J = 11.4 Hz, CH2OH), 3.55 (1H, d, J = 11.4 Hz, CH₂OH), 4.27 (1H, d, J = 9.2 Hz, OCH2), 4.36 (1H, d, J = 9.2 Hz, OCH2), 3.48 (1H, d, J = 11.4 Hz, CH2OH), 3.55 (1H, d, J = 11.4 Hz, CH₂OH), 6.78 (1H, dd, J = 2.0 and 8.2 Hz, benzyl Ar-H), 6.91 (1H, dd, J = 2.0 and 9.3 Hz, CHCF), 7.19-7.20 (4H, m, Ar-H), 7.31 (1H, dd, J = 7.1 and 8.2 Hz, CHCBR), 7.46 (1H, dd, J = 1.1 and 8.0 Hz, H5), 7.50-7.53 (1H, m, H6), 7.85 (1H, dd, J = 1.1 and 7.4 Hz, H7); δC (125 MHz CDCl3): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 42.0 (cyclopropyl), 67.8 (CH2OH), 68.0 (OCH2), 94.4 (hemi-aminal), 107.9 (d, J = 20.7 Hz, C-Br), 117.2 (d, J = 22.5 Hz, CHCF), 122.4 (C4), 125.6 (d, J = 3.4 Hz, benzyl Ar-H), 128.2, 128.5, 129.8, 132.0 (C5), 133.1 (CHCBr), 134.0, 134.0, 138.7 (d, J = 6.4 Hz, benzyl C), 134.9, 140.6, 158.6 (d, J = 246.5 Hz, C-F), 166.7 (C=O); δF (470 MHz CDCl3): -107.2; LCMS Rf = 1.75 min, (3); HRMS m/z: Calc. for C26H22BrCl3FNO3: 564.0139 [M+H]^+; Found 564.0137 [M+H]^+; Analytical HPLC: 95.3% purity

3-Fluoro-4-iodobenzylamine (277)

To a solution of 3-fluoro-4-iodobenzonitrile (500 mg, 2.02 mmol, 1 eq) in THF (1 mL) was added 1M BH3·THF in THF (8.1 mL, 8.10 mmol, 4 eq) and the reaction stirred at RT for 30 min then heated to reflux for 18 h, cooled, diluted with 1:1 AcOH (10 mL) and water (10 mL) added dropwise with stirring for 15 min before concentrated in vacuo. The resulting solid was cooled in an ice-bath, diluted with water (10 mL) and basified to pH 9 using sat. NaHCO3 solution, and extracted using EtOAc (3 x 70 mL). The combined organic extracts were washed with brine (50 mL) and dried over MgSO4. Chromatography (SP4, amino silica, 60-100% EtOAc, PE) to give 277 as a white solid (414 mg, 82%). Rf = 0.46 (Amino silica) (9:1 EtOAc:PE);
mp = 96.9-99.3 °C; UV \( \lambda_{\text{max}} \) (EtOH): 230.0 nm; IR \( \nu_{\text{max}} \) 3283 (N-H), 3.84 (2H, s, NH2), 6.87 (1H, d, \( J = 8.0 \) Hz, CHCl), 7.06 (1H, d, \( J = 8.7 \) Hz, CHCF), 7.67 (1H, dd, \( J = 6.7 \) and 7.8 Hz, Ar-H); \( \delta_{\text{C}} \) (125 MHz CDCl3): 45.5 (CH2), 78.6 (d, \( J = 26.3 \) Hz, Cl), 114.3 (d, \( J = 24.0 \) Hz, CHCF) 124.4 (d, \( J = 3.8 \) Hz, Ar-H), 139.2 (d, \( J = 1.3 \) Hz, CHCl), 146.4 (d, \( J = 6.3 \) Hz, CCH2), 161.9 (d, 243.8 Hz, CF); \( \delta_{\text{F}} \) (470 MHz CDCl3): -94.2; LCMS R\(_t\) = 0.61 min, (3); MS (ESI+) m/z 252.0 [M+H]+.

4-Chloro-3-(chlorophenyl)-3-hydroxy-2-(3-fluoro-4-iodobenzyl)isoindolin-1-one (280)

**General procedure B:** sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (818 mg, 2.58 mmol) and 3-fluoro-4-iodobenzylamine (712 mg, 2.84 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) to give 280 as a off-white solid (443 mg, 33%). \( R_f = 0.48 \) (3:7 EtOAc:PE); mp = 197.5-200.0 °C; UV \( \lambda_{\text{max}} \) (EtOH): 227.0 nm; IR \( \nu_{\text{max}} \) 3264 (OH), 2362, 1683 (C-O), 1587 (Amide C-N), 1092 (C-F), 816, 757 (C-Cl) cm\(^{-1}\); \( \delta_{\text{H}} \) (500 MHz CDCl3): 3.32 (1H, s, OH), 4.02 (1H, d, \( J = 15.2 \) Hz, NCH2), 4.43 (1H, d, \( J = 15.2 \) Hz, NCH2), 6.63 (1H, dd, \( J = 1.6 \) and 8.1 Hz, benzyl Ar-H), 6.81 (1H, dd, \( J = 1.6 \) and 8.7 Hz, benzyl Ar-H), 7.17-7.7.41 (6H, m, Ar-H), 7.44 (1H. dd. \( J = 6.7 \) and 7.9 Hz, \( H^6 \)), 7.65 (1H, dd, \( J = 2.1 \) and 6.3 Hz, \( H^7 \)); \( \delta_{\text{C}} \) (125 MHz CDCl3): 42.0 (NCH2), 90.8 (hemiaminal), 116.0 (d, \( J = 24.1 \) Hz, benzyl Ar-H), 122.2, 126.1 (d, \( J = 3.4 \)Hz, benzyl Ar-H), 128.2, 128.6, 129.6, 131.7, 132.8, 132.2, 134.7, 135.0, 139.0, 140.5 (d, \( J = 7.4 \) Hz, benzyl Ar-H)143.7, 161.4 (d, \( J = 245.2 \) Hz, C-F), 166.1 (C-O); \( \delta_{\text{F}} \) (470 MHz CDCl3): -93.9; LCMS R\(_t\) = 1.72 min, (4); MS (ESI+) m/z 528.1[M+H]+; HRMS m/z: Calc. for C\(_{21}\)H\(_{14}\)\(^{35}\)Cl\(_2\)FINO\(_3\): 527.9425 [M+H]+. Found 527.9411 [M+H]+.
4-Chloro-3-(4-chlorophenyl)-3-((1-(hydroxymethyl)cyclopropane)methoxy)-2-(3-fluoro-4-iodobenzyl)isoindolin-1-one (282)

**General procedure C:** 4-chloro-3-(chlorophenyl)-3-hydroxy-2-(3-fluoro-4-iodobenzyl)isoindolin-1-one (332 mg, 0.63 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.12 mL, 1.26 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 282 as a colourless oil (138 mg, 36%). $R_t = 0.22$ (3:7 EtOAc:PE); UV $\lambda_{max}$ (EtOH): 227.5 nm; IR $\nu_{max}$: 3481 (OH), 2924, 2876, 2158, 1693 (C=O), 1588 (Amide C-N), 1012 (C-F), 727 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.16-0.19 (1H, m, cyclopropyl), 0.31-0.34 (1H, m, cyclopropyl), 0.44-0.49 (2H, m, cyclopropyl), 1.73 (1H, s, OH), 2.86 (1H, d, $J = 9.1$ Hz, OCH$_2$), 2.91 (1H, d, $J = 9.1$ Hz, OCH$_2$), 3.48 (1H, d, $J = 11.4$, CH$_2$OH), 3.54 (1H, d, $J = 11.4$ Hz, CH$_2$OH), 4.27 (1H, d, $J = 15.1$, NCH$_2$), 4.35 (1H, d, $J = 15.1$ Hz, NCH$_2$), 6.65 (1H, dd, $J = 1.8$ and $8.1$ Hz, benzyl Ar-H), 6.77 (1H, dd, $J = 1.8$ and $8.7$ Hz, CHCF), 7.17-7.18 (4H, m, Ar-H), 7.45-7.52 (3H, m, $H^5$, $H^6$ and CHCl), 7.84 (1H, dd, $J = 0.7$ and $7.4$ Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 8.5 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 41.9 (NCH$_2$), 67.1 (CH$_2$OH), 67.9 (OCH$_2$), 79.7 (d, $J = 25.4$ Hz, C-I), 94.4 (hemi-aminal), 116.4 (d, $J = 24.1$ Hz, CHCF), 122.4, 126.4 (d, $J = 3.2$ Hz, benzyl C-H), 128.3, 128.5, 129.8, 131.9, 134.0, 134.9 (d, $J = 2.8$ benzyl CCH$_2$), 139.8 (d, $J = 6.6$ Hz, CHCl), 139.8, 139.9, 140.6, 161.3 (d, $J = 244.7$ Hz, C-F), 166.7 (C=O); $\delta_F$ (470 MHz CDCl$_3$): -93.8; HRMS m/z: Calc. for C$_{26}$H$_{22}$Cl$_2$FINO$_3$: 612.0001 [M+H]$^+$; Found 612.0001 [M+H]$^+$; Analytical HPLC: 99.2% purity
4-Chloro-3-(4-chlorophenyl)-2-(3-fluoro-4-((4-triisopropylsilyl)ethynyl)benzyl)-3-hydroxyisoindolin-1-one (283)

**General procedure F**: 4-chloro-3-(chlorophenyl)-3-hydroxy-2-(3-fluoro-4-iodobenzyl)isoindolin-1-one (250 mg, 0.47 mmol) and triisopropylsilylacetylene (138 µL, 0.62 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) to give 283 as an oily brown solid (261 mg, 95%). $R_f = 0.66$ (3:7 EtOAc:PE); mp = 180.0-182.2 °C; UV $\lambda_{max}$ (EtOH): 224.0 nm; IR $\nu_{max}$ 3305 (OH), 2159 (Ethynyl stretch), 1687 (C=O), 1588 (Amide C-N), 1092 (C-F), 757 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.04 (18H, s, CH$_3$), 3.51 (1H, s, OH), 3.99 (1H, d, $J = 15.3$ Hz, NCH$_2$), 4.45 (1H, d, $J = 15.3$ Hz, NCH$_2$), 6.76-6.79 (2H, m, Ar-H), 7.13-7.19 (5H, m, Ar-H), 7.35-7.40 ($H^5$ and $H^6$), 7.62 (1H, dd, $J = 2.2$ and 6.2 Hz); $\delta_C$ (125 MHz CDCl$_3$): 11.2 (CH$_3$), 18.6 ($CH(CH_3)_2$), 42.3 (NCH$_2$), 90.0 (C(Si(CH$_3$)$_3$)$_3$), 96.9 (CCSi(CH$_3$)$_3$)$_3$, 99.9 (C-CCSi(CH$_3$)$_3$)$_3$, 115.6 (d, $J = 21.3$ Hz, benzyl Ar-H), 122.1, 124.0, 124.0 (d, $J = 8.0$ Hz, benzyl Ar-H), 128.2, 128.6, 129.6, 131.6, 132.7, 133.5, 134.1, 134.8 (d, $J = 14.2$ Hz, benzyl Ar-H), 140.1 (d, $J = 7.2$ Hz, benzyl Ar-H), 143.8, 166.2 (C=O); $\delta_F$ (470 MHz CDCl$_3$): -109.6; LCMS $R_t = 2.25$ min, (4); MS (ESI+) $m/z$ 582.4 [M+H]$^+$; HRMS $m/z$: Calc. C$_{32}$H$_{35}$ClFNO$_2$Si: 582.1793 [M+H]$^+$. Found 582.1780 [M+H]$^+$. 

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4-Chloro-3-(4-chlorophenyl)-2-(3-fluoro-4-((triisopropylsilyl)ethynyl)benzyl)-3-(1-
((hydroxymethyl)cyclopropane)methoxy)isoindolin-1-one (284)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-2-(3-fluoro-4-((4-
triisopropylsilyl)ethynyl)benzyl)-3-hydroxyisoindolin-1-one (261 mg, 0.45 mmol)
and 1,1-bis(hydroxymethyl)cyclopropane (0.09 mL, 0.45 mmol). Chromatography
(SP4, silica, 7-60% EtOAc, PE) gave 284 as a colourless oil (77 mg, 26%). $R_f = 0.23$
(3:7 EtOAc:PE); UV $\lambda_{\text{max}}$ (EtOH): 260.5 nm; IR $\nu_{\text{max}}$ 3397, 2943, 2865, 2160, 1698
(C=O), 1588 (Amide C-N), 1013 (C-F), 760 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.13-
0.16 (1H, m, cyclopropyl), 0.26-0.28 (1H, m, cyclopropyl), 0.37-0.41 (2H, m, cyclopropyl),
1.04 (18H, s, CH$_3$), 1.73 (1H, s, OH), 2.81 (1H, d, $J = 9.2$ Hz, OCH$_2$),
2.84 (1H, d, $J = 9.2$ Hz, OCH$_2$), 3.42 (1H, d, $J = 11.4$ Hz, CH$_2$OH), 3.48 (1H, d, $J =$
11.4 Hz, CH$_2$OH), 4.23 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.29 (1H, d, $J = 15.0$ Hz, NCH$_2$),
6.73 (1H, dd, $J = 1.4$ and 8.0 Hz benzyl Ar-H), 6.76 (1H, dd, $J = 1.4$ and 9.8 Hz,
CHCF), 7.10-7.15 (5H, m, Ar-H), 7.38 (1H, dd, $J = 1.0$ and 8.0 Hz, $H^8$), 7.42-7.45
(1H, m, $H^6$), 7.78 (1H, dd, $J = 1.0$ and 7.4 Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 7.5
(cyclopropyl), 7.6 (cyclopropyl), 10.2 (CH$_3$), 17.6 ($CH(CH_3)_2$), 21.1 (cyclopropyl),
41.2 (NCH$_2$), 66.7 (CH$_2$OH), 66.9 (OCH$_2$), 93.4 (ethynyl), 95.9 (d, $J = 3.2$ Hz, ethynyl),
98.5 (hemiaminal), 110.1 (d, $J = 16.1$ Hz, C-CC), 115.0 (d, $J = 21.4$ Hz,
CHCF), 121.3, 123.3 (d, $J = 3.3$ Hz, benzyl Ar-H), 127.2, 127.4, 128.7, 130.9, 132.4,
132.9, 133.0, 133.8 (d, $J = 6.8$ Hz, benzyl Ar-H), 138.4 (d, $J = 7.1$ Hz, benzyl Ar-H),
139.6, 161.7 (d, $J = 251.2$ Hz, C-F), 165.6 (C=O); $\delta_F$ (470 MHz CDCl$_3$): -109.6;
LCMS $R_t = 2.33$ min, (5); MS (ESI+) m/z 666.5 [M+H]$^+$; HRMS m/z: Calc. for
C$_{37}$H$_{43}^{35}$Cl$_2$FNO$_2$Si: 666.2368 [M+H]$^+$ Found 666.2362 [M+H]$^+$
7. MDM2/p53 Experimental Procedures

4-Chloro-3-(4-chlorophenyl)-2-(4-ethynyl-3-fluorobenzyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)isoindolin-1-one (285)

