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Inhibition of protein-peptide interactions by small molecules

A thesis
Submitted for the Degree of
Doctor of Philosophy
by
Li-Hsuan Yen, Msc (Bsc)

Structural Biochemistry Group
Institute of Structural and Molecular Biology
School of Biological Sciences
University of Edinburgh
Scotland
United Kingdom
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Abstract

In all kinds of disease models, many proteins involved in protein-protein interactions (PPIs) are mutated and do not function properly. The important role of PPIs in disease makes the design of small molecule inhibition an interesting proposition. This project looks at mouse double minute 2 (MDM2) and mouse double minute X (MDMX) which binds and inhibits the tumour suppressor protein p53. MDM2 and MDMX are therefore attractive therapeutic targets due to their role in tumour progression. The aim is to identify small molecule dual inhibitors that are able to disrupt MDM2 and MDMX from binding to p53.

Both N-terminal MDM2 and MDMX were successfully expressed and purified with high purity and decent yield. These proteins were used to develop Fluorescence Polarization (FP) and Capillary Electrophoresis (CE) assays for small molecule inhibitors screening. This work has successfully developed FP and CE assays for detecting weakly interacting fragments. The CE assay is a novel method for detecting weak fragments for protein-protein interactions, which are a challenging target.

Two approaches were employed to identify small molecule inhibitors for MDM2-N/p53 interaction. At first, small molecules were identified using in silico screening and these hits were verified using FP and CE assays. Second, analogue exploration was applied to identify fragments from the small molecule inhibitors discovered from the in silico screening.

Diphenylamine and oxindole fragments were identified as the most potent. However, diphenylamine fragment was discovered to aggregate MDM2-N and was ranked as a false positive hit. No protein aggregation was found when incubated with the oxindole fragment. Therefore oxindole can provide a good starting point for the design of higher affinity analogues.

Studying the interaction of MDMX has only recently been undertaken. MDMX contains a high homology binding site with MDM2. Hence, developing a dual MDM2/MDMX inhibitor has become an attractive target to focus on. FP and CE assays were developed to screen compounds against MDMX-N. In silico screening against MDM2-N and MDMX-N found several hits. One compound was discovered as a dual binder to MDM2-N and MDMX-N with low μM affinity. This novel hit is potentially a good starting point for the design of higher affinity analogues.
Declaration

The work presented in this thesis is the original work of the author. This thesis has been composed by the author and has not been submitted in whole or in part for any other degree.

Li-Hsuan Yen
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Abbreviation list

ABC   Ammonium bicarbonate
ACE   Affinity capillary electrophoresis
ACN   Acetonitrile
ATM   Ataxia telangiectasia mutated
BSA   Bovine serum albumin
CE    Capillary Electrophoresis
CHCA  a-cyano-4-hydroxycinnamic acid
CODASS COmbine Docking And Similarity Search
CPS   Counts per second
DLS   Dynamic Light Scattering
DMSO  Dimethyl sulfoxide
DNA   Deoxyribonucleic acid
DNA-PK DNA dependent protein kinase
DTT   Dithiothreitol
E. coli  Escherichia coli
EDULISS Edinburgh University Ligand Selection System
FAM   Fluorescein amidite
FBDD  Fragment Based Drug Discovery
FP    Fluorescence Polarization
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA   Iodoacetamide
IC50  Half maximal inhibitory concentration
ITC   Isothermal Titration Calorimetry
IMAC  Immobilised Metal Affinity Chromatography
IPTG  Isopropyl β-D-1thiogalactopyranoside
Kd    Equilibrium dissociation constant
kDa   Kilodalton
Ki    Binding affinity of the inhibitor
LB    Luria-Bertani broth
LE    Ligand Efficiency
LIDAEUS Ligand Discovery At Edinburgh UniverSity
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<tr>
<th>Acronym</th>
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<tr>
<td>LIF</td>
<td>Laser Induced Fluorescence</td>
</tr>
<tr>
<td>MALDI MS</td>
<td>Matrix Assisted Laser Desorption Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse Double Minute 2</td>
</tr>
<tr>
<td>MDMX</td>
<td>Mouse Double Minute X</td>
</tr>
<tr>
<td>NBS</td>
<td>Non-binding Surface Plate</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>P53-F</td>
<td>Fluorescently Labelled P53 Peptide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-Protein Interaction</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root Mean Square Deviation</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
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<tr>
<td>SDS-PAGE</td>
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</tr>
<tr>
<td>TB</td>
<td>Terrific Broth</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>UFSRAT</td>
<td>Ultra Fast Shape Recognition Atom Type</td>
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Chapter 1 Introduction

1.1 The importance of protein-protein interactions (PPI)

Proteins are responsible for many biological systems in the cell. Some proteins perform their functions independently but most proteins perform their functions by interacting with others (Gadek and Nicholas, 2003, White et al., 2008). Many of the fundamental processes in cells are controlled by protein-protein interactions (PPI) which play key roles in the signal transduction pathway. Figure 1.1 shows one part of the signal network driven by the protein-protein interaction to control cell proliferation and apoptosis.

Living cells are constantly exposed to signals from the environment. These signals are detected by receptors on cell surfaces which are processed and transduced through a signalling cascade. The proteins translocate in the cell through PPI (Figure 1.1) and uncontrolled protein-protein interactions may lead to diseases such as cancer. In cancer the oncogenic and tumour suppressor proteins are overexpressed and mutated respectively which leads to uncontrolled cell proliferation (Ivanov et al., 2013). Therefore the design of small molecules to inhibit specific protein-protein interactions has become a major focus of current biomedical and pharmaceutical research.

1.2 Challenges of designing small molecule inhibitors for protein-protein interactions

Designing a small molecule to bind to a protein-protein interaction site holds many challenges because naturally occurring small molecules that bind to PPI sites are rare, unlike enzymes which mostly have substrates that can be used as template for drug design. Peptide inhibitors are often used to target protein-protein interactions, however peptides are considered poor drug candidates due to the low oral bioavailability or short half-life. One possible starting point to design small molecules for a particular protein-protein interface is to create a small peptide from one of the proteins and understand the mode of binding. Another challenge of small molecule design for PPI is that the interaction sites are often flat with a large surface area which is many times larger than a small molecule, therefore making it difficult to develop high affinity
Figure 1.1 Signalling transduction pathways to control cell proliferation and apoptosis (Hanahan and Weinberg, 2000).

When a stress signal is detected the cell converts the signal from the environment and transports from the cell surface into the nucleus through protein-protein interactions.

inhibitors (Arkin and Wells, 2004, White et al., 2008). As well as the shallow interface the protein-protein interaction site commonly contains a deep hydrophobic groove. The inhibitor design often ended up with a large complex structure to fill up the whole PPI site which resulted in the inhibitors to be hydrophobic and insoluble (White et al., 2008). However, by combining X-ray crystallography with computer-aided drug discovery it is now possible to design small molecules to target the crucial interaction spots in the PPI site and generate high affinity inhibitors (Table 1.1). In Table 1.1 these non-peptide inhibitors were discovered using different approaches. IL-2/IL-2α inhibitor was designed using combination of X-ray crystallography, NMR and structural activity studies (Tilley et al., 1997). LFA-1/ICAM, Bak-BH3/Bcl-xL, MDM2/p53 and HIV1/CCR5 inhibitors were identified by high throughput screening from a diverse library of compounds (Vassilev et al., 2004, Alexei et al., 2001, Baba et al., 1999, Kelly et al., 1999).
<table>
<thead>
<tr>
<th>Target protein</th>
<th>Protein partner</th>
<th>Binding affinity</th>
<th>Reference</th>
<th>Methods of Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>ICAM</td>
<td>$K_d = 26\text{nM}$</td>
<td>(Last-Barney et al., 2001)</td>
<td>HTS</td>
</tr>
<tr>
<td>Interleukin-2 (IL-2)</td>
<td>IL-2 receptor $\alpha$</td>
<td>$IC_{50} = 3\mu\text{M}$</td>
<td>(Braisted et al., 2003)</td>
<td>Combine crystallography, NMR and structural activity studies</td>
</tr>
<tr>
<td>MDM2</td>
<td>p53</td>
<td>$IC_{50} = 90\text{nM}$</td>
<td>(Vassilev et al., 2004)</td>
<td>HTS</td>
</tr>
<tr>
<td>Bak-BH3</td>
<td>Bcl-xL</td>
<td>$K_i = 3.3\mu\text{M}$</td>
<td>(Alexei et al., 2001)</td>
<td>HTS</td>
</tr>
<tr>
<td>HIV1</td>
<td>CCR5</td>
<td>$K_i = 1.1\text{nM}$</td>
<td>(Baba et al., 1999)</td>
<td>HTS</td>
</tr>
</tbody>
</table>

Table 1.1 Examples of PPI inhibitors (Pagliaro et al., 2004).

The table shows some examples of small molecule inhibitors capable of inhibiting protein-protein interactions.
1.3 Fragment based drug design (FBDD)

Fragment based drug design is a new emerging approach for drug discovery (Hajduk and Greer, 2007). The method uses small molecular weight molecules (<250Da) to interact in the binding grooves. These fragments are more soluble and provide a good starting point for subsequent lead optimisation. FBDD is gradually used in developing inhibitors of PPIs because the hits identified from the conventional high throughput screening (HTS) often brings large and insoluble molecular weight molecules (>400Da). Therefore FBDD provides a higher chance of finding soluble hits for optimisation. However these weak binding affinity fragments (µM to mM) require highly sensitive techniques in order to detect weak interactions (Table 1.2).