General procedure G: 4-chloro-3-(4-chlorophenyl)-2-(3-fluoro(4-((triisopropylsilyl)ethynyl)benzyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)isoindolin-1-one (90 mg, 0.14 mmol). Chromatography (SP4, silica, 7-60 % EtOAc, PE) gave the title compound as colourless oil (44 mg, 62%). Rf = 0.22 (3:7 EtOAc:PE); UV λ max (EtOH): 230.0 nm; IR ν max (cm⁻¹): 3296 (OH), 2361 (Ethynyl stretch), 1696 (C=O), 1588 (Amide C-N), 1012 (C-F), 761 (C-Cl); δH (500 MHz CDCl₃): 0.15-0.18 (1H, m, cyclopropyl), 0.31-0.33 (1H, m, cyclopropyl), 0.44-0.49 (2H, m, cyclopropyl), 1.73 (1H, s, OH), 2.85 (1H, d, J = 9.1 Hz, OCH₂), 2.92 (1H, d, J = 9.1 Hz, OCH₂), 3.26 (1H, s, ethynyl), 3.47 (1H, d, J = 11.3 Hz, CH₂OH), 3.54 (1H, d, J = 11.3 Hz, CH₂OH), 4.26 (1H, d, J = 15.1 Hz, NCH₂), 4.40 (1H, d, J = 15.1 Hz, NCH₂), 6.64-6.88 (2H, m, benzyl Ar-H), 7.19-7.26 (5H, m, Ar-H), 7.46 (1H, dd, J = 1.0 and 8.0 Hz, H₅), 7.50-7.53 (1H, m, H₆), 7.85 (1H, dd, J = 1.0 and 7.4 Hz, H₇); δC (125 MHz CDCl₃): 8.5 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 42.2 (NCH₂), 67.7 (CH₂OH), 68.0 (OCH₂), 76.7 (ethynyl), 82.6 (d, J = 3.2 Hz, ethynyl), 94.5 (hemi-aminal), 109.6 (d, J = 15.6 Hz, C-CCH), 116.1 (d, J = 21.3 Hz, CHCF), 122.4, 124.5 (d, J = 3.3 Hz, benzyl Ar-H), 128.3, 128.5, 129.8, 131.9, 133.6, 134.0, 134.0, 134.9 (d, J = 3.6 Hz, benzyl Ar-H), 140.3 (d, J = 7.5 Hz, benzyl Ar-H), 140.7, 162.9 (d, J = 251.3 Hz, C-F), 166.7 (carbonyl); δF (470 MHz CDCl₃): -110.0; HRMS m/z: Calc. for C₂₈H₂₂⁵Cl₂FNO₃: 510.1034 [M+H]⁺. Found 510.1034 [M+H]⁺; Analytical HPLC: 98.1% purity
Benzo[c][1,2,5]oxadiazol-5-ylmethanamine (288)

To a solution of 5-(bromomethyl)-2,1,3-benzoxadiazole (500 mg, 2.35 mmol, 1 eq) in DMF (6 mL) was added potassium phthalimide (435 mg, 2.35 mmol, 1 eq) and the reaction was stirred at room temperature for 16 h. The solvent was removed in vacuo, and the resulting white solid was suspended in water (20 mL) and extracted into EtOAc (3 x 20 mL). The organic extracts were dried over MgSO₄, and concentrated in vacuo. The resulting solid was dissolved in EtOH (5 mL) and 40% aq. methylamine (5 mL) added and the reaction was stirred for 25 h, concentrating in vacuo, the white solid was suspended in water (50 mL) and extracting with EtOAc (3 x 50 mL). The combined organic extract was dried (MgSO₄) and concentrated in vacuo.

Chromatography (SP4, silica, 2-20% MeOH, EtOAc) gave 288 as an orange gummy solid (186 mg, 53%). Rᵣ = 0.07 (1:9 MeOH:EtOAc); UV λₘₐₓ (EtOH): 289.5 nm; IR νₘₐₓ 2891, 2358, 2159, 1977, 1521 (Oxadiazole ring), 878, 800 cm⁻¹; δH (500 MHz CDCl₃): 1.55 (2H, s, NH₂), 4.02 (2H, s, CH₂), 7.40 (1H, dd, J = 1.1 and 9.2 Hz, CHCCH₂), 7.77-7.78 (1H, m, NCCHCCH₂), 7.80 (1H, d, J = 14.3, NCCHCH); δC (125 MHz CDCl₃): 46.2 (CH₂), 111.7 (NCCHCCH₂), 116.3 (NCCHCH), 132.7 (CHCCH₂), 147.0 (CCH₂), 148.9 (C=N), 149.5 (C=N)

2-(Benzo[c][1,2,5]oxadiazol-5-ylmethyl)-4-chloro-3-(4-chlorophenyl)-3-hydroxyisooindolin-1-one (290)

General procedure B: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (504 mg, mmol) and benzo[c][1,2,5]oxadiazol-5-ylmethanamine (261 mg, 1.75 mmol).

Chromatography (SP4, silica, 7-60% EtOAc) and (silica, 1:1 EtOAc:PE) gave 290 as a gummy yellow solid (100 mg, 15%). Rᵣ = 0.61 (1:1 EtOAc:PE); UV λₘₐₓ (EtOH): 271.5 nm; IR νₘₐₓ 3285 (OH), 2158, 2018, 1691 (C=O), 1665 (Oxadiazole ring), 1586
(Amide C-N), 820, 760 (C-Cl) cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 4.14 (1H, d, \(J = 15.8\) Hz, NCH\(_2\)), 4.48 (1H, d, \(J = 15.8\) Hz, NCH\(_2\)), 5.25 (1H, s, OH), 7.10-7.13 (3H, m, Ar-H), 7.17-7.19 (2H, m, Ar-H), 7.29-7.32 (2H, m, Ar-H), 7.35 (1H, dd, 1.0 and 7.9 Hz, \(H^5\)), 7.51 (1H, dd, \(J = 0.6\) and 9.4 Hz, \(H^6\)), 7.53 (1H, dd, \(J = 1.0\) and 7.3 Hz, \(H^7\)); \(\delta_C\) (125 MHz CDCl\(_3\)): 42.7 (NCH\(_2\)), 91.0 (hemi-aminal), 114.3 (benzyl Ar-H), 116.1 (benzyl Ar-H), 121.9, 128.2, 128.6, 129.8, 131.6, 132.4, 132.9, 134.3, 134.8, 135.0, 141.4, 144.0, 148.3 (C=N), 148.7 (C=N), 166.7 (C=O); LCMS \(R_t = 1.55\) min, (3); MS (ESI+) m/z 426.2 [M+H]\(^+\) HRMS m/z: Calc. for C\(_{21}\)H\(_{14}\)Cl\(_2\)N\(_3\)O\(_3\): 426.0407 [M+H]\(^+\). Found 426.0405 [M+H]\(^+\).

2-(Benzo[c][1,2,5]oxadiazol-5-ylmethyl)-4-chloro-3-(4-chlorophenyl)-3-((1-(hydroxymethyl)cyclopropyl)methoxy)isoindolin-1-one (291)

General procedure D: compound 290 (80 mg, 0.19 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.18 mL, 1.88 mmol, 10 eq). Further additions of BF\(_3\).OEt\(_2\) at 0 °C occurred after 18 h and stirred for a further 2 days. Chromatography (SP4, silica, 17-100 % EtOAc, PE) followed by semi-preparative HPLC (95-0% acetonitrile, 0.1% formic acid (aq)) to give 291 as a colourless oil (35 mg 36%). \(R_f = 0.55\) (3:7 EtOAc:PE); UV \(\lambda_{max}\) (EtOH) 268.0 nm; IR \(\nu_{max}\) 3465 (OH), 2922, 2159, 2032, 1699 (C=O), 1588 (Amide C-N),1011, 818, 760 (C-Cl) cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 0.17-0.24 (1H, m, cyclopropyl), 0.36-0.41 (1H, m, cyclopropyl), 0.43-0.48 (2H, m, cyclopropyl), 1.62 (1H, s, OH), 2.90 (1H, d, \(J = 9.1\) Hz, \(CH_2OH\)), 4.60 (1H, d, \(J = 9.1\) Hz, \(CH_2OH\)), 3.50-3.55 (2H, m, OCH\(_2\)), 4.41 (1H, d, \(J = 15.3\) Hz, NCH\(_2\)), 4.57 (1H, d, \(J = 15.3\) Hz, NCH\(_2\)), 7.07-7.26 (4H, m, Ar-H), 7.32 (1H, dd, \(J = 1.1\) and 9.3 Hz, benzyl \(CHCCH_2\)), 7.37 (1H, s, benzyl CCHCN), 7.27 (1H, dd, \(J = 0.6\) and 7.9 Hz, \(H^6\)), 7.31-7.34 (1H, m, \(H^6\)), 7.40 (1H, \(J = 9.3\) Hz, benzyl CHCN), 7.66 (1H, dd, \(J = 0.6\) and 7.4 Hz, \(H^7\)); \(\delta_C\) (125 MHz CDCl\(_3\)): 8.5 (cyclopropyl), 8.7 (cyclopropyl),
22.2 (cyclopropyl), 42.6 (NCH₂), 67.5 (CH₂OH), 67.7 (OCH₂), 94.3 (hemi-aminal), 115.3 (benzyl CCHCN), 116.4 (CHCN), 122.4 (C⃗), 128.2, 128.5, 129.9, 132.1 (C⃗), 133.1 (benzyl CHCCH₂), 133.8, 134.2 (C⃗), 135.0, 140.6, 140.7, 148.2 (C=N), 148.8 (C=N), 166.8 (C=O); LCMS Rₜ = 1.58 min, (3); MS (ESI+) m/z 510.3 [M+H]⁺; HRMS m/z: Calc. for C₂₆H₂₂Cl₂N₃O₄: 510.0982 [M+H]⁺. Found: 510.0979 [M+H]⁺.;

Analytical HPLC: 98.1% purity

4-Chloro-3-(4-chlorophenyl)-2-(4-iodobenzyl)-3-((1-(hydroxymethyl)cyclopropyl) methoxy)isoindolin-1-one (296)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-2-(4-iodobenzyl)-3-((1-(hydroxymethyl)cyclopropyl) methoxy)isoindolin-1-one (120 mg, 0.24 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.045 mL, 0.47 mmol). Chromatography (SP4, silica, 7-60% EtOAc, petrol) gave 296 as a colourless oil (75 mg, 52%). Rₜ = 0.33 (7:3 PE:EtOAc); UV λmax (EtOH): 227.0 nm; IR vmax 2360, 2160, 1684 (C=O), 1588 (Amide C-N) cm⁻¹; δH (500 MHz CDCl₃): 0.11-0.13 (1H, m, cyclopropyl), 0.28-0.32 (1H, m, cyclopropyl), 0.42-0.47 (2H, m, cyclopropyl), 2.80 (1H, d, J = 9.1 Hz, OCH₂), 2.89 (1H, d, J = 9.1 Hz, OCH₂), 3.43 (1H, d, J = 11.2 Hz, CH₂OH), (1H, d, J = 11.2 Hz, CH₂OH), 4.16 (1H, d, J = 15.0 Hz, NCH₂), 4.41 (1H, d, J = 15.0 Hz, NCH₂), 6.88 (2H, d, J = 8.3 Hz, Ar-H), 7.18-7.20 (4H, m, Ar-H), 7.44-7.52 (4H, m, Ar-H), 7.84 (1H, dd, J = 7.4 and 0.8 Hz, H') δC (125 MHz CDCl₃): 8.6 (cyclopropyl), 22.1 (cyclopropyl), 42.4 (NCH₂), 67.0 (CH₂OH), 68.8 (OCH₂), 92.9 9 (Ar-I), 94.6 (hemi-aminal C), 122.3, 128.3, 128.5, 129.7, 131.0, 131.8, 133.9, 134.1, 134.8, 135.0, 136.7, 137.3, 140.7, 166.7 (carbonyl)
4-Azidobenzyl amine (299)

To a solution of 4-aminobenzyl alcohol (100 mg, 0.81 mmol, 1 eq) in 5 M aqueous HCl (3 mL) at 0 °C was added dropwise over 30 min an aqueous solution of NaNO₂ (71 mg, 1.03 mmol, 1.27 eq in 2 mL of water), followed by NaN₃ (CAUTION) (228 mg, 3.51 mmol, 4.3 eq) in portions over a further 30 min. The reaction mixture was stirred at 0 °C for a further 1 h before diluting with water (15 mL) and basifying with saturated aqueous solution of NaHCO₃ to around pH 8, then extracted into EtOAc (3 x 25 mL), dried over MgSO₄ and concentrated in vacuo. Chromatography (SP4, silica, 7-60% EtOAc, PE) gave the title compound as a colourless oil which solidified on standing to give a white solid which was stored in the dark at 4 °C (118 mg, 98%). Rᵣ = 0.43 (3:7 EtOAc:PE); UV λ_max (EtOH): 251.5 nm; IR ν_max 3300 (OH), 2927, 2863, 2415, 2103 (very strong, N₃), 1505 cm⁻¹; δ_H (500 MHz CDCl₃): 1.84 (1H, s, OH), 4.58 (2H, s, CH₂), 6.94 (2H, d, J = 8.5 Hz, CH-CN₃), 7.27 (2H, d, J = 8.5 Hz, CH-CN₂); δ_C (125 MHz CDCl₃): 64.7 (CH₂), 119.1 (CH-CN₂), 128.5 (CH-CN₃), 137.6 (CN₃), 139.4 (CCH₂)

4-Azidobenzyl bromide (300)

To a solution of 4-azidobenzyl alcohol (334 mg, 2.24 mmol, 1 eq) in diethyl ether (2mL) at 0°C was added PBr₃ (168 µL, 1.79 mmol, 0.8 eq) in diethyl ether (2 mL) over 30 min. The reaction was stirred for 15 min at 0 °C, then room temperature for 30 min, diluted with water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic extracts were washed with sat. NaHCO₃ (30 mL) and brine (30 mL), dried over MgSO₄ and concentrated in vacuo. Chromatography (SP4, silica, 1-10% EtOAc, PE) gave 300 as a colourless oil (363 mg, 76%). Rᵣ = 0.75 (1:9 EtOAc:PE); UV λ_max (EtOH): 379.0, 262.0, 209.0 nm; IR ν_max 2104 (very strong, N₃).
1606, 1504 cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 4.40 (2H, s, CH\(_2\)), 6.92 (2H, d, \(J = 8.5\) Hz, CHCN\(_3\)), 7.30 (2H, d, \(J = 8.5\) Hz, CHCCH\(_2\)); \(\delta_C\) (125 MHz CDCl\(_3\)): 32.9 (CH\(_2\)), 119.4 (CHCCH\(_2\)), 130.6 (CHCN\(_3\)), 134.5 (CN\(_3\)), 140.2 (CCH\(_2\))

4-Azidobenzylamine (295)

To benzyl bromide 289 (91 mg, 0.43 mmol, 1 eq) in acetonitrile (4 mL) was added 18-crown-6 (14 mg, 0.05 mmol, 1 eq) and the reaction was stirred at room temperature for 30 min, then sodium diformylamide (110 mg, 1.16 mmol, 2.7 eq) was added and the reaction heated to 95 \(^\circ\)C for 24 h before cooling to room temperature. 5\% HCl in EtOH (3 mL) was added and the reaction stirred for 3 days, then concentrated \textit{in vacuo}. The residues were diluted with sat. NaHCO\(_3\) (15 mL) and extraction with EtOAc (3 x 25 mL). The combined organic extracts were dried (MgSO\(_4\)) and concentrated \textit{in vacuo}. Chromatography (SP4, silica, 7-60\% EtOAc, PE) gave 295 as a white oily solid (61 mg, 96\%). \(R_t\) = 0.41 (3:7 EtOAc:PE); UV \(\lambda_{\text{max}}\) (EtOH): 251.5 nm; IR \(\nu_{\text{max}}\) 3307 (NH), 2101 (very strong, N\(_3\)), 1506, 1279 cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 4.66 (2H, s, CH\(_2\)), 7.03 (2H, d, \(J = 8.5\) Hz, CHCN\(_3\)), 7.36 (2H, d, \(J = 8.5\) Hz, CHCCH\(_2\)); \(\delta_C\) (125 MHz CDCl\(_3\)): 64.7 (CH\(_2\)), 119.1 (CHCCH\(_2\)), 128.6 (CHCN\(_3\)), 137.6 (CN\(_3\)), 139.4 (CCH\(_2\))

2-(4-Azidobenzyl)-3-(4-chlorophenyl)-3-hydroxyisoindolinone (301)

\textbf{General procedure B}: 2-(4-chlorobenzoyl)benzoic acid (185 mg,0.71 mmol, 1 eq) and 4-azidobenzyl amine (116 mg, 0.78 mmol). The reaction was undertaken in the dark. Chromatography (SP4, silica, 7-60\% EtOAc, PE) gave the title compound as a yellow oil (216 mg, 78\%). \(R_t\) = 0.79 (3:7 EtOAc:PE); UV \(\lambda_{\text{max}}\) (EtOH): 252.5, 225.5
nm; IR \( \nu_{\text{max}} \) 2110 (very strong, \( \text{N}_{3} \)), 1775 (C=O), 1606 (Amide C-N), 1092 cm\(^{-1}\); \( \delta_H \)

(500 MHz CDCl\(_3\)): 4.28 (1H, d, \( J = 11.1 \) Hz, NCH\(_2\)), 4.45 (1H, d, \( J = 11.1 \) Hz, NCH\(_2\)), 4.45 (1H, d, \( J = 11.1 \) Hz, NCH\(_2\)), 6.89 (2H, d, \( J = 8.6 \) Hz, CHCN\(_3\)), 7.19 (2H, dt, \( J = 2.5 \) and 8.6 Hz, benzyl Ar-H), 7.28 (2H, dt, \( J = 2.0 \) and 8.8 Hz, Ar-H), 7.39 (1H, d, \( J = 7.7 \) Hz, \( H^5 \)), 7.46 (2H, dt, \( J = 2.0 \) and 8.8 Hz, Ar-H), 7.52 (1H, ap. td, \( J = 0.9 \) and 7.5 Hz, \( H^5 \)), 7.60 (1H, ap. td, \( J = 1.1 \) and 7.5 Hz, \( H^6 \)), 7.86 (1H, ap. td, \( J = 0.8 \) and 7.6 Hz, \( H^7 \)); \( \delta_C \) (125 MHz CDCl\(_3\)): 108.1 (hemi-aminal), 119.1, 123.3, 125.8, 126.3, 127.3, 129.0, 129.5, 130.9, 133.3, 135.5, 136.3, 139.8 (C-N\(_3\)), 147.9, 168.1 (C=O)

3-(4-Chlorophenyl)-3-hydroxy-2-(4-nitrobenzyl)isoindolin-1-one (304)

**General procedure B:** 2-(4-chlorobenzoyl)benzoic acid (750 mg, 2.88 mmol) and 4-nitrobenzylamine hydrochloride (597 mg, 3.16 mmol). Chromatography (silica, 1:1 EtOAc:PE) gave 304 as a white solid (714 mg, 63%). \( R_t = 0.66 \) (1:1 EtOAc:PE); mp = 178.3-182.1 \( ^{\circ} \)C (lit 148-149 \( ^{\circ} \)C)\(^{192}\); UV \( \lambda_{\text{max}} \) (EtOH): 257.5 nm; IR \( \nu_{\text{max}} \) 3190 (OH), 2928, 1679 (C=O), 1608 (Amide C-N),1342 (NO\(_2\) stretch), 709, 693 (C-Cl) cm\(^{-1}\); \( \delta_H \)

(500 MHz CDCl\(_3\)): 3.40 (1H, s, OH), 4.16 (1H, d, \( J = 15.4 \) Hz, NCH\(_2\)), 4.58 (1H, d, \( J = 15.4 \) Hz, NCH\(_2\)), 7.13-7.20 (5H, m, Ar-H), 2.25 (2H, d, \( J = 8.7 \) Hz, benzyl Ar-H), 7.43 (2H, ap. dtd, \( J = 1.3 \), 7.4 and 18.2 Hz, \( H^5 \) and \( H^6 \)), 7.71 (1H, d, \( J = 6.9 \) Hz, \( H^7 \)), 7.91 (2H, d, \( J = 8.7 \) Hz, CHCNO\(_2\)); \( \delta_C \) (125 MHz CDCl\(_3\)): 42.3 (NCH\(_2\)), 91.1 (hemi-aminal), 122.8, 123.4 (CHCNO\(_2\)), 123.7 (\( H^7 \)), 127.8, 128.8, 129.5, 129.8 (benzyl Ar-H), 130.1, 133.4, 134.9, 136.4, 145.2, 147.0, 148.3, 167.8 (C=O); LCMS \( R_t = 1.52 \) min, (3); MS (ESI+) m/z 395.3 [M+H]\(^{+}\)
3-(4-Chlorophenyl)-3-((1-(hydroxymethyl)cyclopropyl)methoxy)-2-(4-nitrobenzyl)isoindolin-1-one (195)

Using commercial Vilsmeier reagent

To a solution of 304 (100 mg, 0.25 mmol, 1 eq) in THF (3 mL) was added (chloromethylene)dimethyliminium chloride (44 mg, 0.38 mmol, 1.5 eq) and the reaction stirred for 4 h at room temperature, then concentrated in vacuo. The residue was dissolved in THF (3 mL) and K$_2$CO$_3$ (74 mg, 0.54 mmol, 2.1 eq) and 1,1-bis(hydroxymethyl)cyclopropane (0.05 mL, 0.51 mmol, 2 eq) added. The reaction was stirred for 16 h then concentrated in vacuo. The residue was suspended in water (30 mL) and extracted with EtOAc (3 x 50 mL). The combined organic extracts were washed with brine and dried (Na$_2$SO$_4$) and concentrated in vacuo. Chromatography (SP4, silica, 12-100% EtOAc, PE) gave 195 as a white oily solid (26 mg, 22%).

Using oxalyl chloride

To a solution of 304 (100 mg, 0.25 mmol, 2 eq) in DCM (2 mL) was added (COCl)$_2$ (0.11 mL, 1.27 mmol, 5 eq) dropwise, and the mixture was stirred for 2 h, then concentrated in vacuo. The residue was redissolved in DCM (2 mL), K$_2$CO$_3$ (73 mg, 0.55 mmol, 2.2 eq) and 1,1-bis(hydroxymethyl)cyclopropane (0.05 mL, 0.51 mmol, 2 eq) was added and the reaction stirred for 15 h, then concentrated in vacuo. The residue was suspended in water (30 mL) before extracting with EtOAc (3 x 30 mL). The combined organic extracts were washed with Na$_2$SO$_4$, and concentrated in vacuo. Chromatography (SP4, silica, 12-100% EtOAc, PE) gave 195 as an oily solid (74 mg, 62%).
$R_f = 0.56$ (1:1 EtOAc:PE); UV $\lambda_{max}$ (EtOH): 267.5 nm; IR $\nu_{max}$ 3445 (OH), 2923, 2875, 1689 (C=O), 1605 (Amide C-N), 1314 (NO$_2$) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 014-0.16 (1H, m, cyclopropyl), 0.19-0.21 (1H, m, cyclopropyl), 0.41-0.46 (2H, m, cyclopropyl), 1.57 (1H, t, $J = 2.4$ Hz OH), 2.80 (1H, d, $J = 9.5$ Hz, OCH$_2$), 2.82 (1H, d, $J = 9.5$ Hz, OCH$_2$), 3.45 (1H, dd, $J = 2.4$ and 11.2 Hz, CH$_2$OH), 4.47 (1H, d, $J = 15.2$ Hz, NCH$_2$), 4.52 (1H, d, $J = 15.2$ Hz, NCH$_2$), 4.57-7.17 (4H, m, Ar-H), 7.32 (2H, d, $J = 6.9$ Hz, Ar-H), 7.53-7.57 (2H, m, Ar-H), 7.91-7.93 (1H, m, $H^3$), 8.01 (1H, d, $J = 8.8$ Hz, CHCNO$_2$); $\delta_C$ (125 MHz CDCl$_3$): 8.6 (cyclopropyl), 8.7 (cyclopropyl), 22.3 (cyclopropyl), 42.3 (cyclopropyl), 67.5 (CH$_2$OH), 67.7 (OCH$_2$), 94.5 (hemi-aminal), 123.1, 123.4, 123.9, 127.9, 128.7, 129.8, 130.2, 131.2, 133.3, 134.8, 136.8, 144.7, 145.0 (C-NO$_2$), 147.1, 168.3; LCMS $R_t = 1.59$ min, (3); MS (ESI+) m/z 479.2 [M+H]$^+$

4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(3-iodobenzyl)isoindolin-1-one (307)

**General procedure B:** sodium 3-chloro-2-(4-chlorobenzoyle)benzoate (440 mg, 1.39 mmol) and 3-iodobenzylation hydrochloride (750 mg, 2.78 mmol). Chromatography (SP4, silica, 7-60 % EtOAc, PE) gave 307 as a white solid (263 mg, 37%). $R_f = 0.5$ (3:7 EtOAc:PE); mp = 183.9-185.3 °C; UV $\lambda_{max}$ (EtOH): 225.0 nm; IR $\nu_{max}$ 3278 (OH), 1679 (C=O), 1587 (Amide C-N), 813, 764 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 3.56 (1H, s, OH), 4.10 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.31 (1H, d, $J = 15.1$ Hz, NCH$_2$), 7.07 (1H, d, $J = 7.8$ benzyl Ar-H), 7.13-7.19 (4H, m, Ar-H), 7.22-7.23 (1H, m, Ar-H), 7.36-7.40 (3H, m, Ar-H), 7.61-7.65 (1H, m, $H^3$); $\delta_C$ (125 MHz CDCl$_3$): 42.0 (NCH$_2$), 90.8 (Cl), 95.1 (hemi-aminal), 122.2, 128.1, 128.2, 128.5, 129.7, 129.9, 131.6, 132.8, 134.1, 134.7, 134.8, 136.2, 137.6, 139.6, 143.8, 166.2 (C=O); LCMS $R_t = 1.72$ min, (5); MS (ESI+) m/z 510.1 [M+H]$^+$; HRMS m/z: Calc. for C$_{21}$H$_{15}$Cl$_2$INO$_2$: 509.9519 [M+H]$^+$. Found 509.9515 [M+H]$^+$. 

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4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(3-((triisopropylsilyl)ethynyl)benzyl)isoindolinone (308)

**General procedure F:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(3-iodobenzyl)isoindolin-1-one (263 mg, 0.52 mmol, 1 eq) and triisopropylsilylacetylene (150 µL, 0.67 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave the title compound as a white solid (234 mg, 80%). Rf = 0.51 (3:7 EtOAc:PE); mp = 206.9-208.8 °C; UV λ_max (EtOH): 262.0, 250.5 nm; IR υ_max 3373 (C=O), 2943, 2864, 2159 (Ethynyl), 1695 (C=O), 1587 (Amide C-N), 760 (C-Cl) cm⁻¹; δ_H (500 MHz CDCl₃): 1.05 (18H, s, CH₃), 3.59 (1H, s, OH), 4.05 (1H, d, J = 15.1 Hz, NCH₂), 4.39 (1H, d, J = 15.1 Hz, NCH₂), 6.78-7.05 (3H, m, Ar-H), 7.09-7.18 (5H, m, Ar-H), 7.34-7.35 (2H, m, H⁵ and H⁶), 7.60-7.62 (1H, m, H⁷); δ_C (125 MHz CDCl₃): 11.3 (CH₃), 18.7 (CH), 42.4 (NCH₂), 90.6 (ethynyl), 90.9 (ethynyl), 106.7 (hemi-aminal), 122.0 (C-ethynyl), 123.5, 128.1, 128.2, 128.4, 129.7, 131.0, 131.5, 132.0, 132.9, 134.0, 134.7, 134.8, 137.5, 143.9, 166.2 (C=O); LCMS R_t = 2.26 min, (5); MS (ESI+) m/z 564.4 [M+H]^+; HRMS m/z: Calc. for C₃₂H₃₆Cl₂NO₂Si: 564.1887 [M+H]^+. Found 562.1883 [M+H]^+.

4-Chloro-3-(4-chlorophenyl)-3-(1((hydroxymethyl)cyclopropane)methoxy)-2-(3-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (309)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(3-((triisopropylsilyl)ethynyl)benzyl)isoindolinone (167 mg, 0.30 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.06 mL, 0.59 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave the title compound as a colourless oil (67mg, 37%). R_f = 0.29 (3:7 EtOAc:PE); UV λ_max (EtOH): 262.0, 250.5 nm; IR υ_max 2942, 2864, 2158.
(Ethynyl), 1708 (C=O), 763 (C-Cl) cm\(^{-1}\); \(\delta_H\) (500 MHz CDCl\(_3\)): 0.11-0.14 (1H, m, cyclopropyl), 0.26-0.28 (1H, m, cyclopropyl), 0.38-0.45 (2H, m, cyclopropyl), 1.74 (1H, s, OH), 2.78 (1H, d, \(J = 9.2\) Hz, OCH\(_2\)), 2.94 (1H, d, \(J = 9.2\) Hz, OCH\(_2\)), 3.42 (1H, d, \(J = 11.4\) Hz, CH\(_2\)OH), 3.54 (1H, d, \(J = 11.4\) Hz, CH\(_2\)OH), 4.14 (1H, d, \(J = 15.0\) Hz, NCH\(_3\)), 4.48 (1H, d, \(J = 15.0\) Hz, NCH\(_2\)), 7.09-7.19 (6H, m, Ar-H), 7.29 (1H, d, \(J = 7.5\) Hz, benzyl Ar-H), 7.44 (1H, d, \(J = 7.9\) Hz, \(H^5\)), 7.48-7.51 (1H, m, \(H^6\)), 7.86 (1H, d, \(J = 7.3\) Hz, \(H^7\)); \(\delta_C\) (125 MHz CDCl\(_3\)): 8.5 (cyclopropyl), 11.3 (cyclopropyl), 18.7 (CH\(_3\)), 22.1 (cyclopropyl), 46.7 (NCH\(_2\)), 67.8 (CH\(_2\)OH), 67.9 (OCH\(_2\)), 90.8 (ethynyl), 94.7 (ethynyl), 106.6 (hemi-aminal), 122.3, 123.5, 128.2, 128.3, 128.4, 129.1, 129.7, 131.2, 131.8, 132.2, 133.9, 134.2, 134.7, 135.0, 137.1, 140.8, 166.7 (C=O); LCMS \(R_t = 2.36\) min, (5); HRMS m/z: Calc. for C\(_{37}\)H\(_{44}\)Cl\(_2\)NO\(_3\)Si: 648.2462 [M+H]\(^+\). Found 648.2463 [M+H]\(^+\).