Capillary electrophoresis (CE) is one of the few techniques that is able to detect the weak fragment interaction (Table 1.2). Table 1.2 shows the advantages and disadvantages of several techniques that were used in FBDD. CE possesses several advantages over other common techniques (SPR, NMR or ITC) such as there is no protein size limit and the protein does not need to be modified or tethered and the assay development time is relatively fast. CE is applicable for studying protein-protein and protein-ligand interactions and requires little sample consumption. It should be noted that occasionally false positive can occur when the protein is aggregated by the compounds and there are some techniques that are able to detect protein conformation change listed in Table 1.2.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Throughput</th>
</tr>
</thead>
<tbody>
<tr>
<td>capillary electrophoresis (CE)</td>
<td>*Solution phase</td>
<td>*Cannot determine on/off rate</td>
<td>medium</td>
</tr>
<tr>
<td></td>
<td>*Fast assay development time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Low protein consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*No protein size limit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Applicable to protein-protein interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can obtain IC50 value</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Label free technique</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscale thermophoresis (MST)</td>
<td>*Solution phase</td>
<td>*Label free not usable with PPI</td>
<td>low</td>
</tr>
<tr>
<td></td>
<td>*Label free and fluorescent machine available</td>
<td>*Not automated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*High salinity tolerance</td>
<td>*Cannot determine on/off rate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can obtain IC50 value</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can be used for membrane proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface plasmon resonance (SPR)</td>
<td>*High information binding data (on/off rates and stoichiometry of binding)</td>
<td>modified tethered - may affect activity</td>
<td>medium</td>
</tr>
<tr>
<td></td>
<td>*Low protein consumption</td>
<td>*High ligand concentrations can give high false positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Fast screening time</td>
<td>*Protein size limit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can obtain Kd value</td>
<td>*Long assay development time</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Automated</td>
<td>*High instrument entry cost</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Protein needs to be stable for days</td>
<td></td>
</tr>
<tr>
<td>Nuclear magnetic resonance (NMR)</td>
<td>*Clear binding information/interaction with key residues obtained</td>
<td>*High protein consumption</td>
<td>medium</td>
</tr>
<tr>
<td></td>
<td>*No modification of protein required</td>
<td>*Protein size limit (≤30kDa)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Ligand and protein state monitored</td>
<td>*Expensive instrumentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Site localisation</td>
<td>*Resource intensive interpretation requires NMR expert</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Automated</td>
<td>Labelling of protein required</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can detect protein conformation change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray crystallography</td>
<td>*Full site specific structural information</td>
<td>*Soakable crystal form required: Not all target crystallise</td>
<td>low</td>
</tr>
<tr>
<td></td>
<td>*Screening of mixtures possible</td>
<td>*Require specialist equipment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*No need for immobilising/labelling</td>
<td>*Requires intensive time for data interpretation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can obtain Kd value</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can detect protein conformation change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isothermal titration calorimetry (ITC)</td>
<td>*High information binding data (stoichiometry of binding, enthalpy/entropy driven binding)</td>
<td>*High protein consumption</td>
<td>low</td>
</tr>
<tr>
<td></td>
<td>*No need for immobilising/labelling</td>
<td>*Low automation throughput</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can obtain Kd value</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Can detect protein conformation change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen/Deuterium exchange (H/D)</td>
<td>*Low protein consumption</td>
<td>*Require specialist equipment</td>
<td>low</td>
</tr>
<tr>
<td></td>
<td>*Can detect protein conformation change</td>
<td>*Requires intensive time for data interpretation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRET</td>
<td>*Can be used for HTS</td>
<td>*Must incorporate the distance of spacers and limit the range of fluorophores</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td>*Low sample consumption</td>
<td>*Changing the environment can alter the fluorescent properties of the probe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Easily adapted to any instruments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramen spectroscopy</td>
<td>*Solution phase</td>
<td>*Sample heating through intense laser can destroy the sample</td>
<td>medium</td>
</tr>
<tr>
<td></td>
<td>*Low sample consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*No sample preparation needed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Ramen spectra are acquired quickly</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The advantages and disadvantages of the techniques that can be used in FBDD
1.3.1 Capillary electrophoresis for small molecule screening

In CE, electrophoresis is carried out in the interior of a narrow glass capillary. CE has the ability to separate ions based on their electrophoretic mobility with the use of the applied voltage. The electrophoretic mobility will be affected by the differences in the charge to size ratio of the analyte ion (Coulter, 1994). Therefore it is possible to separate mixtures of various ions by mass and charge ratio under the same electric field strength. Affinity capillary electrophoresis (ACE) is a technique that is used to monitor the migration patterns of the interacting molecules in the capillary and can be used in protein-ligand study (Austin et al., 2012). Hence CE was applied in this work to identify small molecule inhibitors to inhibit protein-protein interactions (See Chapter 5).

1.4 MDM2/p53 interaction as a promising cancer therapeutic target

1.4.1 The role of p53

Cell replication is carried out in an event called the cell cycle where the cell duplicates into two daughter cells. The cell cycle consists of four stages. During Gap 1 (G1) phase the cell grows and organises chromosomes ready for replication. This is followed by DNA duplication in the Synthesis (S) phase. The cell then prepares for mitosis in Gap 2 (G2) phase followed by the mitosis (M) phase in which the cell divides into two identical cells. During cell replication there are multiple checkpoint proteins that constantly monitor the replication process and ensure the fidelity of the replication. If the checkpoint detects damage then the cell cycle is postponed until the repairs are made or if the damage cannot be repaired then the activation of the p53 protein is triggered. p53 functions as a transcription factor that simultaneously activates proteins to inhibit the cell cycle progression and sequentially targets the cell for destruction via apoptosis (Figure 1.2). This protects cell populations from harbouring mutations and thus p53 has been recognised as “the guardian of the genome” (Lane, 1992).
The activity of p53 is tightly regulated by post translational modification and interaction with other proteins (Prives and Hall, 1999). It was shown that over 50% of cancers involved a mutation in the p53 gene in order to evade the p53 apoptotic and growth arrest activities and hence manifest its role as a tumour suppressor (Petitjean et al., 2007). Even when certain tumours retain wild type p53, it may also show mutations in other proteins that inhibit p53 activities (Oliner et al., 1992a).

Multiple studies have demonstrated that reactivation of the p53 protein is destructive to cancer cells and hence reactivation of p53 in cancer becomes an attractive therapeutic approach (Teodoro et al., 2007). There are studies showing that in some cancer models p53 has retained the wild type form but the activities have been inhibited by p53 degradation (Reifenberger et al., 1993) (See section 1.9).

Huge efforts have been put into restoring the p53 activities and one of these is the use of small molecules to inhibit MDM2 that has been known to be a major negative regulator of p53 (Zhao et al., 2013). MDM2 binds to the N-terminal domain of p53 in the cell and carries p53 to the proteasome (Figure 1.4). Recent research shows that MDM2 also contain another p53 binding site where the acidic domain of MDM2 interacts with the DNA binding domain of p53 (Hernychova et al., 2013). Hence the

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**Figure 1.2 Activation of p53**

In the cell p53 is activated upon stresses and various pathways will result in the release of p53 from the MDM2 binding. Activation of p53 leads to cell cycle arrest to repair damaged DNA or apoptosis to destroy the damaged cell.
instability of p53 is largely caused by MDM2 and results in p53 with a short life time in a normal cell (Vogelstein et al., 2000).

1.4.2 MDM2 regulates the tumour suppressor p53
MDM2 along with another two MDM genes were originally isolated from the transformed mouse cell line 3T3-DM (Cahilly-Snyder et al., 1987). MDM2 has been discovered to be amplified and overexpressed along with other oncogenes and is therefore associated with tumour progression. This supports the role of MDM2 as an oncogene. Transgenic mice overexpressing MDM2 demonstrated a 100% incidence of tumour formation with amplification in over one-third of osteosarcomas and other soft tissue sarcomas (Jones et al., 1998).

To determine the relationship of MDM2 with p53 in humans, a co-immunoprecipitation experiment was carried out on MDM2 with p53 and confirmed the formation of the MDM2-p53 complex (Oliner et al., 1992b).

Further studies have discovered that MDM2 is an E3 ubiquitin ligase and has been confirmed as a key regulator of the tumour suppressor p53 protein in the cell and responsible for p53 degradation (Momand et al., 1992).

Not only being responsible for the ubiquitination of p53, MDM2 also disrupts p53 activity through binding to the N-terminal transactivation domain of p53 (Moll and Petrenko, 2003). In the stressed condition that leads to an increase of p53 level, p53 also induces the expression of MDM2 through an autoregulatory feedback loop and hence p53 in the cell is maintained at low levels (Figure 1.4). Through this autoregulatory loop MDM2 expression is also reduced to control its expression levels in the cell. The p53 protein activity and level in unstressed cells is regulated by the MDM2-ubiquitin-proteasome degradation pathway (Moll and Petrenko, 2003, Alarcon-Vargas and Ronai, 2002). MDM2 promotes p53 degradation through the E3 ligase function on the RING finger domain located at the C-terminal end of the protein. Figure 1.3 shows the domain organisation of MDM2 and p53 and studies have shown that there are two interaction sites between MDM2 and p53. The first interaction site is at the hydrophobic site of MDM2 and the N-terminal transactivation domain of p53. The second interaction site is between the acid domain of MDM2 and the DNA binding domain of p53 (Hernychova et al., 2013).
Figure 1.3 Domain structure of MDM2 and p53.
There are two interaction sites between MDM2 and p53 that are indicated using arrows and the domains are coloured in light blue and green in the structures.

Figure 1.4 MDM2/p53 autoregulatory feedback loop
The levels of MDM2 and p53 in cells are regulated by the autoregulatory feedback loop. The increase in the level of p53 will also trigger the expression of MDM2 leading to p53 degradation in the proteasome.
1.5 Ligand and peptide binding induce MDM2-N conformational changes

There are many studies suggesting the presence of a ‘lid’ at the N-terminal domain of MDM2 that may be involved in controlling the stability of the MDM2-p53 complex (Shimizu and Hupp, 2003, Schon et al., 2004).

In 2005, Uhrinova S. et al. was able to solve the first apo-MDM2 structure using solution NMR and revealed the open conformation of the MDM2-lid (PDB structure 1Z1M) (Uhrinova et al., 2005). In the open conformation the lid moved outward from the hydrophobic pocket (Figure 1.5 top) to allow interaction with the p53 protein.

In 2008, Showalter S. A. et al. used NMR to demonstrate the difference in the lid state upon binding to p53 peptide and Nutlin-3. When MDM2 bound to the p53 peptide the lid is competed out from the cleft and becomes more dynamic (Showalter et al., 2008). Whereas when MDM2 bound to the Nutlin-3, the surface of the cleft is not fully occupied which allows the lid to interact and resulted in semi-closed state (Showalter et al., 2008).

The structure solved by Michelsen K. et al. (PDB structure 4HBM) was the first structure of MDM2-lid with ligand bound and the structure shows that in the presence of inhibitor piperidinones the lid closes the hydrophobic pocket by fitting over it (Michelsen et al., 2012). This conformationally flexible MDM2-lid is a highly ordered structural element which contains a β sheet (Thr10 – Thr16 (coloured yellow in Figure 1.5 bottom)) and β turn (Ser17 – Pro20). In the closed state the lid is stabilized using Thr16 to form a hydrogen bond with the imidazole functional group on His96.
Figure 1.5 Structures of MDM2-lid

Top) NMR structure of MDM2-lid in open state (1Z1M). The lid is coloured in yellow which swings away from the binding site.

Bottom) The crystal structure of MDM2-lid with piperidinones inhibitor bound (4HBM). Piperidinones is shown as stick and coloured in pink while the lid is coloured in yellow. The lid covers the binding pocket and His96 residue has oriented for interaction with the lid. The phosphorylation site S\textsuperscript{17}Q\textsuperscript{18} is coloured in red.
It was found that DNA dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated (ATM) were involved in activating p53 pathway through phosphorylation of p53 causing release of the bound MDM2 (McCoy et al., 2003, Shimizu and Hupp, 2003). DNA-PK and ATM are both able to recognize and phosphorylate serine on the sequences that contain “SQ”.

The MDM2-lid contains a S^{17}Q^{18} sequence at the β loop and phosphorylation of Ser17 causes a conformational shift of the lid towards the hydrophobic pocket (Shimizu and Hupp, 2003).