4-Chloro-3-(4-chlorophenyl)-2-(3-ethynylbenzyl)-(1-((hydroxymethyl)cyclopropyl) methoxy)isoindolin-2-one (310)

\[
\begin{align*}
\text{General procedure G:} & 4\text{-chloro-3-(4-chlorophenyl)-3-(1((hydroxymethyl) cyclopropane)methoxy)-2-(3-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (76 mg, 0.12 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 310 as a colourless oil (36 mg, 61\%). R}_f = 0.2 (3:7 EtOAc:PE); UV \text{\(\lambda_{max}\) (EtOH): 246.0, 225.0, 211.0 nm; IR \text{\(\nu_{max}\) 3300 (OH), 2927, 2874, 2361, 2160 (Ethynyl), 1695 (C=O), 1587 (Amide C-N), 1074 cm}^{-1}; \delta_H (500 MHz CDCl\(_3\)): 0.10-0.12 (1H, m, cyclopropyl), 0.26-0.29 (1H, m, cyclopropyl), 0.39-0.45 (2H, m, cyclopropyl), 1.70 (1H, t, \(J = 5.6\), OH), 2.80 (1H, d, \(J = 9.2\) Hz, OCH\(_2\)), 2.93 (1H, d, \(J = 9.2\) Hz, OCH\(_2\)), 3.04 (1H, s, ethynyl), 3.43 (1H, dd, \(J = 4.2\) and 11.4 Hz, CH\(_2\)OH), 4.43 (1H, dd, \(J = 4.2\) and 11.4 Hz, CH\(_2\)OH), 4.18 (1H, d, \(J = 14.9\) Hz, NCH\(_2\)), 4.46 (1H, d, \(J = 14.9\) Hz, NCH\(_2\)), 7.11-7.19 (7H, m, Ar-H), 7.30 (1H, d, \(J = 7.6\) Hz, Ar-H), 7.45 (1H, dd, \(J = 0.9\) and 7.9, \(H^5\)), 7.49-7.52 (1H, m, \(H^6\)), 7.85 (1H, dd, \(J = 0.8\) and 7.4 Hz, \(H^7\)); \delta_C (125 MHz CDCl\(_3\)): 8.5 (cyclopropyl), 11.3 (cyclopropyl), 18.7 (CH\(_3\)), 22.1 (cyclopropyl), 46.7 (NCH\(_2\)), 67.8 (CH\(_2\)OH), 67.9 (OCH\(_2\)), 90.8 (ethynyl), 94.7 (ethynyl), 106.6 (hemi-aminal), 122.3, 123.5, 128.2, 128.3, 128.4, 129.1, 129.7, 131.2, 131.8, 132.2, 133.9, 134.2, 134.7, 135.0, 137.1, 140.8, 166.7 (C=O); LCMS \(R_t = 2.36\) min, (5); HRMS m/z: Calc. for C\(_{37}\)H\(_{44}\)Cl\(_2\)NO\(_3\)Si: 648.2462 [M+H]\(^+\). Found 648.2463 [M+H]\(^+\).}
CDCl$_3$): 8.5 (cyclopropyl), 22.1 (cyclopropyl), 42.5 (NCH$_2$), 67.8 (CH$_2$OH), 67.9 (OCH$_2$), 77.4 (ethynyl), 83.1 (ethynyl), 96.6 (hemi-aminal), 122.1, 122.3, 128.3, 128.5, 129.6, 129.7, 131.0, 131.8, 132.6, 133.9, 134.1, 134.7, 135.0, 137.3, 140.8, 166.7 (carbonyl); LCMS $R_t$ = 1.67 min, (4); HRMS $m/z$: Calc. for C$_{28}$H$_{24}$Cl$_2$NO$_3$: 492.1128 [M+H]$^+$. Found 492.1123 [M+H]$^+$.; Analytical HPLC: 98.7% purity

4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-(prop-1-ynyl)benzyl)isoindolin-1-one (313)

To a solution of 252 (383 mg, 0.75 mmol, 1 eq) in THF (4 mL) was added CuI (9 mg, 0.05 mmol, 0.06 eq) and Pd(PPh$_3$)$_2$Cl$_2$ (16 mg, 0.02 mmol, 0.03 eq) and the solution degassed for 5 min. Et$_3$N (0.26 mL, 1.88 mmol, 2.5 eq) was added and the solution degassed for a further 15 min and the reaction flack sealed. The reaction mixture was cooled to -78 °C and propyne (in excess) was added via cannula, then gradually warmed to room temperature and stirred for 18 h, filtered through Celite, washed with methanol (2 x 10 mL) and concentrated in vacuo. Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 313 as an off-white solid (294 mg, 94%). $R_t$ = 0.5 (3:7 EtOAc:PE); mp = 224.0-225.7 °C; UV $\lambda_{\text{max}}$ (EtOH): 232.0 nm; IR $\nu_{\text{max}}$ 3178 (OH), 2159 (Ethynyl), 1979, 1676 (C=O), 1658 (Amide C-N), 1406, 815, 770 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 2.05 (3H, s, CH$_3$), 3.30 (1H, s, OH), 4.05 (1H, d, $J$ = 15.1 Hz, NCH$_2$), 4.62 (1H, d, $J$ = 15.1 Hz, NCH$_2$), 7.11 (2H, d, $J$ = 8.3 Hz, benzyl Ar-H), 7.19 (2H, d, $J$ = 8.3 Hz, benzyl Ar-H), 7.27-7.28 (4H, m, Ar-H), 7.45-7.47 (2H, m, H$_5$ and H$_6$), 7.76 (1H, dd, $J$ = 1.8 and 6.7 Hz, H$_7$); $\delta_C$ (125 MHz CDCl$_3$): 4.4 (CH$_3$), 42.6 (NCH$_2$), 79.5 (ethynyl), 86.0 (ethynyl), 91.0 (hemi-aminal), 122.1, 122.9, 128.3, 128.5, 128.6, 129.6, 131.4, 131.6, 133.0, 134.0, 134.7, 134.9, 137.0, 143.8, 166.1; LCMS $R_t$ = 1.68 min, (4); MS (ESI+) $m/z$ 422.2 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{24}$H$_{18}$Cl$_2$NO$_2$: 422.0709 [M+H]$^+$. Found: 422.0711 [M+H]$^+$. 291
4-Chloro-3-(4-chlorophenyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)-2-(4-(prop-1-ynyl)benzyl)isoindolin-1-one (311)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-(prop-1-ynyl)benzyl)isoindolin-1-one (294 mg, 0.70 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.14 mL, 1.39 mmol). Chromatography (SP4, silica, 6-70% EtOAc, PE) followed by (SP4, silica, 12-100% EtOAc, PE) gave 311 as a colourless oil (72 mg, 20%). $R_t = 0.21$ (3:7 EtOAc:PE); UV $\lambda_{max}$ (EtOH): 229.0 nm; IR $\nu_{max}$ 3423 (OH), 2920, 2159 (Ethylnyl), 1703 (C=O), 1587 (Amide C-N), 760 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.02-0.08 (1H, m cyclopropyl), 0.22-0.28 (1H, m, cyclopropyl), 0.38-0.42 (2H, m, cyclopropyl), 1.67 (1H, s, OH), 2.02 (3H, s, CH$_3$), 2.77 (1H, d, $J = 9.1$ Hz, OCH$_2$), 2.87 (1H, d, $J = 9.1$ Hz, OCH$_2$). 3.38 (1H, dd, $J = 3.5$ and 11.4 Hz, CH$_2$OH), 3.47 (1H, dd, $J = 4.8$ Hz and 11.4 Hz, CH$_2$OH), 4.06 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.53 (1H, d, $J = 15.0$ Hz, NCH$_2$), 7.09 (2H, d, $J = 8.2$ Hz, benzyl Ar-H), 7.19-7.26 (6H, m, Ar-H), 7.44 (1H, dd, $J = 1.0$ and 8.0 Hz, $H^4$), 7.48-7.51 (1H, m, $H^6$), 7.90 (1H, dd, $J = 1.0$ and 7.4 Hz, $H^5$); $\delta_C$ (125 MHz CDCl$_3$): 4.4 (CH$_3$), 8.5 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 42.7 (NCH$_2$), 67.8 (CH$_2$OH), 68.0 (OCH$_2$), 79.4 (CCCH$_3$), 86.1 (CCCH$_3$), 94.8 (hemi-aminial), 122.3, 123.3, 128.4, 128.9, 129.1, 129.7, 131.3, 133.9, 134.7, 135.0, 136.4, 140.8, 166.8 (C=O); LCMS $R_t = 1.73$ min, (3); MS (ESI+) $m/z$ 506.2 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{29}$H$_{26}$Cl$_2$NO$_3$: 506.1284 [M+H]$^+$. Found: 506.1285 [M+H]$^+$; Analytical HPLC: 95.3% purity.
4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-(phenylethynyl)benzyl)isoindolin-1-one (314)

General procedure F: 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-iodobenzyl)-isoindolin-1-one (241 mg, 0.47 mmol) and phenylacetylene (67 µL, 0.61 mmol). Chromatography (SP4, silica, 7-60% PE, EtOAc) and (silica, 3:7 EtOAc:PE) gave 314 as a white solid (208 mg, 91%). $R_f = 0.43$ (3:7 EtOAc:PE); mp = 210.2-214.0 °C; UV $\lambda_{\text{max}}$ (EtOH): 302.0, 284.0 nm; IR $\nu_{\text{max}}$ 3186 (OH), 2158 (Ethylnyl), 1657 (CO), 1589 (Amide C-N), 1406, 751 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 3.22 (1H, s, OH), 4.02 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.54 (1H, d, $J = 15.1$ Hz, NCH$_2$), 7.08 (2H, d, $J = 8.2$ Hz, benzyl Ar-H), 7.18-7.28 (9H, m, Ar-H), 7.35-7.740 (2H, m, $H^5$ and $H^6$), 7.41-7.44 (2H, m, Ar-H), 7.66 (1H, dd, $J = 1.7$ and 6.7 Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 42.7 (NCH$_2$), 89.1 (alkyne), 89.5 (alkyne), 100.0 (hemi-aminal), 122.1 (C-alkyne), 122.2 (C-alkyne), 123.2, 128.3, 128.3, 128.6, 128.7, 129.6, 131.5, 131.6, 131.6, 133.0, 134.0, 134.8, 134.9, 137.8, 143.8, 166.1 (C=O); LCMS $R_t = 1.82$ min, (3); MS (ESI+) $m/z$ 484.2 [M+H]$^+$; HRMS $m/z$: Calc. for C$_{29}$H$_{20}$Cl$_2$NO$_2$ 484.0866 [M+H]$^+$. Found 484.0861 [M+H]$^+$. 

7. MDM2/p53 Experimental Procedures
4-Chloro-3-(4-chlorophenyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)-2-(4-(phenylethynyl)benzyl)isoindolin-1-one (312)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-(phenylethynyl)benzyl)isoindolin-1-one (208 mg, 0.43 mmol) and 1,1-bis(hydroxymethyl)cyclopropane (0.08 mL, 0.86 mmol). Chromatography (SP4, silica, 7-60 % EtOAc, PE) followed by (SP4, silica, 12-100 % EtOAc, PE) gave 312 as a colourless oil (89 mg, 36%). \( R_f = 0.21 \) (3:7 EtOAc:PE); UV \( \lambda_{\text{max}} \) (EtOH): 302.0, 284.0 nm; IR \( \nu_{\text{max}} \) 3465 (OH), 2919, 1704 (C=O), 1587 (Amide C-N), 755 (C-Cl) cm\(^{-1}\); \( \delta \)H (500 MHz): 0.09-0.13 (1H, m, cyclopropyl), 0.27-0.29 (1H, m, cyclopropyl), 0.40-0.45 (2H, m, cyclopropyl), 1.70 (1H, s, OH), 2.82 (1H, d, \( J = 9.1 \) Hz, OCH\(_2\)), 2.90 (1H, d, \( J = 9.1 \) Hz, OCH\(_2\)), 3.40-3.41 (1H, m, \( CH_2OH \)), 3.50 (1H, dd, \( J = 3.9 \) and 11.4 Hz, \( CH_2OH \)), 4.14 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 4.54 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 7.15 (2H, d, \( J = 8.2 \) Hz, Ar-H), 7.22-7.35 (9H, m, Ar-H), 7.45 (1H, dd, \( J = 1.0 \) and 8.0 Hz, \( H_5^\beta \)), 7.49-7.52 (3H, m, Ar-H), 7.86 (1H, dd, \( J = 1.0 \) and 7.4 Hz, \( H_7^\beta \)); \( \delta \)C (125 MHz): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 42.8 (NCH\(_2\)), 67.9 (\( CH_2OH \)), 68.2 (OCH\(_2\)), 89.0 (ethynyl), 89.6 (ethynyl), 95.8 (hemiacetal), 122.3, 122.4, 123.2, 128.3, 128.5, 129.0, 129.7, 131.5, 131.6, 131.9, 133.9, 134.4, 134.7, 135.0, 137.2, 140.8, 156.8, 166.8 (C=O); LCMS \( R_t = 1.91 \) min, (4); MS (ESI+) m/z 568.3 [M+H]\(^+\); HRMS m/z: Calc. for C\(_{34}H_{28}^{35}\)Cl\(_2\)NO\(_3\): 568.1441 [M+H]\(^+\). Found 568.1440 [M+H]\(^+\); Analytical HPLC: 94.8% purity
4-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-nitrobenzyl)isoindolin-2-one (321)

General procedure B: sodium 3-chloro-2-(4-chlorobenzoyl)benzoate (500 mg, 1.58 mmol) and 4-nitrobenzylamine hydrochloride (328 mg, 1.74 mmol). Chromatography (SP4, silica, 12-100% EtOAc, petrol) gave 321 as a white solid (187 mg, 28%). $R_t = 0.59$ (1:1 EtOAc:PE); mp. = 202.2-202.9 °C (lit. 202-203 °C)$^{240}$; UV $\lambda_{max}$ (EtOH): 272.0 nm; IR $\upsilon_{max}$ 1686 (C=O), 1589 (Amide C-N), 1515 cm$^{-1}$; $\delta_H$ (500 MHz $d_6$-DMSO): 4.41 (1H, d, $J = 16.2$ Hz, NCH$_2$), 4.54 (1H, d, $J = 16.2$ Hz, NCH$_2$), 7.28-7.33 (4H, m, Ar-H), 7.40 (2H, d, $J = 8.2$ Hz, Ar-H), 7.55 (1H, s, OH), 7.63-7.66 (2H, m, Ar-H), 7.72 (1H, d, $J = 5.5$ Hz, $H^7$), 8.04 (2H, d, $J = 8.2$ Hz, Ar-H); $\delta_C$ (125 MHz $d_3$-MeCN): 41.3 (NCH$_2$), 89.9 (hemi-aminal), 121.4, 122.7, 127.8, 128.2, 128.7, 128.9, 131.6, 133.0, 135.5, 135.8, 143.5(CCl), 145.4 (CH$_2$C), 146.5 (Ar-NO$_2$), 165.6 (carbonyl carbon); LCMS $R_t = 3.63$ min (1); MS (ESI+) $m/z$ 429.07 [M+H]$^+$

(3-Hydroxymethyloxetan-3-yl)-methanol (318)

A mixture of 3-bromo-2,2-bis-hydroxymethylpropan-1-ol (100 mg, 0.50 mmol, 1 eq), ethanol (0.9 mL) and potassium hydroxide (32 mg, 0.60 mmol, 1.15 eq) was stirred for 2h at room temperature then reflux for 15 min, cooled to room temperature and filtered. The solid was washed with methanol (10 mL). The filtrate was evaporated in vacuo and residue purified by chromatography (silica, 10:90 MeOH:EtOAc) to give 318 as a colourless liquid (46 mg, 78%). $R_t = 0.3$ (1:9 MeOH:EtOAc); IR $\upsilon_{max}$ 3280 (OH), 2954, 2881 cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 2.20 (2H, s, OH), 3.95 (4H, s, CH$_2$OH), 4.41 (4H, s, CH$_2$O); $\delta_C$ (125 MHz CDCl$_3$): 44.6, (quaternary carbon), 66.7 (CH$_2$OH), 76.3 (CH$_3$O)
3-(2-Bromomethyl-3-hydroxy-2-hydroxymethyl-propoxy)-4-chloro-3-(4-chlorophenyl)-2-(4-nitrobenzyl)isoindolin-1-one (322)

**General procedure C:** 4-chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-nitrobenzyl)isoindolin-2-one (382 mg, 0.89 mmol, 1 eq) and 2-(bromomethyl)-2-hydroxymethylpropan-1,2-diol (354 mg, 1.78 mmol, 2 eq). Chromatography (SP4, silica, 17-100 % EtOAc, petrol) gave 322 as a white solid (227 mg, 42%). $R_f = 0.45$ (3:7 EtOAc:PE); mp = 185.7-185.4 °C; UV $\lambda_{\text{max}}$ (EtOH): 262.0, 250.0 nm; IR $\nu_{\text{max}}$ 3376 (C=O), 2942, 2160 (Ethynyl), 1695 (C=O), 1588 (Amide C-N), 757 (C-Cl), 655 cm$^{-1}$; $\delta_H$ (500 MHz $d_6$): 2.86 (1H, d, $J = 8.5$ Hz, OCH$_2$), 3.24 (1H, d, $J = 8.5$ Hz OCH$_2$), 3.52-3.60 (4H, m, CH$_2$OH), 3.67 (1H, s, CH$_2$Br), 4.37 (1H, d, $J = 16.0$ Hz, NCH$_2$), 4.70 (1H, $J = 4.9$ Hz, OH), 4.76 (1H, t, $J = 4.8$ Hz, OH), 4.90 (1H, d, $J = 16.0$ Hz, NCH$_2$), 7.20-7.24 (6H, m, Ar-H), 7.76 (2H, d, $J = 4.1$ Hz, Ar-H), 7.94-7.98 (3H, m, Ar-H); $\delta_C$ (125 MHz $d_6$ DMSO): 36.7 (CBr), 41.1 (C(CH$_3$)$_4$), 44.6 (NCH$_2$), 60.4 (CH$_2$OH), 60.4 (CH$_2$OH), 62.0 (OCH$_2$), 93.0 (hemi-aminal carbon), 122.1, 122.8, 127.9, 128.4, 129.2, 132.9, 133.2, 133.8, 134.1, 135.3, 139.8, 144.8, 146.0 (CNO$_2$), 165.8 (C=O) ; LCMS $R_t = 1.56$ min, (3); MS (ES-) m/z 655 [M+HCOO]$^-$; HMRS: Calculated for C$_{26}$H$_{27}$Br$^{35}$Cl$_2$N$_3$O$_6$ 626.0455 [M+NH$_4]^+$ Found 626.0437 [M+NH$_4]^+$
7. MDM2/p53 Experimental Procedures

4-Chloro-3-(4-chlorophenyl)-3-hydroxymethyloxetan-3-ylmethoxy)-2-(4-nitrobenzyl)isoindolin-2-one (317)

General procedure H: 3-(2-bromomethyl-3-hydroxy-2-hydroxymethyl-propoxy)-4-chloro-3-(4-chlorophenyl)-2-(4-nitrobenzyl)isoindolin-1-one (63 mg, 0.10 mmol). Chromatography (SP4, silica, 17-100% EtOAc, PE) gave the title compound as colourless oil (36 mg, 68%). \( R_f = 0.27 \) (3:7 EtOAc:PE); mp = 207.8-208.3 °C; UV \( \lambda_{\text{max}} \) (EtOH): 267.0 nm; IR \( \nu_{\text{max}} \) 3408 (OH), 2875, 2163, 1687 (C=O), 1518 (Amide C-N), 1339 (NO\( _2 \)) cm\(^{-1} \); \( \delta_H \) (500 MHz CDCl\( _3 \)): 1.72 (1H, s, OH), 3.07 (1H, d, \( J = 9.0 \) Hz, OCH\( _2 \)), 3.31 (1H, d, \( J = 9.0 \) Hz, OCH\( _2 \)), 3.86 (2H, s, CH\( _2 \)OH), 4.36-4.44 (4H, m, oxetane CH\( _2 \)), 4.42 (1H, d, \( J = 15.0 \) Hz, NCH\( _2 \)), 4.52 (1H, d, \( J = 15.0 \) Hz, NCH\( _2 \)), 7.16-7.26 (6H, m, Ar-H), 7.50-7.51 (1H, m, \( H^5 \)), 7.55-7.58 (1H, m, \( H^6 \)), 7.90 (1H, dd, \( J = 3.5 \) and 7.5 Hz, \( H^7 \)), 7.98 (2H, d, 8.5 Hz, CHCNO\( _2 \)); \( \delta_C \) (125 MHz CDCl\( _3 \)): 42.2 (NCH\( _2 \)), 44.0 (oxetane OCH\( _2 \)C), 64.4 (OCH\( _2 \)), 64.8 (CH\( _2 \)OH), 75.6 (oxetane CH\( _2 \)), 75.6 (oxetane CH\( _2 \)), 94.4 (semi-aminal), 122.6, 123.4, 128.8, 128.6, 129.6, 129.8, 132.3, 133.9, 134.3, 134.6, 135.1, 140.3, 144.1, 147.1 (C-NO\( _2 \)), 166.8 (C=O); LCMS \( R_t = 1.48 \) min, (3); MS (ESI+) m/z 573 [M+HCOOH]\(^+\); HRMS m/z: Calc. for C\(_{26}\)H\(_{26}\)Cl\(_2\)N\(_3\)O\(_6\) 546.1193 [M+NH\(_4\)]\(^+\). Found: 546.1190 [M+NH\(_4\)]\(^+\).; Analytical HPLC: 97.9% purity

Chiral Separation mobile phase hexane:ethanol 85:15:

\textbf{326} Peak 1: (50 mg, 49%); Specific rotation \([\alpha] = -10.7^\circ\) (at 24.4 °C, wavelength = 589 nm, tube length = 0.1 dm, concentration = 0.560 g per 100 mL);

\textbf{327} Peak 2: (50 mg, 49%); Specific rotation \([\alpha] = +30.3^\circ\) (at 24.5 °C, wavelength = 589 nm tube length = 0.1 dm, concentration = 0.462 g per 100 mL)
3-(2-Bromomethyl-3-hydroxy-2-hydroxymethyl-proxy)-4-chloro-3-(4-chlorophenyl)-2-((4-triisopropylsilyl)ethynyl)benzyl)isoindolin-2-one (323)

![Structure of 323]

**General procedure C:** 3-Chloro-3-(4-chlorophenyl)-3-hydroxy-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (334 mg, 0.59 mmol) and 2-(bromomethyl)-2-(hydroxymethyl)-1,3-propan-diol (236 mg, 1.19 mmol). Chromatography (SP4, silica, 10-80% EtOAc, PE) gave 323 as a colourless oil (135 mg, 31%). $R_t = 0.12$ (3:7 EtOAc:PE); UV $\lambda_{\text{max}}$ (EtOH): 267.0, 255.0 nm; IR $\nu_{\text{max}}$ 3342 (OH), 2942, 2865, 2361, 2156 (Ethynyl), 1685 (C=O), 1589 (Amide C-N), 1041, 761 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.04 (18H, s, CH$_3$), 2.13 (1H, s, OH), 2.18 (1H, s, OH), 2.73 (1H, s, $J = 8.9$ Hz, CH$_2$Br), 3.10 (1H, d, $J = 8.9$ Hz, CH$_2$Br), 3.42 (1H, d, $J = 10.5$ Hz, OCH$_2$), 3.46 (1H, d, $J = 10.5$ Hz, OCH$_2$), 3.57-3.66 (4H, m, CH$_2$OH), 4.24 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.45 (1H, d, $J = 15.1$ Hz, NCH$_2$), 6.86 (2H, d, $J = 8.2$ Hz, benzyl Ar-H), 7.05-7.14 (6H, m, Ar-H), 7.40 (1H, dd, $J = 1.0$ and 8.0 Hz, $H^5$), 7.44-7.47 (1H, m, $H^5$), 7.79 (1H, dd, $J = 1.0$ and 7.4 Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 11.3 (CH$_3$), 18.7 (CH(CH$_2$)$_2$), 34.7 (CH$_2$Br), 42.8 (C(CH$_3$)$_3$), 44.5 (NCH$_2$), 63.05 (OCH$_2$), 64.2 (CH$_2$OH), 64.5 (CH$_2$OH), 90.9 (ethynyl), 94.4 (ethynyl), 106.7 (hemi-aminal), 122.4, 122.5, 128.1, 128.4, 128.7, 129.7, 131.8, 132.2, 134.1, 134.2, 134.6, 134.9, 136.9, 140.2, 166.8 (C=O); LCMS $R_t = 2.26$ min, (4); HRMS $m/z$: Calc. for C$_{37}$H$_{48}$Br$^{79}$Cl$_2$NO$_4$Si: 761.1938 [M+NH$_4$]$^+$; Found 761.1934 [M+NH$_4$]$^+$. 

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4-Chloro-3-(4-chlorophenyl)-3-(3-hydroxymethyl-oxetan-3-ylmethoxy)-2-(((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (324)

**General procedure H:** 3-(2-bromomethyl-3-hydroxy-2-hydroxymethyl-propoxy)-4-chloro-3-(4-chlorophenyl)-2-(((4-triisopropylsilyl)ethynyl)benzyl)isoindolin-2-one (750 mg, 2.88 mmol). Chromatography (SP4, silica, 12-100% EtOAc, PE) gave 324 as a colourless oil (142 mg, 63%). \( R_f = 0.18 \) (1:1 EtOAc:PE); UV \( \lambda_{\text{max}} \) (EtOH): 262.0, 250.5 nm; IR \( \nu_{\text{max}} \) 3362 (OH), 2842, 2865, 2156 (Ethynyl), 1694 (C=O), 1590 (Amide C-N), 815, 761 (C-Cl) cm\(^{-1}\); \( \delta_H \) (500 MHz CDCl\(_3\)): 1.10 (18H, s, CH\(_3\)), 1.76 (1H, t, 4.5 Hz, OH), 2.99 (1H, d, \( J = 9.0 \) Hz, OCH\(_2\)), 3.17 (1H, d, \( J = 9.0 \)Hz, OCH\(_2\)), 3.71 (1H, dd, \( J = 4.5 \) and 10.8 Hz, \( CH_2OH \)), 3.79 (1H, dd, \( J = 4.5 \) and 10.8 Hz, \( CH_2OH \)), 4.20 (1H, d, \( J = 15.0 \) Hz, NCH\(_2\)), 4.29 (1H, d, \( J = 6.25 \) Hz, oxetane \( CH_2 \)), 4.37-4.41 (3H, m, oxetane \( CH_2 \)), 4.49 (1H, d, \( J = 15.0 \) Hz, NCH\(_2\)), 7.04 (2H, d, \( J = 8.2 \) Hz, benzyl Ar-H), 7.19-7.20 (4H, m, Ar-H), 7.25 (2H, d, \( J = 8.2 \) Hz, benzyl Ar-H), 7.46 (1H, dd, \( J = 0.8 \) and 8.0 Hz, \( H^5 \)), 7.51-7.54 (1H, m, \( H^6 \)), 7.87 (1H, dd, \( J = 0.8 \) and 7.4 Hz, \( H^7 \)); \( \delta_C \) (125 MHz CDCl\(_3\)): 11.3 (CH\(_3\)), 18.7 (\( CH(CH_3)_2 \)), 42.8 (NCH\(_2\)), 43.9 (oxetane quat. C), 64.3 (OCH\(_2\)), 65.0 (CH\(_2OH\)), 75.4 (oxetane \( CH_2 \)), 75.6 (oxetane \( CH_2 \)), 91.1 (CC-Si), 94.7 (CC-Si), 106.6 (hemi-aminial), 122.5 (C-CC-Si), 122.7, 128.3, 128.6, 128.8, 129.6, 131.7, 132.1, 134.1, 134.4, 134.6, 134.9, 137.0, 140.0, 166.7 (C=O); LCMS \( R_t = 2.20 \) min, (5); MS (ESI+) \( m/z \) 664.5 [M+H]^+
4-Chloro-3-(4-chlorophenyl)-2-(4-ethynylbenzyl)-3-(3-hydroxymethyl-oxetan-3-ylmethoxy)isoindolin-1-one (325)

**General procedure G:** 4-chloro-3-(4-chlorophenyl)-3-(3-hydroxymethyl-oxetan-3-ylmethoxy)-2-(((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (142 mg, 0.21 mmol. Chromatography (SP4, silica, 12-100% EtOAc, PE) gave 325 as a colourless oil (95 mg, 89%). $R_f$ = 0.18 (1:1, EtOAc:PE); mp = 182.2-182.9 °C; UV $\lambda_{\text{max}}$ (EtOH): 229.0 nm; IR $\nu_{\text{max}}$ (3447 (OH), 3296, 2924, 2872, 2360, 2162 (Ethynyl), 2026, 1709, 1683 (C=O), 1587 (Amide C-N), 762 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.58 (1H, t, $J = 5.3$ Hz, OH), 3.02 (1H, d, $J = 8.9$, OCH$_2$), 3.07 (1H, s, CCH), 3.21 (1H, d, $J = 8.9$ Hz, OCH$_2$), 3.74 (1H, dd, $J = 5.3$ and 10.9 Hz, CH$_2$OH), 3.80 (1H, dd, $J = 5.3$ and 10.9 Hz, CH$_2$OH), 4.17 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.31-4.40 (4H, m, oxetane CH$_2$), 4.58 (1H, d, $J = 15.1$ Hz, NCH$_2$), 7.13 (2H, d, $J = 8.2$ Hz, benzyl Ar-H), 7.24-7.43 (6H, m, Ar-H), 7.50 (1H, dd, $J = 0.9$ and 7.9 Hz, $H^6$), 7.55-7.59 (1H, m, $H^6$), 7.92 (1H, dd, $J = 0.9$ and 7.4 Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 42.8 (NCH$_2$), 43.9 (oxetane OCH$_2$C), 64.4 (OCH$_2$), 65.0 (CH$_2$OH), 75.4 (oxetane CH$_2$), 75.6 (oxetane CH$_2$), 77.6 (CCH), 83.1 (CCH), 94.8 (hemiacetal), 121.3, 122.5, 128.6, 128.9, 129.6, 132.1, 132.1, 134.1, 134.1, 134.6, 134.9, 137.7, 140.4, 166.8 (C=O); LCMS $R_t$ =1.53 min, (3); MS (ESI+) m/z 552 [M+HCOO]$^+$; HRMS m/z: Calc. for C$_{28}$H$_{33}$Cl$_2$NO$_4$ 508.1077 [M+H]$^+$; Found 508.1074 [M+H]$^+$; Analytical HPLC: 99.2% purity

Chiral Separation mobile phase tert butyl methyl ether:isopropanol 95:5:

328 Peak 1: (38 mg, 42%); Specific rotation $[\alpha] = -7.74 ^\circ$ (at 25.6 °C, wavelength = 589 nm, tube length = 0.1 dm, concentration = 0.517 g per 100 mL);
329 Peak 2: (37 mg, 41%); Specific rotation $[\alpha] = +4.65 ^\circ$ (at 25.6 °C, wavelength = 589 nm tube length = 0.1 dm, concentration = 0.43 g per 100 mL)
**5-**tert-Butyl-2-hydroxybenzaldehyde (340)**

![Chemical Structure](image)

To a solution of 4-**tert**butylphenol (2.00 g, 13.3 mmol, 1 eq) in TFA (20 mL) was added hexamethylenetetramine (1.86 g, 13.3 mmol, 1 eq) and the reaction was heated to 120 °C for 30 min with microwave heating, cooled in an ice-bath, diluted with water (40 mL) and neutralised using K₂CO₃. The resulting aqueous solution was extracted with EtOAc (3 x 60 mL) and combined organic extracts dried over MgSO₄ and concentrated *in vacuo*. Chromatography (SP4, silica, 1-10% EtOAc, PE) gave 340 as a white oily solid (1.09 g, 46%). \( R_f = 0.57 \) (5:95 EtOAc:PE); UV \( \lambda_{max} \) (EtOH): 335.0, 258.5, 220.0 nm; IR \( \nu_{max} \) (CH₃): 2963 (OH), 2160, 1652 (C=O), 1483 cm⁻¹; \( \delta_H \) (500 MHz CDCl₃): 1.25 (9H, s, CH₃), 6.86 (1H, d, \( J = 8.8 \) Hz, \( H^3 \)), 7.44 (1H, d, \( J = 2.5 \) Hz, \( H^6 \)), 7.51 (1H, dd, \( J = 2.5 \) and 8.8 Hz, \( H^5 \)), 9.81 (1H, d, OH), 10.80 (1H, s, CHO); \( \delta_C \) (125 MHz CDCl₃): 31.4 (CH₃), 34.1 (C(CH₃)₃), 117.2 (C), 120.0, 129.8, 134.8, 142.8 (CC(CH₃)₃), 157.8 (COH), 196.9 (C=O); LCMS \( R_t = 1.52 \) min, (3), MS (ESI+) \( m/z \) 179.2 [M+H]+