![Figure 1.6 Position of MDM2 Tyr100](image)

Apo-MDM2 structure is shown in yellow (1Z1M), MDM2 with piperidinones bound is shown in blue (4HBM), MDM2 with MI63 inhibitor (3LBL) is shown in green, MDM2 interaction with Nutlin-2 (1RV1) is shown in light blue, MDM2 with Nutlin-3a (4HG7) bound is shown in grey, MDM2 with benzodiazepine inhibitor (1T4E) is shown in magentas, MDM2 with central valine inhibitor (4DIJ) is shown in lime green, MDM2 bound with chlorobenzyl inhibitor (3TJ2) is shown in dark blue, MDM2 interaction with D-peptide (3IWY) is shown in tints, MDM2 interaction with stapled peptide (3V3B) is shown in orange and MDM2/p53 peptide complex is shown in pale yellow (1YCR). For clarity ligands are not shown in the figure.
In the apo-MDM2 structure (Figure 1.6) it has been discovered that the binding site is rather dynamic and flexible (Schon et al., 2004, Uhrinova et al., 2005). The binding site may undergo chemical shift upon binding to p53 peptide. Certain residues in the binding site can be affected when the binding site is occupied. The conformation change of these residues can open or close the binding site. X-ray crystal structures also showed that Tyr100 may also pose as intermediate position upon binding to small molecules and peptide inhibitors (Figure 1.6). There are several papers suggesting that Tyr100 is flexible and can exist as a closed and open conformation (Uhrinova et al., 2005). When the different states of MDM2 structure are superimposed a clear positional difference can be seen in His96 and Tyr100 (Figure 1.6).

In the unbound structure (yellow in Figure 1.6) the residues His96 and Tyr100 are in a closed position where the side chains tilt into the binding site. In the presence of small molecule and peptide inhibitors the side chain of Tyr100 rotates outward by 6.4 – 7.5 Å and the side chain of His96 rotates outward by 3.8 – 4.9 Å (Figure 1.6).

Upon binding to p53 peptide (pale yellow in Figure 1.6) the side chain of Tyr100 rotates outward by 10 Å whereas the imidazole ring of His96 has tilted outward by 5 Å. In the presence of the ligand the side chain of Tyr100 and His96 becomes semi-open and tilted outward by 6 Å and 3 Å respectively. The imidazole ring of His96 also rotates by 90° to provide π-π interaction and hydrogen bond interaction with the ligand (Figure 1.7).
1.6 MDM2 and MDMX coordinate to regulate p53 in the cell

Several studies have identified that MDM2 is not the only negative regulator to inhibit p53 tumor suppressor activity in both unstressed and cancer cells. MDMX (also known as MDM4) is a homolog of MDM2 which also binds to the N-terminal transactivation domain of p53 but it does not contain E3 ubiquitin ligase function and the expression level is not p53 dependent. MDM2 contains 491 residues and MDMX contains 490 residues. They contain three well conserved regions: the p53 binding site at N-terminal domain (residues 18-101), a zinc finger domain (residues 289-331) and a RING domain (residues 436-482) (Figure 1.8 bottom).

The N-terminal domains of MDM2 and MDMX are structurally related with a high similarity of 54% and the amino acids required to interact with p53 are highly conserved.

MDM2 also contains a nuclear localisation signal (NLS) (residues 179-185) and nuclear export signal (NES) (residues 190-202) which is absent in MDMX. Therefore MDM2 is able to travel in between the nucleus and the cytoplasm. Through binding to p53 protein MDM2 exports p53 from nucleus into the cytoplasm for degradation.
MDMX however does not contain NLS and NES in the sequence and hence its subcellular localisation depends on the interaction with other proteins. There is evidence to indicate that the RING domain at the C-termini of MDM2 and MDMX is essential for heterodimer and homodimer formation (Tanimura et al., 1999). Dimerization of MDM2-MDMX or MDM2-MDM2 is required for E3 ubiquitin ligase activity. There is no MDMX-MDMX homodimer found in vitro or in vivo studies which suggest that free MDMX may be monomeric or heterodimeric with MDM2 (Wade and Wahl, 2009). MDMX heterodimerization with MDM2 helps to stabilize MDM2 from auto-ubiquitination (Gu et al., 2002).
Figure 1.8 Sequence comparison of MDM2 and MDMX

**Top** Sequence alignment on MDM2 and MDMX. The identical residues are highlighted in red. Most conserved residues are located at the N-terminal and C-terminal. The sequences of p53 binding site, Nuclear Localisation signal/Nuclear export signal, acid domain, zinc finger, Nucleolar localisation signal and RING domains are circled in light blue, black, green, grey, brown and purple respectively.

**Bottom** Comparison of MDM2 and MDMX structure. High conserved regions of MDM2 and MDMX p53 binding site, Acid domain, Zinc finger, Nucleolar localisation signal and RING domains are coloured in light blue, green, grey, brown and purple respectively.
1.7 Structure of the N-terminal domain of MDM2

The N-terminal domain of MDM2 (MDM2-N) is the most studied because it is directly involved in the interaction with the p53 transactivation domain and inhibits its tumour suppressor activities. The crystal structure of the N-terminal domain of MDM2 solved by Kussie et al. confirmed MDM2-N can bind to the transactivation domain of p53 (Kussie et al., 1996). The crystal structure of the 109 residues of MDM2-N reveals two highly similar structure of portions that consist of two helices and two beta strands in each portion (Figure 1.9 left). Each portion contains a short α helix named α1, a long α helix, α2 and two β strand, β1 and β2. The other portion uses the apostrophe symbol to distinguish. The α1 and α1’ helices sit antiparallel to each other at the bottom of the hydrophobic cleft while the α2 and α2’ form the left and right side of the cleft (Figure 1.9 right).

![Figure 1.9 The structure of MDM2-N showing two highly similar portions](image)

**Left)** The crystal structure of MDM2-N solved by Kussie et al. (PDB: 1YCR) is displayed in cartoon form (light blue colour). The second repeat of the α and β strands are distinguished using the apostrophe symbol. The picture is generated using VMD software (Humphrey et al., 1996).

**Right)** The structure of MDM2-N is displayed in surface form with 20% transparency (grey colour) and the secondary structure is displayed in green colour. The α helices have been labelled. The p53 peptide bound to the MDM2-N binding site is coloured in yellow. The picture was generated using PyMOL (Delano, 2002).
The sequence of MDM2-N is shown in Figure 1.10 top and the residues highlighted in grey are the residues that form the MDM2-N binding pocket. The sequence reveals that the binding site is mainly comprised of hydrophobic residues. A hydrophobicity surface generated using Discovery Studio 4.0 shows the binding site is highly hydrophobic; hydrophilic residues can be seen on the exterior of the pocket (Figure 1.10 bottom).

\[
\begin{align*}
10 & \quad 20 & \quad 30 & \quad 40 & \quad 50 \\
SQIPASEQET & \quad LVRKPOLLK & \quad LLKSVGQAQKD & \quad TYTMEVLFY & \quad LGQYIMTKRL \\
60 & \quad 70 & \quad 80 & \quad 90 & \quad 100 \\
YDEKQHIYV & \quad CSNDLGDF & \quad GVPSFSVYKEH & \quad RKIYTMYRN & \quad LVVQNQESS \\
\end{align*}
\]

DSGTVSENN

**Figure 1.10 The hydrophobicity of MDM2-N binding site.**

The hydrophobic binding site is formed by various hydrophobic residues. The hydrophobicity of the binding site is generated using Discovery Studio 4.0 (Accelrys, 2013). Brown colour is ranked as hydrophobic and blue colour is ranked as hydrophilic.
1.8 MDM2-N binds to a p53 helical stretch

The p53 peptide that binds to MDM2-N in the crystal structure is 13 residues long (residues 17 – 29) and fills the whole entrance of the binding site (Figure 1.11). The position of the p53 peptide allows the hydrophobic side chains of Phe19, Trp23 and Leu26 to insert deep into the hydrophobic pocket (Figure 1.11). The deepest hydrophobic groove lies in the middle accommodating the Trp23 side chain, whereas the Phe19 and Leu26 side chains bind into the other two shallower hydrophobic grooves on the flank.

Phe19 of p53 forms van der Waals interactions with Gly58 and Ile61 of MDM2-N while Trp23 forms van der Waals interaction with Phe86. The Leu26 of p53 also forms van der Waals interaction with Ile99. Additionally there are also tight hydrogen bond interactions present between the amide backbone of Phe19 of p53 with the oxygen atom of side chain Gln72 and the nitrogen atom of p53 side chain of Trp23 with the oxygen atom on the backbone carbonyl of Leu54. These three residues, Phe19, Trp23 and Leu26 are the three principle residues that contribute to the binding. The three hydrophobic grooves that are occupied by these residues are therefore named after these residues.
The helix of p53 peptide17-29 (coloured in yellow) binds to a deep MDM2-N hydrophobic pocket using Phe19, Trp23 and Leu26 residues. Dots are displayed for the p53 peptide to show that the peptide has filled up the pocket.

1.9 Drug discovery on disrupting the MDM2/p53 interaction

In the initial MDM2/p53 small molecule drug discovery the work focused on using known inhibitors with anti-tumour effects, however this was unsuccessful (Stoll et al., 2001).

The first crystal structure of p53 peptide interaction with MDM2 (1YCR) has unveiled a deep hydrophobic pocket in MDM2. More importantly the p53 peptide filled the three hydrophobic grooves in MDM2 using the side chains of Phe19, Trp23 and Leu26 residues (Figure 1.12). This provides a novel strategy for designing potent MDM2 inhibitors. Many known published inhibitors eg. Nutlin-3, Mi63, isoindolinones, benzodiazepinediones and WK298 mimic the binding of p53.
Figure 1.12 p53 peptide\textsuperscript{17-29} uses three hydrophobic residues to interact with MDM2.  
The helix of p53 peptide is displayed and the three residues that bind to the three hydrophobic pockets of MDM2-N are circled using yellow for the Leu26 site, blue for the Trp23 site and green for the Phe19 site.