**4-Chlorobenzoic acid (4-**tert**-butyl-2-hydroxy-benzylidene)hydrazine (341)**

![Chemical Structure](image)

To a solution of 340 (1.06 g, 5.96 mmol, 1 eq) in AcOH (20 mL) was added 4-chlorobenzhydrazide (1.02 g, 5.96 mmol, 1 eq) and the reaction stirred at room temperature for 15 min, then added dropwise to ice-cold water (20 mL). The precipitate was collected by filtration, washed with ice-cold water (20 mL) and petrol, (10 mL) and dried over P₂O₅ to give 341 as a white solid (1.85 g, 94%). \( R_t = 0.26 \) (1:4 EtOAc:PE); mp = 225.2-228.0 °C; UV \( \lambda_{max} \) (EtOH): 338.5, 290.5, 241.0 nm; IR \( \nu_{max} \) 2953 (OH), 2159 (C=N), 1642 (C=O), 826, 750 (C-Cl) cm⁻¹; \( \delta_H \) (500 MHz d₆ DMSO): 1.29 (9H, s, CH₃), 6.89 (1H, dd, \( J = 8.6 \) Hz, \( H^6 \)), 7.36 (1H, dd, \( J = 2.2 \) and 8.6 Hz, \( H^5 \)), 7.53 (1H, d, \( J = 2.2 \) Hz, \( H^3 \)), 7.64 (2H, d, \( J = 8.1 \) Hz, CHCCl), 7.98 (2H,
d, J = 8.1 Hz, CHCCO), 8.66 (1H, s, CHN), 11.02 (1H, s, OH), 12.12 (1H, s, NH); δC (125 MHz d6 DMSO): 31.2 (CH3), 33.8 (C(CH3)3), 116.1 (C6), 17.9, 125.4 (C3), 128.6, 128.7, 129.6 (CHCl), 131.6 (CHCCO), 136.7, 141.5, 148.8 (C=N), 155.3 (CO), 161.8 (C=O); LCMS Rf = 1.60 min, (3); MS (ESI+) m/z 331.2 [M+H]+; HRMS m/z: Calc. for C18H20Cl3N2O2: 331.1208 [M+H]+. Found 331.1207 [M+H]+.

5-tert-Butyl-2-(4-chlorobenzoyl)benzaldehyde (342)

To 341 (500 mg, 1.51 mmol, 1 eq) in THF (11 mL) at 0 °C was added Pb(OAc)4 (670 mg, 1.51 mmol, 1 eq) portionwise and stirred for 2 h at 0 °C, then concentrated in vacuo. The residue was dissolved in EtOAc (30 mL), filtered through Celite and washed with EtOAc (20 mL). The filtrate was washed with sat. NaHCO3 solution (30 mL) and brine (30 mL), dried over Na2SO4 and concentrated in vacuo. Chromatography (SP4, silica, 2-20% EtOAc, PE) gave 342 as a white solid (331 mg, 73%). Rf = 0.51 (1:9 EtOAc:PE); mp = 116.3-117.1 °C; UV λmax (EtOH): 261.0 nm; IR νmax 2963 (Aldehyde C-H), 2160, 1688 (Ketone C=O), 1583 cm⁻¹; δH (500 MHz CDCl3): 1.34 (9H, s, CH3), 7.35-7.38 (3H, m, Ar-H), 7.64 (1H, dd, J = 2.0 and 8.0 Hz, H5), 7.68 (2H, d, J = 8.7 Hz, Ar-H), 7.97 (1H, d, J = 2.0 Hz, H7), 9.96 (1H, s, CHO); δC (125 MHz CDCl3): 31.1 (CH3), 35.2 (C(CH3)3), 127.4, 129.0, 129.0, 130.5, 131.3, 135.5, 135.7, 138.0, 140.0 (CCI), 154.7 (CC(CH3)3), 191.0 (CHO), 195.4 (CO); LCMS Rf = 1.80 min, (3); MS (ESI+) m/z 301.2 [M+H]+; HRMS m/z: Calc. for C18H18Cl3O2: 301.0990 [M+H]+. Found 301.0997 [M+H]+.

6-tert-Butyl-2-(4-chlorobenzoyl)benzoic acid (343)

To a solution of 5-tert-butyl-2-(4-chlorobenzoyl)benzaldehyde (606 mg, 2.01 mmol, 1 eq) in MeCN (12 mL) was added sulfamic acid (364 mg, 2.62 mmol, 1.3 eq) in water (2 mL) and the reaction was stirred for 10 min before NaClO2 (254 mg, 4.03
mmol, 2 eq) in water (2 mL) was added dropwise, and stirred 1.5 h, then concentrated in vacuo. The residue was suspended in water (30 mL) and extracted with EtOAc (3 x 40 mL). The combined organic layers were dried over Na₂SO₄ and evaporated in vacuo. Recrystallisation (EtOAc and petrol) gave 343 as a white fluffy solid (529 mg, 83%). Rf = 0.06 (3:7 EtOAc:PE); mp = 218.1-219.6 °C; UV λmax (EtOH): 254.5 nm; IR v max (cm⁻¹) 2965 (OH), 2161, 2032, 1679 (Ketone C=O), 1584 (Acid C=O), 757 (C-Cl) cm⁻¹; δH (500 MHz d₆ DMSO): 1.36 (9H, s, CH₃), 7.39 (1H, d, J = 8.0 Hz, H³), 7.58 (2H, d, J = 8.5 Hz, CHCl), 7.63 (2H, d, J = 8.5 Hz, CHCO), 7.78 (1H, d, J = 8.0 Hz, H⁴), 7.98 (1H, s, H⁶); δC (125 MHz CDCl₃): 30.8 (CH₃), 34.6 (C(CH₃)₃), 126.2 (C⁶), 127.5 (C⁵), 128.8, 129.4 (CHCl), 129.9 (C⁴), 130.5 (CHCO), 135.9 (CCOOH), 137.9 (CCO), 138.2 (CCl), 152.7 (CC(CH₃)₃), 167.1 (COOH), 195.3 (CO); LCMS Rt = 1.58 min, (3); MS (ESI+) m/z 317.2 [M+H]+; HRMS m/z: Calc. for C₁₈H₁₈ClO₃: 317.0939 [M+H]+. Found 317.0942 [M+H]+.

6-tert-Butyl-3-(chlorophenyl)-3-hydroxy-2-(4-iodobenzyl)isoindolin-2-one (334)

[Diagram]

General procedure B: 6-tert-butyl-2-(4-chlorobenzoyl)-benzoic acid (500 mg, 1.58 mmol) and 4-iodobenzylamine hydrochloride (468 mg, 1.74 mmol). Chromatography (SP4, silica, 7-60 % EtOAc, PE) gave 334 as a white solid (724 mg, 86%). Rf = 0.68 (3:7 EtOAc:PE); mp = 223.6-226.1 °C; UV λmax (EtOH): 228.5 nm; IR v max 3253 (OH), 2970, 2159, 1664 (C=O), 1623, 835, 695 (C-Cl) cm⁻¹; δH (500 MHz CDCl₃): 1.26 (9H, s, CH₃), 3.25 (1H, s, OH), 3.88 (1H, d, J = 15.1 Hz, NCH₂), 4.38 (1H, d, J = 15.1 Hz, NCH₂), 6.80 (2H, d, J = 8.3 Hz, benzyl Ar-H), 7.09-7.13 (5H, m, Ar-H), 7.37 (2H, d, J = 8.3 Hz, benzyl Ar-H), 7.47 (1H, dd, J = 1.8 and 8.1 Hz, H⁵), 7.81 (1H, d, J = 1.8 Hz, H³); δC (125 MHz CDCl₃): 31.4 (CH₃), 35.2 (C(CH₃)₃), 42.4 (NCH₂), 91.0 (Cl), 92.6 (hemi-aminal), 120.5, 122.3, 127.9, 128.6, 129.7, 130.5, 130.7, 134.5, 136.8, 137.3, 137.6, 145.7, 153.7 (CC(CH₃)₃), 168.1 (C=O); LCMS Rt = 1.87 min, (5); MS (ESI+) m/z 532.2 [M+H]+; HRMS m/z: Calc. for C₂₅H₂₃ClNO₂ 532.0535 [M+H]+. Found 532.0532 [M+H]+.
6-tert-Butyl-3-(4-chlorophenyl)-3-hydroxy-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (335)

![Chemical structure of 335]

**General procedure F: 334** (700 mg, 1.32 mmol, 1 eq) and triisopropylsilylacetylene (384 µL, 1.71 mmol, 1.3 eq). Chromatography (SP4, silica, 3-30% EtOAc, PE) gave 335 as a white solid (708 mg, 91%). $R_f = 0.34$ (15:85 EtOAc:PE); mp = 165.8-167.7 $^\circ$C; UV $\lambda_{max}$ (EtOH): 266.0, 254.5 nm; IR $\nu_{max}$ (OH): 3285 (OH), 2945, 2866, 2153 (Ethynyl), 1677 (C=O), 838 cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.01 (18H, s, Si(CH$_3$)$_2$), 1.23 (9H, s, C(CH$_3$)$_3$), 3.78 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.16 (1H, d, $J = 15.1$ Hz, NCH$_2$), 4.55 (1H, s, OH), 6.80 (2H, d, $J = 8.2$ Hz, benzyl Ar-H), 7.02-7.09 (6H, m, Ar-H), 7.42 (1H, dd, $J = 1.6$ and 8.1 Hz, $H^5$), 7.76 (1H, d, 1.6 Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 11.3 (Si(CH(CH$_3$)$_3$)$_2$), 18.7 (Si(CH(CH$_3$)$_3$)$_3$), 31.4 (C(CH$_3$)$_3$), 35.1 (C(CH$_3$)$_3$), 42.6 (NCH$_2$), 90.4 (ethynyl), 90.9 (ethynyl), 107.0 (hemi-aminal), 120.4 (C-ethynyl), 122.1, 127.9, 128.4, 128.6, 129.8, 130.4, 131.7, 134.4, 137.1, 137.9146.1, 153.3 (CC(CH$_3$)$_3$), 168.5 (C=O); LCMS $R_t = 2.50$ min, (5); MS (ESI+) m/z 586.4 [M+H]$^+$; HRMS m/z: Calc. for C$_{36}$H$_{45}^{35}$ClNO$_2$Si: 586.2903 [M+H]$^+$. Found 586.2901 [M+H]$^+$.

6-tert-Butyl-3-(4-chlorophenyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (336)

![Chemical structure of 336]
**General procedure C:** 6-tert-butyl-3-(4-chlorophenyl)-3-hydroxy-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (354 mg, 0.60 mmol, 1 eq) and bis(hydroxymethyl)cyclopropane (0.12 mL, 1.21 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 336 as a colourless oil (200 mg, 50%). \( R_f = 0.53 \) (3:7 EtOAc:PE); \( \lambda_{\text{max}} \) (EtOH): 266.5, 254.5 nm; \( \nu_{\text{max}} \) (EtOH): 1685 (C=O) cm\(^{-1}\); \( \delta_H \) (500 MHz CDCl\(_3\)): 0.35-0.41 (2H, m, cyclopropyl), 1.33 (9H, s, C(CH\(_3\))\(_3\)), 1.61 (1H, t, \( J = 5.5 \) Hz, OH), 2.82 (1H, d, \( J = 9.5 \) Hz, OCH\(_2\)), 3.34 (1H, dd, \( J = 5.5 \) and 11.3 Hz, CH\(_2\)OH), 3.46 (1H, dd, \( J = 5.5 \) and 11.3 Hz, CH\(_2\)OH), 4.16 (1H, d, \( J = 15.0 \) Hz, NCH\(_2\)), 4.55 (1H, d, \( J = 15.0 \) Hz, NCH\(_2\)), 7.03 (1H, d, \( J = 8.0 \) Hz, \( H^1 \)), 7.11 (2H, d, \( J = 8.2 \) Hz, benzyl Ar-H), 7.15-7.19 (4H, m, Ar-H), 7.24 (2H, d, \( J = 1.6 \) Hz, Ar-H), 7.52 (1H, dd, \( J = 1.8 \) and 8.0 Hz, \( H^5 \)), 7.90 (1H, d, \( J = 1.8 \) Hz, \( H^7 \)); \( \delta_C \) (125 MHz CDCl\(_3\)): 8.6 (cyclopropyl), 8.7 (cyclopropyl), 11.3 (Si(CH(CH\(_3\))\(_2\))), 18.7 (Si(CH(CH\(_3\))\(_2\))), 22.1 (cyclopropyl), 31.4 (C(CH\(_3\))\(_3\)), 35.2 (C(CH\(_3\))\(_3\)), 42.8 (NCH\(_2\)), 67.8 (CH\(_2\)OH), 68.1 (OCH\(_2\)), 90.7 (ethynyl), 94.7 (ethynyl), 106.8 (hemi-aminal), 120.6, 122.4, 122.5, 127.9, 128.6, 129.0, 130.3, 131.3, 131.8, 137.3, 137.7, 143.2, 153.8 (CC(CH\(_3\))\(_3\)), 168.7 (C=O); LCMS \( R_t = 2.66 \) min, (5); MS (ESI+) \( m/z \) 670.5 [M+H]\(^+\); HRMS \( m/z \): Calc. for C\(_{41}\)H\(_{53}\)ClNO\(_3\)Si: 670.3478 [M+H]\(^+\). Found 670.3477 [M+H]\(^+\).

6-tert-Butyl-3-(4-chlorophenyl)-2-(4-ethynylbenzyl)-3-(1-((hydroxymethyl)cyclopropyl)methoxy)isoindolin-1-one (330)

**General procedure G:** 6-tert-butyl-3-(4-chlorophenyl)-3-(1-((hydroxymethyl)cyclopropane)methoxy)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (150 mg, 0.22 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 330 as a colourless oil (91 mg, 80%). \( R_f = 0.36 \) (3:7 EtOAc:PE); \( \lambda_{\text{max}} \) (EtOH): 230.0 nm;
IR $\nu_{\text{max}}$ 3494 (OH), 3295, 2962, 2869, 2158 (Ethynyl), 2030, 1686 (C=O), 1489 cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.08-0.11 (1H, m, cyclopropyl), 0.12-0.15 (1H, m, cyclopropyl), 0.36-0.42 (2H, m, cyclopropyl), 1.35 (9H, s, C(CH$_3$)$_3$), 1.67 (1H, s, OH), 2.63 (1H, d, $J = 9.5$ Hz, OCH$_2$), 2.84 (1H, d, $J = 9.5$ Hz, OCH$_2$), 3.04 (1H, s, CCH), 3.35 (1H, d, $J = 11.0$ Hz, CH$_2$OH), 3.47 (1H, d, $J = 11.0$ Hz, CH$_2$OH), 4.16 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.59 (1H, d, $J = 15.0$ Hz, NCH$_2$), 7.05 (1H, d, $J = 8.0$ Hz, $H^4$), 7.16-7.20 (6H, m, Ar-H), 7.30 (2H, d, $J = 8.2$ Hz, Ar-H), 7.54 (1H, dd, $J = 1.7$ and 8.0 Hz, $H^5$), 7.92 (1H, d, $J = 1.3$ Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 31.4 (C(CH$_3$)$_3$), 35.2 (C(CH$_3$)$_3$), 42.8, (NCH$_2$), 67.8 (CH$_2$OH), 68.0 (OCH$_2$), 83.4 (ethynyl), 94.8 (hemiaminal) 120.6 (C-CCH), 121.0, 122.4, 127.9, 128.6, 129.1, 130.3, 131.3, 131.9, 134.5, 137.3, 138.4, 142.3, 153.8 (C-tBu), 168.7 (C=O); LCMS $R_t$ = 2.66 min, (5); HRMS $m/z$: Calc. for C$_{32}$H$_{32}$ClNO$_3$: 514.2143 [M+H]$^+$. Found: 514.2140 [M+H]$^+$.; Analytical HPLC: 95.6% purity

3-(2-Bromomethyl-2-hydroxymethyl-propoxy)-6-tert-butyl-3-(4-chlorophenyl)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (349)

**General procedure D:** 334 (266 mg, 0.45 mmol), 2-(bromomethyl)-2-(hydroxymethyl)propan-1,3-diol (902 mg, 4.34 mmol) in THF (5 mL). Reaction quenched after 3.5 h at 0°C and 1 h at RT. Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 349 as a colourless oil (300 mg, 87%). $R_t$ = 0.26 (3:7 EtOAc:PE); UV $\lambda_{\text{max}}$ (EtOH): 267.0, 255.0 nm; IR $\nu_{\text{max}}$ 3567 (OH), 2941, 2865, 2365, 2157 (Ethynyl), 1685 (C=O), 839 cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 0.04 (18H, s, Si(CH(CH$_3$)$_2$)$_3$), 1.29 (9H, s, C(CH$_3$)$_3$), 1.96 (2H, s, OH), 2.74 (1H, d, $J = 9.1$ Hz, CH$_2$Br), 2.88 (1H, d, $J = 9.1$ Hz, CH$_2$Br), 3.40 (1H, d, $J = 10.4$ Hz, OCH$_2$), 3.44 (1H, s, CH$_2$OH), 3.79 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.59 (1H, d, $J = 15.0$ Hz, NCH$_2$), 7.05 (1H, d, $J = 8.0$ Hz, $H^4$), 7.16-7.20 (6H, m, Ar-H), 7.30 (2H, d, $J = 8.2$ Hz, Ar-H), 7.54 (1H, dd, $J = 1.7$ and 8.0 Hz, $H^5$), 7.92 (1H, d, $J = 1.3$ Hz, $H^7$); $\delta_C$ (125 MHz CDCl$_3$): 8.6 (cyclopropyl), 8.6 (cyclopropyl), 22.1 (cyclopropyl), 31.4 (C(CH$_3$)$_3$), 35.2 (C(CH$_3$)$_3$), 42.8, (NCH$_2$), 67.8 (CH$_2$OH), 68.0 (OCH$_2$), 83.4 (ethynyl), 94.8 (hemiaminal) 120.6 (C-CCH), 121.0, 122.4, 127.9, 128.6, 129.1, 130.3, 131.3, 131.9, 134.5, 137.3, 138.4, 142.3, 153.8 (C-tBu), 168.7 (C=O); LCMS $R_t$ = 2.66 min, (5); HRMS $m/z$: Calc. for C$_{32}$H$_{32}$ClNO$_3$: 514.2143 [M+H]$^+$. Found: 514.2140 [M+H]$^+$.; Analytical HPLC: 95.6% purity
d, \( J = 10.4 \) Hz, OCH\(_2\)), 3.49-3.52 (4H, m, CH\(_2\)OH), 4.28 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 4.40 (1H, d, \( J = 15.1 \) Hz, NCH\(_2\)), 6.98-7.00 (3H, m, Ar-H), 7.02 (2H, d, \( J = 8.6 \) Hz, Ar-H), 7.09 (2H, d, \( J = 8.9 \) Hz, Ar-H), 7.17-7.19 (2H, m, Ar-H), 7.48 (1H, dd, \( J = 1.8 \) and 8.0 Hz, \( H^5 \)), 7.82 (1H, d, \( J = 1.8 \) Hz, \( H^7 \)); \( \delta_{C} \) (125 MHz CDCl\(_3\)): 11.3 (Si(CH\((CH_3)_2\))\(_3\)), 18.7 (Si(CH\((CH_3)_2\))\(_3\)), 31.3 (C(CH\(_3\)_3)), 34.6 (CH\(_2\)Br), 35.2 (C(CH\(_3\)_3)), 42.9 (NCH\(_2\)), 44.4 (OCH\(_2\)C(CH\(_3\)_3)), 62.7 (OCH\(_2\)), 64.6 (CH\(_2\)OH), 64.7 (CH\(_2\)OH), 90.9 (ethynyl), 94.7 (ethynyl), 106.7 (hemi-aminal), 120.6 (C\(^\gamma\)), 122.6, 122.6, 127.8, 128.6, 128.8, 130.4, 131.3, 131.8, 134.6, 137.0, 137.5, 141.6, 154.1 (C(CH\(_3\)_3)), 168.8 (C=O); LCMS \( R_t = 2.50 \) min, (5); HRMS m/z: Calc. for C\(_{41}\)H\(_{54}\)Br\(^{35}\)ClNO\(_4\)Si: 766.2689 [M+H]\(^+\). Found 766.2690 [M+H]\(^+\).

6-tert-Butyl-3-(4-chlorophenyl)-3-(3-hydroxymethyl-oxetan-3-ylmethoxy)-2-(4-(((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (350)

![Chemical Structure 350]

**General procedure H: 349** (250 mg, 0.33 mmol). Chromatography (SP4, silica, 7-60% EtOAc, PE) gave 350 as a colourless oil (176 mg, 78%). \( R_f = 0.13 \) (3:7 EtOAc:PE); UV \( \lambda_{max} \) (EtOH): 266.5, 254.5 nm; IR \( \nu_{max} \) 2944, 2866, 1689 (C=O), 728 C-Cl cm\(^{-1}\); \( \delta_H \) (500 MHz CDCl\(_3\)): 1.04 (18H, s, Si(CH\((CH_3)_2\))\(_3\)), 1.29 (9H, s, C(CH\(_3\)_3)), 1.62 (1H, s, OH), 2.94 (2H, s, OCH\(_2\)), 3.60 (1H, d, \( J = 10.8 \) Hz, CH\(_2\)OH), 4.13 (1H, d, \( J = 15.0 \) Hz, NCH\(_2\)), 4.22-4.30 (4H, m, oxetane CH\(_2\)), 4.50 (1H, d, \( J = 15.0 \) Hz), 6.94 (1H, d, \( J = 8.0 \) Hz, H\(^4\)), 7.03 (2H, d, J = 8.0 Hz, benzyl Ar-H), 7.08 (2H, d, \( J = 8.4 \) Hz, Ar-H), 7.13 (2H, d, J = 8.4 Hz, Ar-H), 7.14 (2H, d, \( J = 8.0 \) benzyl Ar-H), 7.48 (1H, dd, \( J = 1.3 \) and 7.9 Hz, H\(^5\)), 7.89 (1H, d, \( J = 1.3 \) Hz, H\(^7\)); \( \delta_{C} \) (125 MHz CDCl\(_3\)): 11.3 (Si(CH\((CH_3)_2\))\(_3\)), 18.7 (Si(CH\((CH_3)_2\))\(_3\)), 31.3 (C(CH\(_3\)_3)), 35.2 (C(CH\(_3\)_3)), 42.9 (NCH\(_2\)), 44.0 (oxetane quat. C), 64.1 (OCH\(_2\)), 65.0 (CH\(_2\)OH), 75.6 (oxetane CH\(_2\)), 75.6 (oxetane CH\(_2\)), 91.0 (ethynyl), 94.8 (ethynyl), 106.7 (hemi-
aminal), 120.8 (C-ethynyl), 122.3, 122.6, 127.8, 128.7, 128.9, 130.5, 131.3, 131.8, 134.6, 136.9, 137.5, 142.0, 154.0 (CC(CH$_3$)$_3$), 168.7 (C=O); LCMS $R_t$ = 2.40 min, (5); MS (ESI+) m/z 686.6 [M+H]$^+$; HRMS m/z: Calc. for C$_{41}$H$_{53}$ClNO$_4$Si: 686.3427 [M+H]$^+$. Found 686.3427 [M+H]$^+$.

6-tert-Butyl-3-(4-chlorophenyl)-2-(4-ethynylbenzyl)-3-(3-hydroxymethyloxetan-3-ylmethoxy)isoindolin-1-one (351)

**General procedure G:** 6-tert-butyl-3-(4-chlorophenyl)-3-(3-hydroxymethyl-oxetan-3-ylmethoxy)-2-(4-((triisopropylsilyl)ethynyl)benzyl)isoindolin-1-one (141 mg, 0.20 mmol). Chromatography (SP4, silica, 12-100 % EtOAc, PE) gave 351 as a colourless oil (62 mg, 59%). $R_f$ = 0.28 (1:1 EtOAc:PE); UV $\lambda_{max}$ (EtOH): 229.5 nm; IR $\nu_{max}$ 3300 (OH), 2963, 2874, 2160 (Ethynyl), 1687 (C=O), 1091, 907, 727 (C-Cl) cm$^{-1}$; $\delta_H$ (500 MHz CDCl$_3$): 1.30 (9H, s, 'tBu), 1.63 (1H, s, OH), 2.92 (2H, s, OCH$_2$), 2.97 (1H, s, ethynyl), 3.59 (1H, d, $J = 11.0$ Hz, CH$_2$OH), 3.62 (1H,d, $J = 11.0$ Hz, CH$_2$OH), 4.08 (1H, d, $J = 15.0$ Hz, NCH$_2$), 4.16 (1H, d, $J = 6.2$ Hz, oxetane CH$_2$), 4.23 (1H, d, $J = 6.2$ Hz, oxetane CH$_2$), 4.25 (1H, d, $J = 6.2$ Hz, oxetane CH$_2$), 4.28 (1H, d, 6.2 Hz, oxetane CH$_2$), 4.56 (1H, d, 14.0 Hz, NCH$_2$), 6.95 (1H, d, $J = 8.0$ Hz, $H^5$), 7.08-7.10 (4H, m, Ar-H), 7.15 (2H, d, $J = 8.9$ Hz, Ar-H), 7.24 (2H, d, $J = 8.2$ Hz, Ar-H), 7.49 (1H, dd, $J = 1.8$ and 8.0 Hz, $H^6$), 7.89 (1H, d, $J = 1.8$ Hz, $H^5$); $\delta_C$ (125 MHz CDCl$_3$): 31.3 (C(CH$_3$)$_3$), 35.2 (C(CH$_3$)$_3$), 42.9 (NCH$_2$), 43.9 (oxetane OCH$_2$C), 64.1 (OCH$_2$), 64.9 (CH$_2$OH), 75.5 (oxetane CH$_2$), 75.6 (oxetane CH$_2$), 77.5 (CCH), 83.2 (CCH), 94.8 (hemi-aminal), 120.8, 121.2, 122.4, 127.8, 128.7, 129.0, 130.6, 131.3, 132.0, 134.7, 136.9, 138.2, 141.9, 154.1, 168.7 (C=O); LCMS $R_t$ = 1.73 min, 2.1(1); MS (ESI+) m/z 530.3 [M+H]$^+$; HRMS m/z: Calc. for C$_{32}$H$_{33}^{35}$ClNO$_4$: 530.2093 [M+H]$^+$. Found 530.2090 [M+H]$^+$; Analytical HPLC: 100.0% purity.
8. Bibliography


Bibliography


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Appendix 1- Protein Crystallography

Methodology

MDM2 residues 17-109 or 17-125 expressed as a GST fusion. To a pellet equivalent to 1 L of culture was added DNAase (2 µL) and lysozme. After 20 min rotation at 4 ºC, sample was lysated on ice for 3 min using sonication in 20 sec cycles with 40 sec rest. Sample centrifuged at 4 ºC for 1 h at 20 000 rpm. Supernatent was loaded into gravity flow glutathione Sepharose column containing 2 mL of resin washed before use with PBS buffer. Column was washed with PBS buffer (20 mL) and protein eluded using 10 mL of glutathione buffer (PBS buffer with 20 mM glutathione). Cleavage was achieved using 1/20th (w/w) 3C protease (prepared in-house as a GST-fusion) overnight at 4 ºC. Further purification achieved using size exclusion chromatography using highload 20/60 Superdex 200 column equilibrated with Hepes buffer.

Table 32 Conditions for crystallisation for each mutant

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Purchased trays</th>
<th>Custom Trays</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>K94A</td>
<td>Molecular Dimension</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1+ 2, Wizard EDL, ST, Proplex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K94E95A</td>
<td>Insufficient protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>AMD, Wizard EDL, ST, JCS6</td>
<td>PEG4000, PEG5000,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEG8000</td>
<td></td>
</tr>
<tr>
<td>E69K70A</td>
<td></td>
<td>PEG4000, 0.2 M (NH₄)₂SO₄, 0.1 M NaOAc (pH adjusted to 4.6) and PEG4000, 0.2 M (NH₄)₂SO₄</td>
<td>Only with inhibitors NCL-00018225 and NCL-00013774 Seeded</td>
</tr>
<tr>
<td>E69A (17-109)</td>
<td>1st Morpheus, PEG/ION</td>
<td>PEG4000, 0.2 M (NH₄)₂SO₄, 0.1 M NaOAc and PEG4000, 0.2 M (NH₄)₂SO₄</td>
<td></td>
</tr>
<tr>
<td>E69A (17-125)</td>
<td>-</td>
<td>PEG4000, 0.2 M (NH₄)₂SO₄, 0.1 M NaOAc (adjusted to pH 4.6)</td>
<td>-</td>
</tr>
<tr>
<td>E69K70E9 4A</td>
<td>-</td>
<td>PEG5000, 0.5-2.0 M (NH₄)₂SO₄ and PEG8000, 0.2-0.35 M (NH₄)₂SO₄</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 33** Conditions for crystallisation tray set-up

<table>
<thead>
<tr>
<th>52%</th>
<th>54%</th>
<th>56%</th>
<th>58%</th>
<th>60%</th>
<th>62%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 215 µL water</td>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 205 µL water</td>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 195 µL water</td>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 185 µL water</td>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 175 µL water</td>
<td>PEG5000, 0.05 M (NH₄)₂SO₄, 165 µL water</td>
</tr>
<tr>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 190 µL water</td>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 180 µL water</td>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 170 µL water</td>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 160 µL water</td>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 150 µL water</td>
<td>PEG5000, 0.1 M (NH₄)₂SO₄, 140 µL water</td>
</tr>
<tr>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 165 µL water</td>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 155 µL water</td>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 145 µL water</td>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 135 µL water</td>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 125 µL water</td>
<td>PEG5000, 0.15 M (NH₄)₂SO₄, 115 µL water</td>
</tr>
<tr>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 140 µL water</td>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 130 µL water</td>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 120 µL water</td>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 110 µL water</td>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 100 µL water</td>
<td>PEG5000, 0.2 M (NH₄)₂SO₄, 90 µL water</td>
</tr>
</tbody>
</table>
Table 34 Thermofluor plate set-up for identification of the optimal dye concentrations

| 7.5 µL 5 x dye, 7.5 µL 3 µM protein | 7.5 µL 5 x dye, 7.5 µL buffer | 7.5 µL 10 x dye, 7.5 µL 3 µM protein | 7.5 µL 15 x dye, 7.5 µL 3 µM protein | 7.5 µL 20 x dye, 7.5 µL 3 µM protein |
| 7.5 µL 10 x dye, 7.5 µL 4 µM protein | 7.5 µL 10 x dye, 7.5 µL buffer | 7.5 µL 15 x dye, 7.5 µL 4 µM protein | 7.5 µL 20 x dye, 7.5 µL 4 µM protein | 7.5 µL 20 x dye, 7.5 µL 4 µM protein |
| 7.5 µL 15 x dye, 7.5 µL 5 µM protein | 7.5 µL 15 x dye, 7.5 µL buffer | 7.5 µL 20 x dye, 7.5 µL 5 µM protein | 7.5 µL 20 x dye, 7.5 µL 5 µM protein | 7.5 µL 20 x dye, 7.5 µL 5 µM protein |
| 7.5 µL 20 x dye, 7.5 µL 6 µM protein | 7.5 µL 20 x dye, 7.5 µL buffer | 7.5 µL 20 x dye, 7.5 µL 6 µM protein | 7.5 µL 20 x dye, 7.5 µL 6 µM protein | 7.5 µL 20 x dye, 7.5 µL 6 µM protein |

Table 35 MDM2 and p53 concentrations to identify optimal conditions for fluorescence

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<tr>
<th>[MDM2] (nM)</th>
<th>Fluorescently labelled p53 concentration (nM)</th>
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<tr>
<td>5</td>
<td>1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34</td>
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<tr>
<td>10</td>
<td>2, 8, 14, 20, 26, 32, 38, 44, 50, 56, 62, 68</td>
</tr>
<tr>
<td>15</td>
<td>4, 16, 28, 40, 52, 64, 76, 88, 100, 112, 124, 136</td>
</tr>
</tbody>
</table>
Appendix 2 Crystallography of 127

Table 1. Crystal data and structure refinement for 127.