1.9.1 Nutlin series

Figure 1.13 Chemical structures of Nutlin series  
The structure of Nutlin1, Nutlin-2 and Nutlin-3a are displayed. The subgroups of Nutlin-3a that occupy the three hydrophobic sites are circled in different colours.
The Nutlin series is the most studied inhibitor of MDM2 and it was identified in high throughput screening (Vassilev et al., 2004). There are three analogues; Nutlin-1, Nutlin-2 and Nutlin-3, with IC₅₀s ranging from 100nM to 300nM and the most potent inhibitor for the Nutlin series is Nutlin-3a (Figure 1.13) with an IC₅₀ of 90nM (Vassilev et al., 2004). The crystal structure of MDM2-Nutlin-2 complex (PDB structure: 1RV1) was the first inhibitor bound MDM2 structure available (Vassilev et al., 2004). Due to the flexible structure of MDM2 it was not until 2013 that a MDM2-Nutlin-3a complex was made (PDB structure: 4HG7) (Anil et al., 2013). Nutlin-3a contains an imidazoline group as the core to connect four hydrophobic groups and it is posed at the centre of the MDM2 binding site. In Nutlin-3a there are two 4-chlorobenzyl groups, the first 4-chlorobenzyl group inserts deep into the Trp23 pocket and the second 4-chlorobenzyl group fits into the Leu26 pocket. The chlorine atom on the 4-chlorobenzyl is positioned at the bottom of the Trp23 pocket which provides a tight interaction with the side chain residues in the pocket. The dimethyl ether group on the Nutlin-3a occupies the third pocket (Phe19). As seen in Figure 1.14, the side chain residues that surround Nutlin-3a are highly hydrophobic which suggest that the tight binding interaction may be driven by the hydrophobic interactions. Disrupting the interaction between MDM2 and p53 by Nutlin-3a enables the stabilization and accumulation of p53. Both in vitro and in vivo studies showed that Nutlin-3a is able to re-activate p53 tumour suppressor activity and results in suppressed tumour growth (Tovar et al., 2006). Currently the Nutlin inhibitor has progressed to Phase I clinical trial (Hoe et al., 2014).
Figure 1.14 Nutlin-3a interaction in the MDM2-N binding groove
Nulin-3a interaction with MDM2 is presented using Discovery studio (Accelrys, 2013) and the protein hydrophobicity surface is shown where brown is hydrophobic and blue is hydrophilic.

1.9.2 MI families

Figure 1.15 Chemical structures of MI63 and MI219.
The structures of MI63 and MI219 are displayed and the subgroups that occupy the three hydrophobic sites are circled in different colours.
The MI series is the second most studied group of MDM2 inhibitors which were developed by Shaomeng Wang’s group (Ding et al., 2006). Structure based de novo design was employed to design compounds based on the spiro-oxindole group. The oxindole group originated from the indole ring of tryptophan to mimic the interaction of Trp23 on p53 (Ding et al., 2005). Both MI63 and MI219 bind tightly to MDM2 with K_i of 3nM and 5nM respectively (Shangary et al., 2008). MI63 has poor pharmacokinetic properties therefore MI219 was developed with improved solubility and hence better bioavailability. Similar to the Nutlin series the MI family of inhibitors also uses a core structure that stretches the subgroup to fit into the Phe19, Trp23 and Leu26 pockets (Figure 1.16).

MI63 and MI219 contain 2-morpholin-4-yl-ethylamine and 1, 2-pentanediol groups respectively (Figure 1.15) that extend to the region between Glu72 and Lys94. This provides additional interactions as well as improving the water accessibility of the compound (Ding et al., 2006). The MI63 bound MDM2 crystal structure 3LBL did not contain this 2-morpholin-4-yl-ethylamine probably due to its flexible structure making it difficult to crystalize (Figure 1.16). The tighter interaction of MI63 may be owing to the hydrogen bond interactions present in the nitrogen atom on the oxindole group with the Leu54 backbone and the oxygen atom on the 2-morpholin-4-yl-ethylamine with His96 (green dash line in Figure 1.16). These hydrogen bond interactions are absent in the Nutlin-3a/MDM2-N interaction.
1.10 MDMX as a target to design a dual inhibitor

It has been confirmed that MDM2 and MDMX are both negative regulators of the tumour suppressor p53. These proteins use their highly similar N-terminal domains to bind to the N-terminal transactivation domain of p53 and inhibit p53 transcriptional activity. The N-terminal domain is therefore an attractive target for drug design (Brown et al., 2009, Brown et al., 2011, Wade and Wahl, 2009).

As of now there are no effective dual inhibitors developed to target both MDM2 and MDMX. There are multiple classes of inhibitors developed to disrupt the MDM2/p53 complex which have proceeded to clinical trials, however they bind at least 1000 times weaker to MDMX (Table 1.3).
There has been several approaches for developing dual inhibitors using phage display and staple peptide to design peptide inhibitors (Bernal et al., 2010, Pazgier et al., 2009, Chang et al., 2013). Pazgier et al. have used phage display to identify a peptide inhibitor with Kd values of 3nM and 4nM affinity for MDM2 and MDMX respectively. However in vitro studies showed this peptide inhibitor is only active in the cell free systems (Pazgier et al., 2009). Bernal F. et al. and Chang Y. et al. have designed stapled peptide named SAH-p53-8 and ATSP-7041 to inhibit both MDM2 and MDMX. ATSP-7041 has progressed into clinical trials but SAH-p53-8 designed by Bernal F. et al. is unable to disrupt p53-MDM2 binding effectively (Bernal et al., 2010, Chang et al., 2013).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki (nM)</th>
<th>Ki (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutlin-3a</td>
<td>36</td>
<td>9.3</td>
<td>(Shangary et al., 2008)</td>
</tr>
<tr>
<td>MI63</td>
<td>3</td>
<td>55</td>
<td>(Shangary et al., 2008)</td>
</tr>
<tr>
<td>MI219</td>
<td>5</td>
<td>55</td>
<td>(Shangary et al., 2008)</td>
</tr>
<tr>
<td>WK298</td>
<td>109</td>
<td>11</td>
<td>(Popowicz et al., 2010)</td>
</tr>
</tbody>
</table>

Table 1.3 Binding constants of MDM2 and MDMX inhibitors
It was not until 2012 when Graves B. et al. identified a new class of small molecule inhibitor RO-2443 that is capable of inhibiting both the MDM2-p53 and MDMX-p53 interaction with 33nM and 41nM affinities respectively (Graves et al., 2012). RO-2443 was identified using HTS on a diverse library comprising ~1 million of organic compounds. Examining RO-2443 (Figure 1.17) we see that its structure is very different from the known MDM2 inhibitors. It does not contain a central group to connect multiple hydrophobic rings together to fit into all three hydrophobic pockets. In fact, when viewing the crystal structure of the MDM2 and MDMX complex with RO-2443, RO-2443 only fits into the Phe19 and Trp23 pocket (Figure 1.18 A and C).

**Figure 1.17 Structure of RO-2443 (left) and RO-5963 (right).**

RO-2443 is able to bind to both MDM2 and MDMX with high potency through a unique mode of binding. It occupies two out of three hydrophobic pockets on the surface of MDM2 and MDMX. Dimerization of the protein occurs when the indolyl hydantoin group forms an aromatic stacking interaction. The indolyl hydantoin also forms a stacking interaction with a tyrosine ring (Tyr67 in MDM2 or Tyr66 in MDMX) and creates four π-π stacked rings (Figure 1.18 B, D). The di-fluoro-phenyl group inserts deep into the Trp23 pocket.

Further chemical optimisation has yielded the inhibitor RO-5963 with improved water solubility and potency (Figure 1.17). Testing RO-5963 in *in vitro* has been shown to re-activate the wild type p53 signalling pathway (Graves et al., 2012).
At the moment the most advanced MDM2/X dual inhibitor is the stapled peptide inhibitor ATSP-7041 developed by Chang Y. S. et al. with Kd values of 0.91nM and 2.31nM against MDM2 and MDMX (Chang et al., 2013). *In vitro* and *in vivo* studies have shown activation of p53 signalling pathway in the MDM2 and MDMX overexpressed cancer models. Whereas for the small molecule inhibitors, the most potent Nutlin family and MI series have advanced into phase I clinical trial and the recently discovered dual action inhibitor RO-5963 is still at the stage of optimisation of the potency and pharmacological properties (Graves et al., 2012, Hoe et al., 2014). The discovery of these small molecule inhibitors has demonstrated that it is possible to use small molecule inhibitors to target both MDM2/p53 and MDMX/p53...
interactions and it is clear that the most potent inhibitors have > 1000 times higher affinity than the p53 peptide. These inhibitors provide a new approach to design potent dual MDM2/MDMX inhibitors.

1.11 Insight into the MDMX/p53 interaction site

Early studies have discovered MDMX as another key gatekeeper of the genome and huge effort has recently been placed into studying MDMX because of its high similarity with MDM2 (Marine et al., 2007). The sequence comparison carried out in Section 1.6 showed that the p53 binding site at the N-terminal domains of MDM2 and MDMX are highly conserved. Popowicz G. et al. have determined the crystal structure of the MDMX-N/p53 (residues 15 – 29) complex (PDB structure 2Z5S) and reveals several features in the MDMX-N structure that restrict the known MDM2-N inhibitors from binding (Popowicz et al., 2007).

The MDMX-N structure is also comprised of two highly similar portions consisting of two α helices and two β strands (Figure 1.19 left) that is similar to the MDM2-N structure (Figure 1.9). The short α helices (α1, α1’) in both portions form the bottom of the binding site and the long α helices from both portions form the side of the binding site.

The surface of MDMX-N is also shown to be highly hydrophobic in the binding site with three hydrophobic grooves similar to the MDM2-N binding pocket (Figure 1.19 right).
Figure 1.19 The secondary structure of MDMX-N (Left) and the hydrophobicity surface of MDMX-N (Right).

Left) The crystal structure of MDMX-N solved by Popowicz G. et al. (PDB: 2Z5S) is displayed. The structure is presented in surface form with 20% transparency (grey colour) and the secondary structure is displayed in green colour. The p53 peptide binds to the MDMX-N binding site and is coloured in light blue. The second repeat of the α and β strands are distinguished using apostrophe symbol. The picture is generated using PyMOL (Delano, 2002).

Right) The hydrophobic binding site of MDMX-N is comprised of various hydrophobic residues and the hydrophobicity of the binding site is generated using Discovery Studio 4.0 (Accelrys, 2013). Brown is ranked as most hydrophobic and blue is ranked as hydrophilic.

The crystal structure shows that the p53 peptide also binds to MDMX-N using the same hydrophobic residues Phe19, Trp23 and Leu26 from a 15 amino acid helical stretch (Figure 1.20). These residues are able to fully occupy the hydrophobic grooves in MDMX-N using the same binding mode as with MDM2-N. The interaction of the p53 peptide with MDMX-N is mainly driven by the hydrophobic contacts that are also observed in the MDM2-N/p53 interaction. The same hydrogen bonds present in the MDM2-N/p53 interaction have also been identified with the MDMX-N/p53 complex, namely p53 Trp23---MDM2/X Leu54 and p53 Phe19---MDM2 Gln72 (MDMX Gln71). At the C-terminal end of p53 peptide there is a different number of hydrogen bonds formed between MDM2-N/p53 and MDMX-N/p53 complexes. In the MDM2-
N/p53 complex there are hydrogen bonds formed at p53 Asn29---MDM2 Tyr100, Tyr104 and Thr26. Whereas in the MDMX-N/p53 complex there is only one hydrogen bond formed at p53 Pro27---MDMX Tyr99.