<table>
<thead>
<tr>
<th>Identification code</th>
<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula (moiety)</td>
<td>C_{12}H_{17}NO</td>
</tr>
<tr>
<td>Chemical formula (total)</td>
<td>C_{12}H_{17}NO</td>
</tr>
<tr>
<td>Formula weight</td>
<td>191.27</td>
</tr>
<tr>
<td>Temperature</td>
<td>293(2) K</td>
</tr>
<tr>
<td>Radiation, wavelength</td>
<td>MoKα, 0.71073 Å</td>
</tr>
<tr>
<td>Crystal system, space group</td>
<td>monoclinic, P12_1/c1</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td>a = 9.4720(4) Å, α = 90°</td>
</tr>
<tr>
<td></td>
<td>b = 10.8606(4) Å, β = 102.654(4)°</td>
</tr>
<tr>
<td></td>
<td>c = 10.5802(4) Å, γ = 90°</td>
</tr>
<tr>
<td>Cell volume</td>
<td>1061.97(7) Å³</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>Calculated density</td>
<td>1.196 g/cm³</td>
</tr>
<tr>
<td>Absorption coefficient μ</td>
<td>0.076 mm⁻¹</td>
</tr>
<tr>
<td>F(000)</td>
<td>416</td>
</tr>
<tr>
<td>Crystal colour and size</td>
<td>colourless, 0.50 × 0.40 × 0.40 mm³</td>
</tr>
<tr>
<td>Reflections for cell refinement</td>
<td>3781 (θ range 3.2 to 27.1°)</td>
</tr>
<tr>
<td>Data collection method</td>
<td>Oxford Diffraction Gemini A Ultra</td>
</tr>
<tr>
<td>d</td>
<td>thin-slice ω scans</td>
</tr>
<tr>
<td>θ range for data collection</td>
<td>3.2 to 27.1°</td>
</tr>
<tr>
<td>Index ranges</td>
<td>h −11 to 9, k −10 to 13, l −13 to 11</td>
</tr>
<tr>
<td>Completeness to θ = 26.0°</td>
<td>87.8 %</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>6016</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>1880 (R_{int} = 0.0172)</td>
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<tr>
<td>Reflections with F^2&gt;2σ</td>
<td>1503</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>semi-empirical from equivalents</td>
</tr>
<tr>
<td>Min. and max. transmission</td>
<td>0.9632 and 0.9704</td>
</tr>
<tr>
<td>Structure solution</td>
<td>direct methods</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F²</td>
</tr>
<tr>
<td>Weighting parameters a, b</td>
<td>0.0456, 0.0840</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>1880 / 0 / 128</td>
</tr>
<tr>
<td>Final R indices [F^2&gt;2σ]</td>
<td>R1 = 0.0310, wR2 = 0.0809</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.0422, wR2 = 0.0839</td>
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<tr>
<td>Goodness-of-fit on F²</td>
<td>1.087</td>
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<tr>
<td>Extinction coefficient</td>
<td>0.022(5)</td>
</tr>
<tr>
<td>Largest and mean shift/su</td>
<td>0.000 and 0.000</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.16 and −0.14 e Å⁻³</td>
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</table>
Table 2. Atomic coordinates and equivalent isotropic displacement parameters (Å²) for 127. \( U_{eq} \) is defined as one third of the trace of the orthogonalized \( U^{ij} \) tensor.

<table>
<thead>
<tr>
<th></th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>( U_{eq} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>O(1)</td>
<td>0.28781(8)</td>
<td>0.03042(7)</td>
<td>0.06200(6)</td>
<td>0.0252(2)</td>
</tr>
<tr>
<td>N(1)</td>
<td>0.42847(10)</td>
<td>-0.18797(9)</td>
<td>0.38777(8)</td>
<td>0.0271(3)</td>
</tr>
<tr>
<td>C(1)</td>
<td>0.37117(11)</td>
<td>-0.07559(10)</td>
<td>0.39158(10)</td>
<td>0.0243(3)</td>
</tr>
<tr>
<td>C(2)</td>
<td>0.32505(11)</td>
<td>-0.00161(10)</td>
<td>0.28520(10)</td>
<td>0.0220(3)</td>
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<tr>
<td>C(3)</td>
<td>0.33582(11)</td>
<td>-0.04560(10)</td>
<td>0.16406(9)</td>
<td>0.0193(3)</td>
</tr>
<tr>
<td>C(4)</td>
<td>0.39456(11)</td>
<td>-0.16148(10)</td>
<td>0.15631(10)</td>
<td>0.0211(3)</td>
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<td>C(5)</td>
<td>0.43863(11)</td>
<td>-0.22702(10)</td>
<td>0.27032(10)</td>
<td>0.0255(3)</td>
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<td>C(6)</td>
<td>0.28700(12)</td>
<td>-0.01564(10)</td>
<td>-0.06602(9)</td>
<td>0.0219(3)</td>
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<tr>
<td>C(7)</td>
<td>0.21730(11)</td>
<td>0.08138(10)</td>
<td>-0.16215(9)</td>
<td>0.0207(3)</td>
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<tr>
<td>C(8)</td>
<td>0.05925(11)</td>
<td>0.10332(10)</td>
<td>-0.15656(10)</td>
<td>0.0236(3)</td>
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<td>C(9)</td>
<td>-0.01086(12)</td>
<td>0.20023(10)</td>
<td>-0.25453(10)</td>
<td>0.0264(3)</td>
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<td>C(10)</td>
<td>0.00011(12)</td>
<td>0.16367(11)</td>
<td>-0.39072(10)</td>
<td>0.0282(3)</td>
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<tr>
<td>C(11)</td>
<td>0.15706(12)</td>
<td>0.14177(11)</td>
<td>-0.39835(10)</td>
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<td>C(12)</td>
<td>0.22849(12)</td>
<td>0.04559(10)</td>
<td>-0.29933(10)</td>
<td>0.0227(3)</td>
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Table 3. Bond lengths [Å] and angles [°] for 127.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length [Å]</th>
<th>Angle [°]</th>
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<tr>
<td>O(1)–C(3)</td>
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<td>N(1)–C(1)</td>
<td>1.3399(15)</td>
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<tr>
<td>C(1)–H(1A)</td>
<td>0.930</td>
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<tr>
<td>C(2)–H(2A)</td>
<td>0.930</td>
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<tr>
<td>C(3)–C(4)</td>
<td>1.3857(15)</td>
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<tr>
<td>C(4)–C(5)</td>
<td>1.3840(15)</td>
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<tr>
<td>C(6)–H(6A)</td>
<td>0.970</td>
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<tr>
<td>C(6)–C(7)</td>
<td>1.5121(14)</td>
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<tr>
<td>C(7)–C(8)</td>
<td>1.5298(15)</td>
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<td>C(8)–H(8A)</td>
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<td>C(9)–C(10A)</td>
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<td>C(10)–C(11)</td>
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<td>C(12)–H(12A)</td>
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<tr>
<td>C(3)–O(1)–C(6)</td>
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<td>N(1)–C(1)–H(1A)</td>
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<td>H(1A)–C(1)–C(2)</td>
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<tr>
<td>C(1)–C(2)–C(3)</td>
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<tr>
<td>O(1)–C(3)–C(2)</td>
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<td>C(2)–C(3)–C(4)</td>
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<td>C(3)–C(4)–C(5)</td>
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<td>O(1)–C(6)–H(6B)</td>
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<td>C(8)–C(7)–C(12)</td>
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C(7)–C(8)–H(8B) 109.3  C(7)–C(8)–C(9) 111.45(9)  
H(8A)–C(8)–H(8B) 108.0  H(8A)–C(8)–C(9) 109.3  
H(8B)–C(8)–C(9) 109.3  C(8)–C(9)–H(9A) 109.5  
C(8)–C(9)–H(9B) 109.5  C(8)–C(9)–C(10) 110.75(9)  
H(9A)–C(9)–H(9B) 108.1  H(9A)–C(9)–C(10) 109.5  
H(9B)–C(9)–C(10) 109.5  C(9)–C(10)–H(10A) 109.4  
C(9)–C(10)–H(10B) 109.4  C(9)–C(10)–C(11) 111.15(9)  
H(10A)–C(10)–H(10B) 108.0  H(10A)–C(10)–C(11) 109.4  
H(10B)–C(10)–C(11) 109.4  C(10)–C(11)–H(11A) 109.4  
C(10)–C(11)–H(11B) 109.4  C(10)–C(11)–C(12) 111.14(9)  
H(11A)–C(11)–H(11B) 108.0  H(11A)–C(11)–C(12) 109.4  
H(11B)–C(11)–C(12) 109.4  C(7)–C(12)–H(12B) 109.4  
C(7)–C(12)–H(12A) 109.3  C(7)–C(12)–H(12B) 109.3  
C(11)–C(12)–H(12A) 109.4  C(11)–C(12)–H(12B) 109.3  
H(12A)–C(12)–H(12B) 108.0

Table 4. Anisotropic displacement parameters (Å²) for 127. The anisotropic displacement factor exponent takes the form: \(-2\pi^2[h^2U_{11} + ... + 2hka*b*U_{12}]\)

<table>
<thead>
<tr>
<th></th>
<th>U¹¹</th>
<th>U¹²</th>
<th>U¹³</th>
<th>U¹³</th>
<th>U¹²</th>
<th>U¹²</th>
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<tr>
<td>O(1)</td>
<td>0.0367(5)</td>
<td>0.0225(4)</td>
<td>0.0158(4)</td>
<td>0.0002(3)</td>
<td>0.0048(3)</td>
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<td>N(1)</td>
<td>0.0280(5)</td>
<td>0.0319(6)</td>
<td>0.0220(5)</td>
<td>0.0046(4)</td>
<td>0.0067(4)</td>
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<tr>
<td>C(1)</td>
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<td>0.0335(7)</td>
<td>0.0175(5)</td>
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<td>C(2)</td>
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<td>0.0226(6)</td>
<td>0.0220(6)</td>
<td>-0.0034(5)</td>
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<tr>
<td>C(3)</td>
<td>0.0176(5)</td>
<td>0.0214(6)</td>
<td>0.0186(5)</td>
<td>0.0010(4)</td>
<td>0.0032(4)</td>
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<tr>
<td>C(4)</td>
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<td>0.0234(6)</td>
<td>0.0182(5)</td>
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<tr>
<td>C(5)</td>
<td>0.0266(6)</td>
<td>0.0236(6)</td>
<td>0.0275(6)</td>
<td>0.0026(5)</td>
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<td>C(6)</td>
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<td>0.0052(4)</td>
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<tr>
<td>C(7)</td>
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<td>0.0196(6)</td>
<td>0.0196(5)</td>
<td>-0.0002(4)</td>
<td>0.0044(4)</td>
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<tr>
<td>C(8)</td>
<td>0.0257(6)</td>
<td>0.0255(6)</td>
<td>0.0208(5)</td>
<td>-0.0012(5)</td>
<td>0.0079(4)</td>
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<tr>
<td>C(9)</td>
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<td>0.0019(5)</td>
<td>0.0057(5)</td>
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<td>C(10)</td>
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<td>0.0294(6)</td>
<td>0.0265(6)</td>
<td>0.0081(5)</td>
<td>0.0015(5)</td>
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<tr>
<td>C(11)</td>
<td>0.0309(7)</td>
<td>0.0292(6)</td>
<td>0.0201(6)</td>
<td>0.0035(5)</td>
<td>0.0068(5)</td>
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<tr>
<td>C(12)</td>
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<td>0.0251(6)</td>
<td>0.0206(6)</td>
<td>0.0006(5)</td>
<td>0.0064(4)</td>
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Table 5. Hydrogen coordinates and isotropic displacement parameters (Å²) for 127.

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<tr>
<th></th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>U</th>
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<tr>
<td>H(1A)</td>
<td>0.3620</td>
<td>−0.0456</td>
<td>0.4717</td>
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<tr>
<td>H(2A)</td>
<td>0.2874</td>
<td>0.0762</td>
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<tr>
<td>H(4A)</td>
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<td>H(5A)</td>
<td>0.4786</td>
<td>−0.3045</td>
<td>0.2647</td>
<td>0.031</td>
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<tr>
<td>H(6A)</td>
<td>0.2326</td>
<td>−0.0919</td>
<td>−0.0812</td>
<td>0.026</td>
</tr>
<tr>
<td>H(6B)</td>
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<td>H(7A)</td>
<td>0.2702</td>
<td>0.1587</td>
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<tr>
<td>H(8A)</td>
<td>0.0060</td>
<td>0.0267</td>
<td>−0.1742</td>
<td>0.028</td>
</tr>
<tr>
<td>H(8B)</td>
<td>0.0545</td>
<td>0.1301</td>
<td>−0.0701</td>
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<tr>
<td>H(9A)</td>
<td>−0.1118</td>
<td>0.2095</td>
<td>−0.2512</td>
<td>0.032</td>
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<tr>
<td>H(9B)</td>
<td>0.0368</td>
<td>0.2788</td>
<td>−0.2324</td>
<td>0.032</td>
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<tr>
<td>H(10A)</td>
<td>−0.0408</td>
<td>0.2284</td>
<td>−0.4509</td>
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<td>H(10B)</td>
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<td>0.0892</td>
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<td>H(11A)</td>
<td>0.2103</td>
<td>0.2185</td>
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<td>H(11B)</td>
<td>0.1604</td>
<td>0.1140</td>
<td>−0.4848</td>
<td>0.032</td>
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<tr>
<td>H(12A)</td>
<td>0.3296</td>
<td>0.0373</td>
<td>−0.3026</td>
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<tr>
<td>H(12B)</td>
<td>0.1821</td>
<td>−0.0335</td>
<td>−0.3214</td>
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Table 6. Torsion angles [°] for 127.

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<th>Bond</th>
<th>Torsion Angle</th>
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<td>C(5)–N(1)–C(1)–C(2)</td>
<td>−0.33(16)</td>
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<td>C(6)–O(1)–C(3)–C(2)</td>
<td>−175.36(9)</td>
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<td>C(1)–C(2)–C(3)–O(1)</td>
<td>179.48(9)</td>
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<td>O(1)–C(3)–C(4)–C(5)</td>
<td>179.85(9)</td>
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<td>C(1)–N(1)–C(5)–C(4)</td>
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<td>C(3)–O(1)–C(6)–C(7)</td>
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<tr>
<td>O(1)–C(6)–C(7)–C(12)</td>
<td>173.38(8)</td>
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<tr>
<td>C(12)–C(7)–C(8)–C(9)</td>
<td>56.06(12)</td>
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<tr>
<td>C(8)–C(9)–C(10)–C(11)</td>
<td>56.28(12)</td>
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<tr>
<td>C(10)–C(11)–C(12)–C(7)</td>
<td>−55.44(12)</td>
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<tr>
<td>C(8)–C(7)–C(12)–C(11)</td>
<td>55.29(12)</td>
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</tbody>
</table>