Figure 1.20 p53 peptide binds to MDMX-N using three hydrophobic residues. Crystal structure of 2Z5S with p53 peptide binding to MDMX-N. The p53 peptide uses three hydrophobic side chains, residues Phe19, Trp23 and Leu26 to interact with MDM2 and MDMX. Dots are displayed for the p53 peptide to represent the peptide has filled up the pocket.
Despite the high level of similarity between the MDM2-N and MDMX-N structures there are still two differences that result in a shallower binding pocket in MDMX-N. First, Leu54 on MDM2-N has been substituted by the Met53 in MDMX-N where the side chain of Met53 is pointed towards the binding site (Figure 1.21). The second variation is the helix loop of His96-Arg97-Lys98-Ile99-Tyr100 in MDM2-N which has been substituted by Pro95-Ser96-Pro97-Leu98-Tyr99 in MDMX-N (Figure 1.21). This helix is situated at the rim of the Leu26 pocket and the Tyr99 residue protrudes into the binding pocket and fixes the closed conformation which results in a shallow Leu26 pocket (Figure 1.22).

With the assistance of high resolution crystal structures we are able to understand the major differences between the MDM2-N and MDMX-N binding grooves and consequently this will assist in the rational development of greater affinity inhibitors toward MDMX-N.

Figure 1.21 An overlap of MDM2 and MDMX N-terminal binding site. 
p53 peptide bound MDMX structure (2Z5S) (light blue) is superimposed onto p53 peptide bound MDM2 structure (1YCR) (green) with a RMS fit of 1.01Å.
Figure 1.22 The p53 binding site MDM2-N and MDMX-N.
Surface representation of MDM2-N (left) and MDMX-N (right) to highlight the size difference in the Leu26 pocket.
1.12 Aim of project

The aim of this project is to identify small molecules to prevent MDM2 and MDMX from binding to the tumour suppressor p53. This is aided using the in house virtual screening script CODASS to search through millions of commercially available compounds in the EDULISS database. There are also other papers that use virtual screening approach in small molecules identification, however the CODASS employed in this project is a combined docking and ligand based virtual screening method which increased the hit rate even when targeting protein-protein interfaces.

The interaction of the small molecules will be validated by fluorescence polarization and capillary electrophoresis assays.

The currently identified inhibitors have high complexity and hydrophobicity which results in poor solubility, preventing further optimization. This project will explore analogues of the virtual screening hits to aid in identifying small molecular weight fragments with higher solubility.

In order to provide a sufficient amount of protein needed for the assays an optimised scale up for protein purification and storage conditions has been developed.
Chapter 2 Protein expression and purification

The expression and purification strategies of MDM2-N and MDMX-N will be discussed in this chapter. In Figure 2.1, a sequence alignment was done on the published structures with the MDM2-N11-130. The published structures of MDM2-N were shorter than the MDM2-N11-130 construct used in the studies. The MDM2-N11-130 construct contains an unstructured tail at the C-terminal end and lid at the N-terminal end which are not present in the published structures and this makes the protein uncrystallizable. Therefore a short MDM2-N18-111 construct was designed for a crystallization trial.

When comparing the sequence of MDMX-N14-111 with the published structures’ sequences in Figure 2.2, the alignment showed that MDMX-N14-111 has more residues at the N-terminal domain. To maximise the chance that the MDMX-N would be crystallisable a short MDMX-N23-109 construct was designed from MDMX-N14-111.

We have also aligned the sequence of MDM2-N11-130 with MDMX-N14-111 using PRALINE sequence alignment tool. The alignment showed MDM2-N11-130 and MDMX-N14-111 has an identity of 54% with only two residue difference in the binding sites of MDM2-N and MDMX-N (Figure 2.3). Therefore designing dual inhibitors against MDM2-N and MDMX-N becomes highly attractive.

The purified MDM2-N11-130 and MDMX-N14-111 were used for biophysical studies and screening against small molecules identified from the in silico screening.
Figure 2.1 MDM2-N sequence alignment.

Sequence alignment of MDM2-N^{11-130} and MDM2-N^{18-111} with other published structures’ sequences. The star sign below the sequences indicate that the residues are identical. The sequences are aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Figure 2.2 MDMX-N sequence alignment.

Sequence alignment of MDMX-N^{14-111} and MDMX-N^{23-109} with other published structures’ sequences. The sequences are aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).
The sequences of MDM2-N11-130 and MDMX-N14-111 are aligned using PRALINE alignment (http://www.ibi.vu.nl/programs/pralinewww/). The conserved sequences are highlighted in different colours from red as highly conserved to blue as unconserved. The clear circle above the sequences are the residues in the binding site of MDM2-N and MDMX-N. The percent sequence identity of MDM2-N\textsuperscript{11-130} and MDMX-N\textsuperscript{14-111} is 54%.

Figure 2.3 Sequence alignment of MDM2-N\textsuperscript{11-130} with MDMX-N\textsuperscript{14-111}.
2.1 Methods

2.1.1 Cloning of MDM2-N and MDMX-N

2.1.1.1 MDM2-N<sub>18-111</sub> and MDMX-N<sub>23-109</sub> cloning

For cloning the MDM2 N-terminal region (Residues 18-111) and MDMX N-terminal region (Residues 23-109), the DNA sequence from Dr. Maria Sanchez’s MDM2-N<sub>11-130</sub> and MDMX-N<sub>14-111</sub> constructs were used as template. Forward and reverse primers were designed to amplify the DNA coding sequence as shown below.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDM2-N&lt;sub&gt;18-111&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDM2-N F1</td>
<td>TEV</td>
<td>CCGAAAACCTGTATTTTCAGGGCTCTCAGATTCCGGGAGCGAA</td>
</tr>
<tr>
<td>MDM2-N F2</td>
<td>AttB1, RBS, ATG, 6x His, TEV</td>
<td>GGGGACAGTTTTGTACAAAGGACGGTTCGAGGAGATACATATGTCTGTACTACATCAATCATCACTCAGATTACCATATCCCACGACCGAAAACCTGTATTTTCAGG</td>
</tr>
<tr>
<td>MDM2-N R</td>
<td>AttB2, CTA</td>
<td>GGGGACACCTTTGTACAAAGGACCTGGGTCTCTAGTCACCACCACCAGGTTACG</td>
</tr>
</tbody>
</table>

| **MDMX-N<sub>23-109</sub>** | | |
| MDMX-N F1 | TEV | CCGAAAACCTGTATTTTCAGGGGCACAAATCAATCAGGTACGACCAAG |
| MDMX-N F2 | AttB1, RBS, ATG, 6x His, TEV | GGGGACAGTTTTGTACAAAGGACGGTTCGAGGAGATACATATGTCTGTACTACATCAATCATCACTCAGATTACCATATCCCACGACCGAAAACCTGTATTTTCAGG |
| MDMX-N R | AttB2, CTA | GGGGACACCTTTGTACAAAGGACCTGGGTCTCTAGTCACCACCACCAGGTTACG |

Two PCR reactions were required to attach the His-tag onto the N-terminal sequence. The first reaction required a forward primer with TEV recognition sequence which was recognised by the second PCR reaction forward primer. The second forward primer contains AttB1, RBS, ATG, 6x His tag and TEV recognition site. Only one reverse primer was used with an AttB2 and a stop codon. The gateway entry vector contained attL recombination site allowing PCR products that contained attB sites to recombine with the entry vector.
PCR cloning

In PCR reactions the templates were a plasmid containing the full length MDMX and N-terminal MDM2 gene which was a gift from Prof. Kathryn Ball and Dr. Maria Sanchez.

The PCR reaction mixtures contained the components in Table 2.1 and Elongase enzyme mix kit (Invitrogen) was used.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (Total 50µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP mix (0.5mM)</td>
<td>20µl</td>
</tr>
<tr>
<td>Forward primer (10µM)</td>
<td>1µl</td>
</tr>
<tr>
<td>Reverse primer (10µM)</td>
<td>1µl</td>
</tr>
<tr>
<td>Template DNA (≥100ng)</td>
<td>1µl</td>
</tr>
<tr>
<td>Elongase enzyme mix</td>
<td>1µl</td>
</tr>
<tr>
<td>5x Buffer A</td>
<td>5µl</td>
</tr>
<tr>
<td>5x Buffer B</td>
<td>5µl</td>
</tr>
<tr>
<td>Water</td>
<td>17µl</td>
</tr>
</tbody>
</table>

Table 2.1 Reaction set up for PCR Elongase enzyme mix.

PCR reaction conditions were:
1. Pre amplification denaturation 94°C for 2min.
2. Thermal cycling: Denaturation; 94°C for 1min
   Annealing; 50 - 55°C for 30sec
   Extension; 68°C for 1min
3. 68°C for 10min.

When the first PCR reaction was finished the PCR products were verified on a 1.2% agarose gel. This separated the PCR products from the mixtures. The band at the expected size was cut out from the agarose gel and purified using a PCR purification kit (QIAquick).

Purified PCR product was used as the template DNA in the second PCR reaction. The second PCR reaction attached the His-tag onto the N-terminal end of the sequence. The PCR reaction was performed by adding the same mixtures and performed in the same reaction conditions. The PCR reaction mix was loaded onto an agarose gel and
the band purified using the PCR purification kit. The purified PCR product contained
the specific gene sequence with the attB sites attached on both end of sequence which
allows insertion into the gateway entry vector.

**Gateway cloning**
The Gateway cloning technology enables transfer of DNA sequence into different
cloning vectors. First, the sequence was inserted into the donor vector using BP
clonase enzyme mix.

The following components were added into a sterile tube:

<table>
<thead>
<tr>
<th></th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB-PCR product (15-150ng)</td>
<td>1-7µl</td>
</tr>
<tr>
<td>pDONR221 vector (150ng/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>TE buffer pH 8.0</td>
<td>To 10µl</td>
</tr>
<tr>
<td>BP clonase enzyme mix</td>
<td>2µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 25°C for 1h. To terminate the reaction 1µl of
Proteinase K was added into the mixture and incubated at 37°C for 10min. Following
the BP clonase reaction mixtures were transformed into the DH5α competent cell for
plasmid production. 2µl BP reaction was added into 50µl DH5α cells, incubated on
ice for 30min followed by heat shock at 42°C for 30seconds. S.O.C. medium (250µl)
was then added and the mixture incubated in the shaking incubator at 37°C for 1h. The
competent cells were spread onto a LB agar plate (30µg/ml Kanamycin) and incubated
at 37°C overnight. A single colony was grown in a 5ml LB medium (30µg/ml
Kanamycin) overnight and the plasmid was harvested using the QIAprep Miniprep kit
(Qiagen).

The donor vector containing the gene of interest was subcloned into the destination
vector pDEST14. This was done by LR clonase enzyme mix (Invitrogen). The mixture
was made and incubated at 25°C for 1h:
<table>
<thead>
<tr>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR221 (15-150ng)</td>
</tr>
<tr>
<td>pDEST14 vector (150ng/µl)</td>
</tr>
<tr>
<td>TE buffer pH 8.0</td>
</tr>
<tr>
<td>BP clonase enzyme mix</td>
</tr>
</tbody>
</table>

The reaction was terminated with Proteinase K and transformed into the DH5α cell for plasmid production.

Sequences of all the constructs designed were verified by sequencing in GenePool.

### 2.1.2 Protein concentration determination

The protein concentration was measured using a NanoVue spectrophotometer measuring the absorbance at 280nm using molar extinction coefficient and molecular weight of the protein. The extinction coefficient and molecular weight of each construct are listed below:

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Extinction coefficient (M⁻¹ cm⁻¹)</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2-N¹¹⁻¹₃₀</td>
<td>10430</td>
<td>15601.7</td>
</tr>
<tr>
<td>MDM2-N¹⁸⁻¹¹₁</td>
<td>16390</td>
<td>14153.3</td>
</tr>
<tr>
<td>MDMX-N¹⁴⁻¹₁₁</td>
<td>7450</td>
<td>13313.3</td>
</tr>
<tr>
<td>MDMX-N²₃⁻¹₀⁹</td>
<td>13410</td>
<td>13001.9</td>
</tr>
</tbody>
</table>

2µl of protein buffer was used as a blank and 2µl of protein sample was used to measure the concentration. The protein was measured in triplicate and the average concentration was calculated.

### 2.1.3 Transformation and expression

The construct along with optimised expression and purification protocols for MDM2-N¹⁸⁻¹₁¹ and MDMX-N¹⁴⁻¹₁₁ were obtained from Dr. Maria Sanchez in the School of Chemistry, University of Edinburgh. All the constructs contain a His-tag and thrombin or TEV cleavage site at N-terminus. The vector used is PET28b and expression is in E. coli BL21 STAR strain.

All the agar plates and mediums contain the specific antibiotics for the constructs.
<table>
<thead>
<tr>
<th>Constructs</th>
<th>Antibiotics used</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2-N&lt;sup&gt;11-130&lt;/sup&gt;</td>
<td>50µg/ml Kanamycin</td>
</tr>
<tr>
<td>MDM2-N&lt;sup&gt;18-111&lt;/sup&gt;</td>
<td>100µg/ml Carbenicillin</td>
</tr>
<tr>
<td>MDMX-N&lt;sup&gt;14-111&lt;/sup&gt;</td>
<td>50µg/ml Kanamycin</td>
</tr>
<tr>
<td>MDMX-N&lt;sup&gt;23-109&lt;/sup&gt;</td>
<td>100µg/ml Carbenicillin</td>
</tr>
</tbody>
</table>

Single colonies of cells were picked up from Luria Bertani (LB) plates and used to inoculate 50ml of Terrific Broth (TB) media. Cultures were grown for ~5 hours at 37°C with shaking (250rpm). This culture was transferred to 1L of TB media and grown at 37°C with shaking (250rpm). When the one litre cultures were grown to OD<sub>600</sub> = 0.3 the temperature was reduced to 25°C. Expression was induced with 0.1mM IPTG when OD<sub>600</sub> reached 0.6 and the culture was incubated overnight at 25°C. The cells were harvested by centrifugation at 8000rpm for 30min at 4°C. One litre cell pellet (weight around 9 grams) was frozen in liquid nitrogen and stored in -80°C freezer.

### 2.1.4 Preparation of cell lysate

The cellular pellet was resuspended in 5ml lysis buffer (50mM HEPES pH 8.5, 300mM NaCl, 1mM DTT, 20mM Imidazole, 1mg/ml Lysozyme, 2.5 units/ml benzonase, 10% glycerol) per 1gram cell pellet and incubated on ice for 30min. The cells were passed through a constant system cell disruptor twice to break open the cells. After lysis the lysate was centrifuged at 16000rpm for 45minutes at 4°C. The supernatant was collected and filtered through 0.2µm filter.

### 2.1.5 Purification strategies for MDM2-N & MDMX-N

All purifications were performed using AKTA purifier system (10ml/min) at 6°C. Two steps of purifications were applied.

#### 2.1.5.1 Immobilized metal ion affinity chromatography (IMAC)

In the IMAC purification a HisTrap column containing an immobilized chelating group which can be charged with metal ions such as Ni, Co, Zn Fe and Cd was used.
Several amino acids bind to divalent metals when exposed on the protein surface. To improve the affinity of binding the protein has fused with a 6xHis tag and this makes the His tag protein the strongest binder. The His tag protein can then be eluted by high concentration of imidazole.

All the constructs that were used in this work have 6xHis-tag fused on the N-terminal for IMAC purification.

Buffer A

50mM HEPES pH8.5, 300mM NaCl, 1mM DTT, 20mM Imidazole (pH 7.5 with 10% (v/v) glycerol when purifying MDMX-N^{14-111} and MDM2-N^{18-111}).

Buffer B

50mM HEPES pH8.5, 300mM NaCl, 1mM DTT, 500mM Imidazole (pH 7.5 with 10% (v/v) glycerol when purifying MDMX-N^{14-111} and MDM2-N^{18-111}).

The supernatant was loaded onto a pre-packed 5ml Ni^{2+} HiTrap IMAC HP Sepharose column (column was pre-charged with Ni^{2+} and equilibrated with Buffer A) at a flow rate of 5ml/min. The flow rate was maintained at 5ml/min throughout the purification process.

The column was washed with 20 column volumes of Buffer A to remove unbound proteins. Then contaminant was removed by washing with 10 column volumes of 15% Buffer B. The protein was eluted through a linear gradient (0% - 100%) at 25 column volumes using Buffer B. The purity of the fractions was confirmed in SDS-PAGE gel and the fractions containing the desired proteins were pooled together.

2.1.5.2 Size exclusion chromatography (SEC)

Size exclusion chromatography is a technique that can separate molecules based on its molecular size in the solution. SEC uses various columns that contains different matrices to provide diverse separation ranges.

HiPrep 16/60 Sephacryl S-100 HR column was used to purify MDM2-N^{11-130} and MDM2-N^{18-111}, while MDMX-N^{14-111} was purified using HiLoad 16/60 Superdex 200 pg. The column was pre-equilibrated with 2 column volumes of gel filtration buffer. The eluted fractions were confirmed by 18% SDS-PAGE gel. Pure MDM2-N^{11-130}
fractions were pooled and concentrated to 1mg/ml using vivaspin column [Molecular weight cut off = 5kDa] and stored at -80°C. Purified MDM2-N^{18-111} and MDMX-N^{14-111} were concentrated to ~0.5mg/ml and stored at -80°C.

Gel filtration buffer
50mM HEPES pH 7.5, 100mM NaCl, 0.1mM EDTA and 2mM DTT (10% (v/v) glycerol was added when purifying MDMX-N^{14-111} and MDM2-N^{18-111}).

2.1.6 Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI MS)
MALDI MS is a technique that was first introduced in 1988 by Franz Hillenkamp and Michael Karas for analysing biomolecules (proteins, peptides or DNA) and large organic molecules (polymer and other macromolecules) (Karas and Hillenkamp, 1988, Tanaka et al., 1988). In MALDI MS the molecule is co-crystallized with matrix compound, sinapinic acid for proteins or a-cyano-4-hydroxycinnamic acid (CHCA) for peptides. As the laser is fired onto the crystallized matrix, the matrix strongly absorbs the energy and undergoes desorption and ionization which function to ionize the molecule. In MALDI Time of Flight (TOF) MS, the ionized molecules are accelerated into the tube and can be separated by the mass to charge ratio. Smaller mass molecules reach the detector first and large molecule takes longer time to reach the detector.

A MALDI-TOF MS experiment was used to compare the mass of the MDM2-N^{11-130} after the His-tag cleavage. The sample was prepared by dropping 0.5µl protein onto the Gold plate and then adding 0.5µl of sinapinic acid. The plate was fitted onto the robot arm and scrolled into the voyager DE STR MALDI-TOF MS (Applied Biosystem Ltd.).

2.1.6.1 In-Gel digest for mass spectrometry
MS was also used as an analytical technique to identify protein from the gel. The protein band was digested using trypsin into many small pieces of peptides. Trypsin cuts at the C-terminal side of lysine and arginine.
The protein band was cut out from the gel and incubated in 300µl NH₄HCO₃ (ABC, 200mM) in 50% acetonitrile (ACN) at room temperature for 30min. The washing was repeated twice to remove the SDS from the band. The band was then incubated in 300µl DTT (20mM), ABC (200mM), ACN (50%) at room temperature for 1hour and followed by washing three times with 300µl ABC (200mM), ACN (50%). The next step is to alkylate cysteines in 100µl iodoacetamide (IAA, 50mM), ACN (50%) at room temperature for 20min. The band was washed three times with 500µl ABC (20mM), ACN (50%). The band was centrifuged at 13,000rpm for 2min and covered with ACN for 5min until the band turned white. The band was extracted and allowed to dry before adding 29µl of ABC (50mM) and 1µl trypsin. The band was incubated with trypsin at 32°C overnight to digest. The sample was sonicated for 10min prior to MS analysis as described above.

2.2 Results and Discussion

2.2.1 Expression & purification of MDM2-N¹¹⁻¹³⁰

2.2.1.1 Expression of MDM2-N¹¹⁻¹³⁰

*E. coli* BL21 STAR cells were transformed with pET28b vector containing the MDM2-N¹¹⁻¹³⁰ gene. The expression of MDM2-N was induced with 0.1mM IPTG at OD₆₀₀ of 0.6 and incubated overnight at 25°C.

An expression trial was carried out in LB and TB media to confirm if the expression can be improved in TB medium. Cells were grown using the same conditions and SDS-PAGE was run to compare the expression level of MDM2-N in LB and TB. The gel showed there was more MDM2-N produced in TB than in LB (Figure 2.4). This could be because TB medium is more nutritious and provides better cell growth, so the higher biomass in TB gives more protein than in LB.
Figure 2.4 Expression trial of MDM2-N in LB and TB medium.

In the gel, a band is present between 15-20kDa markers, indicating presence of MDM2-N\textsuperscript{11-130}. The band in TB medium is more intense than LB showing more MDM2-N\textsuperscript{11-130} produced in TB medium.

2.2.1.2 Purification of His\textsubscript{6}-tag MDM2-N\textsuperscript{11-130}

After optimum storing conditions were found (section 2.2.1.3) it was possible to purify MDM2-N\textsuperscript{11-130} in large scale so that purification did not have to be repeated so often. 4X 1L of MDM2-N pellets were purified and passed through the cell disruptor at 25KPSI. After purifying using Ni\textsuperscript{2+} HisTrap column, fractions from peak 1 and peak 2 were run on SDS-PAGE gel to confirm MDM2-N presence (Figure 2.5). MDM2-N was present in peak 2 and concentration was determined to be 1mg/ml in 100ml. The yield was ~100mg from 4X 1L pellet.
Figure 2.5 First step of purification of MDM2-N.
The soluble fraction was pumped onto a 5ml Ni\textsuperscript{2+} His-Trap column attached to an AKTA system. Two step gradients were applied that elute the contaminant at 7% buffer B, then elute MDM2-N at 100% buffer B.

MDM2-N sample purified from IMAC column was further run through a 26/60 S200 gel filtration column (Figure 2.6). Two peaks eluted at 200ml and 340ml. A SDS-PAGE gel was run on the peaks to confirm the presence of MDM2-N and the purity. MDM2-N was found to be present in peak 1 as can be seen on the gel with pure His-tag MDM2-N present only.

The two batches of MDM2-N purified from gel filtration were concentrated and the concentration was measured. The yield from each gel filtration was 18mg and 16mg so the total yield is 34mg. More than half of the protein was lost in gel filtration. One possibility could have been that the protein precipitated during loading onto the
column, becoming stuck in the column. Another possible reason for the protein to precipitate could have been that the buffer was not suitable. The pH has decreased from 8.5 to 7.5 and the salt concentration has dropped from 300mM down to 100mM. These rapid changes may have caused the protein to become unstable and prone to precipitate.

Even though half of the protein was lost ~34mg of MDM2-N was obtained. The purified MDM2-N was concentrated to 1mg/ml and storing conditions explored.

Figure 2.6 Second step purification of MDM2-N.
MDM2-N was run through a 26/60 S200 Gel filtration column. 1 single peak was eluted out at 200ml in the elution profile and confirmed to be MDM2-N from SDS-PAGE gel.
2.2.1.3 Finding storing condition for MDM2-N

Purification of MDM2-N was shown to be successful with pure His-tag MDM2-N obtained but currently there was no long term storing condition available. MDM2-N precipitated within one week and purification had to be carried out every week which was very inconvenient. Therefore a storing condition has to be identified for storing MDM2-N long term.

Two storing trials were tested. The first sample of 200µl MDM2-N was stored in the freezer for two days and the second sample of 200µl MDM2-N was flash frozen using liquid Nitrogen and then stored in the -20°C freezer. These frozen MDM2-N were thawed and tested in Malvern Zetasizer APS Dynamic Light Scattering (DLS) to confirm if the protein was still folded properly.

Dynamic Light Scattering (DLS) is used to measure hydrodynamic sizes, polydispersities and aggregation effects of protein samples. DLS measures the laser light scattered from the macromolecules in solution. The macromolecules in solution fluctuate due to the Brownian motion so the scattering intensity can be observed. These fluctuations are directly related to the rate of diffusion of the molecules in the solution (Jachimska et al., 2008).

The protein was centrifuged at 12000rpm for 10min to remove any precipitant. Blank was run using Protein Buffer (50mM HEPES pH 7.5, 100mM NaCl, 1mM DTT). 50µl of protein was added into well and the sample was measured three times. Since small molecules diffuse faster, they lead to a faster decay in the measured correlation function.

2.2.1.3.1 DLS on fresh MDM2-N sample

Figure 2.7 shows the size distribution results and there were different peaks present in the size distribution. The peaks present in 0.43 and 0.88nm were small particles in the buffer solution. The peaks at the hydrodynamic radius of 5.3nm was MDM2-N and the DLS estimates the molecular weight to be 15.7kDa. The molecular weight of MDM2-N is 15.6kDa so DLS has accurately estimated the molecular weight of MDM2-N. Multiple peaks were found in hydrodynamic radius over 100nm and this indicates that there were aggregates present in the solution.
Figure 2.7 Size distribution by intensity of fresh MDM2-N protein.

DLS was performed three times on the same sample and multiple peaks were present in the size distribution profile. The peaks at ~5.3nm size is the MDM2-N which estimates the molecular weight to be 15.7kDa.

2.2.1.3.2 DLS on frozen MDM2-N

MDM2-N protein that had been frozen in -20°C for two days was analysed in DLS to confirm if the protein is still active. The size distribution in Figure 2.8 showed a similar trend to the size distribution as found in Figure 2.7 of fresh MDM2-N sample. The peaks at ~5.3nm were MDM2-N and DLS estimated the molecular weight to be 17.9kDa. The molecular weight of MDM2-N is 15.6kDa so this means that MDM2-N did not form aggregates and was stable to use after freezing with only a small amount of aggregate present. However in the MDM2-N protein that was flash frozen using
liquid Nitrogen the size distribution profile showed large aggregates present in the solution (Figure 2.9). This suggests that flash freeze is not an ideal method for storing MDM2-N and storing MDM2-N in -20°C freezer is more appropriate.

**Intensity Distribution Results**

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<th>Peak</th>
<th>%Int</th>
<th>Mean (d.n.a)</th>
<th>Mode (d.n.a)</th>
<th>Std (d.n.a)</th>
<th>%Pd</th>
<th>MW (kDa)*</th>
<th>Polydispers...</th>
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<td>179.5</td>
<td>60.12</td>
<td>30.2</td>
<td>1.59e5</td>
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<tr>
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<td>0.7017</td>
<td>0.07707</td>
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</table>

**Mass Distribution Results**

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<th>Mode (d.n.a)</th>
<th>Std (d.n.a)</th>
<th>%Pd</th>
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<tr>
<td>Peak 2</td>
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<td>17.9</td>
<td></td>
</tr>
<tr>
<td>Peak 3</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.8 Size distribution intensity profile of frozen MDM2-N.**

This is the MDM2-N protein frozen in -20°C freezer for two days and analysed in DLS. The sample was analysed in DLS three times. The peaks at ~5.3nm size are the MDM2-N and DLS estimates the molecular weight to be 17.9kDa.
Figure 2.9 Size distribution intensity profile of flash frozen MDM2-N.
MDM2-N protein was frozen using liquid Nitrogen and stored in -20°C freezer. In the profile, there is only a single peak present at hydrodynamic radius >100nm. This suggest the protein has been aggregated.

2.2.1.4 Using thrombin to cleave off His-tag
A thrombin cleavage trial was set up to try cleaving off the His-tag on MDM2-N. The His-tag is located at the N-terminus of the MDM2-N construct near the hydrophobic pocket and this may interfere compounds binding into the pocket. Therefore it was important to cleave off the His-tag to see whether the His-tag had an effect on the assay.

MDM2-N (1mg) was incubated with 2U thrombin (GE healthcare) overnight at 4°C as recommended by the manual. The protein was then loaded back onto the Ni²⁺ HisTrap column and the flow through was collected. From the chromatogram in Figure 2.10 a large peak was found in the flow through which suggest the His-tag was cleaved off from the MDM2-N and the protein can no longer bind to the HisTrap column. There was only a small peak present in the elution suggesting most of the His-tag was cleaved off from the MDM2-N.
Figure 2.10 MDM2-N incubated with 2U Thrombin was run through Ni²⁺ IMAC column.
A peak came out in the flow through and only a small peak was present in the elution profile indicating that the His-tag was cleaved off and MDM2-N cannot bind to the IMAC column.

2.2.1.5 Matrix assisted laser desorption/ionisation (MALDI) mass spectrometry

His-tag cleavage was further confirmed using MALDI mass spectrometry to identify the molecular weight of MDM2-N sample after incubating with thrombin. Fig. 2.5 shows the results of Mass spectrometry. The top graph shows the control MDM2-N without thrombin incubation and the bottom graph shows MDM2-N after incubating with thrombin. From the mass spectrometry results (Figure 2.11) the molecular mass of control MDM2-N detected in the MS was 15473Da and the molecular mass of His-tag MDM2-N is 15601.7Da. In the MS trace after thrombin treatment (Figure 2.11 bottom), the molecular mass has change to 13721Da. This change in molecular mass indicated the His tag has been cleaved off from MDM2-N as the His-tag MW is 1900Da.
Mass Spectrometry was run on the control with MDM2-N only and on a sample of MDM2-N incubated with Thrombin. From the profile, MDM2-N incubated with Thrombin changed the molecular weight from 15473Da to 13721Da. This confirms that the His-tag was cleaved.

2.2.2 Expression & purification of MDM2-N\textsuperscript{18-111}

A paper published recently by Prof. Martin Noble’s group has found a method to enhance the crystallogenesis of the N-terminal domain of MDM2 (Anil et al., 2013). The group has found that residues 109-125 can form a large unstructured tail and disfavour the crystallization process (Figure 2.1). Alignment was performed on the sequences of multiple published structures with the sequence from Martin Noble (4HG7) and the currently used MDM2-N\textsuperscript{11-130} sequence (Figure 2.1). As shown below the sequences from the published structures and Martin Noble’s structure have the residues 109-125 chopped off. However the construct MDM2-N\textsuperscript{11-130} that was used in the studies has an unstructured tail present and also extra residues on the N-terminal that could be the lid. This could be one of the reasons of no crystal growth in the crystallization trial. Therefore, a new short construct was designed that removed the unstructured tail on residues 109-125 and also the lid residues on the N-terminal.
2.2.2.1 Construct design of MDM2-N\textsuperscript{18-111}

The MDM2-N\textsuperscript{18-111} sequence was cloned by PCR using the MDM2-N\textsuperscript{11-130} plasmid as a template. The PCR product was analysed in a 1.2% agarose gel (Figure 2.12 left) and extracted using QIAquick gel extraction kit (QIAGEN). The extracted MDM2-N\textsuperscript{18-111} sequence was run through a second PCR reaction to attach a His-tag at the N-terminal end. After attaching the His-tag, the length of the sequence increased by \(\sim 100\) bases and this difference can be seen from the gel (Figure 2.12 right).

The sequence was then inserted into gateway donor vector and destination vector before transforming into the E. coli cells.

![Figure 2.12 MDM2-N18-111 PCR reactions' products in 1.2% agarose gel.](image)

Left gel is the samples from the MDM2-N\textsuperscript{18-111} first PCR reaction and the product is \(\sim 350\) bases. The right gel is the samples from the second PCR reaction with the His-tag attached and the product length has increased to \(\sim 450\) bases.

2.2.2.2 Expression of MDM2-N\textsuperscript{18-111}

The condition used to express MDM2-N\textsuperscript{11-130} was also used on MDM2-N\textsuperscript{18-111}. E. coli BL21 (DE3) and BL21 (DE3) STAR cells were investigated. The cells were grown at 37°C until OD\textsubscript{600} reaches 0.3 and the cells were transferred to 25°C. 0.1mM IPTG was added at OD\textsubscript{600} = 0.6 to induce the MDM2-N\textsuperscript{18-111} expression.

The cells were harvested by centrifugation at 8000rpm for 30min and the expression was analysed using SDS PAGE. Expression of MDM2-N\textsuperscript{18-111} was present in both
BL21 (DE3) and BL21 (DE3) STAR induced cells (Figure 2.13). In the uninduced cells there was also a band present in the same position in both BL21 (DE3) and BL21 STAR uninduced cells which may indicate a leaky expression.

![Image of gel electrophoresis](image)

**Figure 2.13 The results of an expression trial in two different competent cells.**

Cells were induced with 0.1mM IPTG and MDM2-N^{18-111} was expressed in both cell lines.

### 2.2.2.3 Purification of MDM2-N^{18-111}

To confirm if the protein is expressed in the folded form the pelleted cells were lysed in 30ml lysis buffer and passed through the Constant Systems cell disruptor. The lysate was centrifuged and MDM2-N was mostly present in the soluble fraction (Figure 2.14).

MDM2-N was purified using a HisTrap column. Some of the contaminants were eluted at 75mM imidazole elution and the MDM2-N protein was eluted at 120mM concentration. However when looking at the proteins collected in the fraction collector
the MDM2-N proteins precipitated in the fractions. The concentration of the MDM2-N measured in the fractions was ~2mg/ml. This suggested that removing the unstructured tail and the N-terminal sequence from the MDM2-N reduced the solubility of the protein. The MDM2-N became prone to precipitation and the purification step had to be modified to prevent precipitation.

![Figure 2.14 First step purification of MDM2-N18-111.](image)

MDM2-N_{18-111} was loaded onto a 1ml Ni^{2+} HisTrap column and there were two peaks present in the elution profile. The peaks in the fractions were verified by SDS PAGE gel. MDM2-N_{18-111} was confirmed to be eluted in the 2nd peak.

To help improve the solubility of the protein 10% glycerol was added into all the buffer solutions. The elution step was modified from a linear gradient into a two steps gradient elution. The first step involved eluting with 75mM imidazole to remove contaminants and the second step used 50CV of linear gradient to elute MDM2-N
protein (Figure 2.15). Elution with 50CV dilutes the protein to make it less concentrated. Unfortunately the MDM2-N protein eluted in the first 75mM imidazole elution but there was no precipitation in the fraction. The glycerol (10%) must have helped stabilized the protein and prevented the protein from precipitating. The protein was polished using HiPrep 16/60 S100 HR gel filtration column and 6mg/L of pure monomeric MDM2-N18-111 was obtained (Figure 2.16).

Figure 2.15 MDM2-N18-111 purification in Ni2+ HisTrap column.

MDM2-N18-111 was purified on an IMAC column using a buffer solution containing glycerol (10%). The first elution step used a 75mM imidazole step gradient and second step used a 50CV linear gradient from 75mM – 500mM imidazole. The MDM2-N was eluted in the first step of elution.
2.2.2.4 Assess MDM2-N_{18-111} in FP binding assay

The purified His tag-MDM2-N_{18-111} was assessed in the FP binding assay with the fluorescent labelled p53 peptide (Method can be found in Chapter 4). Detailed principle and method can be found in Chapter 4. The results of FP assay in Figure 2.17 (A) showed the His tag-MDM2-N_{18-111} affinity for p53-F has a K_d = 2.79µM. This is seven times weaker than binding with the His tag-MDM2-N_{11-130} (Figure 2.17 B). As shown in Figure 2.1, the Histag-MDM2-N_{18-111} has seven residues removed from the N-terminal end and the His-tag becomes very close to the binding site. This can
cause the His tag to interfere the p53-F from binding to the binding site and also disrupt the small molecule from binding.

Figure 2.17 Comparing binding affinity of p53-F against MDM2-N^{18-111} and MDM2-N^{11-130}.

FP binding assays were performed on MDM2-N^{18-111} (A) and MDM2-N^{11-130} (B) against p53-F. p53-F binds seven times weaker to MDM2-N^{18-111} than MDM2-N^{11-130}.

2.2.2.5 His-tag cleavage on MDM2-N^{18-111}

A cleavage trial was carried out to cleave off the His-tag attached on the MDM2-N. A TEV recognition site was present between the His tag and MDM2-N so the cleavage trial can be performed using TEV protease enzyme. 100µg of MDM2-N was incubated overnight at 4°C with 1µg, 2µg and 4µg TEV protease. The cleavage trial resulted in around 50% cleavage using 4:100 ratio (Figure 2.18 left). When attempting to cleave the His tag from the 1.7mg of MDM2-N using 50µg TEV protease (3:100 ratio), only 50µg of MDM2-N had the His tag cleaved off (Figure 2.18 right). The TEV protease cleavage site may have been buried into the binding site and thus hindered the cleavage. Unfortunately the amount of untagged MDM2-N was too small to verify the effect of His tag in the FP assay.
Figure 2.18 SDS-PAGE gel of cleavage trial for His-tag MDM2-N^{18-111}.

(Left) Different concentrations of TEV protease were incubated with MDM2-N^{18-111}. An increase of cleavage when increased the TEV protease and at 4/100 ratio, the cleavage rate was 50%.

(Right) His-tag-MDM2-N^{18-111} was incubated with TEV protease at a ratio of 3/100.

### 2.2.3 Expression & purification of MDMX-N^{14-111}

MDMX has also been identified as a negative regulator of tumor suppressor p53 protein. Both MDM2 and MDMX binds to a short α helix in the transactivation domain of p53. The N-terminal domain of MDMX contains a p53 binding site that has a high homology as MDM2. Design of a dual inhibitor becomes possible to disrupt both MDM2 and MDMX binding to the p53.

To design a dual inhibitor, high purity MDMX-N had to be purified for small molecules screening in different assays.

#### 2.2.3.1 Expression of MDMX-N^{14-111}

MDMX-N^{14-111} plasmid was obtained from Dr. Maria Sanchez (Figure 2.2). The construct was expressed in LB, TB and Enpresso mediums. There was no expression found in the Enpresso medium but the MDMX-N^{14-111} was expressed in the LB and
TB mediums (Figure 2.19). A higher expression was present in the TB medium than the LB medium.

![Image of SDS-PAGE gel showing expression trial of MDMX-N14-111](image)

**Figure 2.19 Expression trial of MDMX-N14-111.**
SDS-PAGE gel showing the expression of MDMX-N14-111 in *E. coli* BL21 DE3 cells grown in LB, TB and Enpresso mediums. Cells were grown at 25°C and induced with 1mM IPTG when OD600 reaches 0.6. The lanes compare the expression level of MDMX-N14-111 in the uninduced cells and induced cells grown in the mediums.

### 2.2.3.2 MDMX-N14-111 purification
The *E. coli* cells containing MDMX-N protein were lysed by resuspending the cells in the lysis buffer and passing through the cell disruptor. The lysate was clarified by centrifugation and the His tag-MDMX-N was loaded onto a 1ml Ni²⁺ HisTrap column. The imidazole gradient to elute contaminants was already known from the previous purification trial (data not shown), so a step gradient (75mM imidazole) was applied to remove contaminants before eluting the MDMX-N protein with a linear gradient
The truncated MDMX-N was separated at higher imidazole concentration.

The next step purification was to separate the contaminants and the truncated MDMX-N using superdex200 16/60 size exclusion column. The truncated MDMX-N is only 5kDa smaller than MDMX-N but the impurities and truncated MDMX-N was still successfully separated from the MDMX-N yielding 1mg/L culture (Figure 2.21).

**Figure 2.20 IMAC purification of MDMX-N14-111.**

The lysate containing MDMX-N was loaded onto the 1ml Ni²⁺ HisTrap column as first purification step. First gradient comprised of 75mM imidazole wash and large amount of impurities was washed off. MDMX-N was eluted in the linear gradient elution from 75mM – 500mM imidazole over 30 column volume. The elution also separated truncated MDMX-N at the peak fraction 3.
SEC was performed using Superdex200 16/60 column. The peak fractions were analysed by SDS-PAGE gel. The gel confirmed peak 3 contains the pure MDMX-N and the shoulder peak 2 close to the peak 3 contains truncated MDMX-N.

### 2.2.4 Expression & purification of MDMX-N^{23-109}

#### 2.2.4.1 MDMX-N^{23-109} construct design

In Figure 2.2, multiple MDMX-N sequences from the PDB structures were compared with the MDMX-N^{14-111} construct. The MDMX-N^{14-111} sequence has a longer sequence at the N-terminal end and is absent in other published structure sequences. It is not clear whether this extended sequence at the N-terminal will affect the crystallization so a shorter construct was designed. The sequence from 3JZO structure was taken for new construct design.
2.2.4.2 Construct design of MDMX-N^{23-109}

MDMX-N^{14-111} plasmid was used as template to design the MDMX-N^{23-109} construct. Two rounds of PCR reactions were performed to insert the His-tag onto the MDMX-N^{23-109} construct. The length of the sequence has increased to ~400 bases when the His-tag was attached onto the N-terminal of MDMX-N^{23-109} construct (Figure 2.22).

The MDMX-N^{23-109} sequence was inserted into the gateway destination vector to be ready to transform into the E. coli cells.

![MDMX-N^{23-109} PCR reactions' products in 1.2% agarose gel.](image)

Sample 1 is the MDMX-N^{23-109} first PCR reaction product with ~300 bases length and sample 2 is the second PCR reaction product with ~400 bases length.

2.2.4.3 MDMX-N^{23-109} expression

The conditions that were used to express MDMX-N^{14-111} and MDM2-N^{11-130} gave a reasonable yield of soluble proteins so these conditions were also used to express MDMX-N^{23-109}.

The plasmid was transformed into E. coli BL21 DE3 and BL21 (DE3) STAR cells. The protein was induced using 0.1mM and 1mM IPTG. The cells were run in the SDS PAGE gel to confirm if there is MDMX-N^{23-109} expression. The MDMX-N^{23-109} molecular weight is 13kDa and should be between 10kDa – 15kDa in the gel but there was no clear indication of MDMX-N^{23-109} expression in this region (Figure 2.23 Top).
The plasmid was also transformed into *E. coli* C41 (DE3), C43 (DE3) and Rosetta 2 strains. *E. coli* C41 (DE3) and C43 (DE3) strains were derived from BL21 (DE3) and it has the characteristic that helps to prevent cell death from the expression of toxic protein. Rosetta 2 is derived from BL21 strain with a supply of seven rare codons to enhance the expression of eukaryote proteins in the *E. coli*. The cells were grown using the same condition and the MDMX-N<sup>23-109</sup> expression was induced using 1mM IPTG. The whole cells were run in the gel and there was still no indication of MDMX-N<sup>23-109</sup> expression (**Figure 2.23 Bottom**).

Further expression trials will need to be carried out to express MDMX-N<sup>23-109</sup>.

**Figure 2.23 SDS PAGE gel showing the trial expression of MDMX-N23-109 construct.**

Top) These lanes show different trial expressions in BL21 DE3 and BL21 STAR competent cells, uninduced cells and cells induced with 0.1mM, 1mM IPTG. There is no clear indication of MDMX-N<sup>23-109</sup> expression at these conditions.

Bottom) This gel shows trial expressions of MDMX-N<sup>23-109</sup> in C41 (DE3), C43 (DE3) and Rosetta 2 competent cells and induced with 1mM IPTG.
2.3 Conclusion of the purification of MDM2-N and MDMX-N constructs

MDM2-N\textsuperscript{11-130} was successfully expressed and purified with a yield of 34mg/4L and a purity >95%. When MDM2-N\textsuperscript{18-111} construct was expressed and purified we are not sure if the His-tag interfered with the binding site and there were also difficulties with cleaving off the His tag. Due to this problem the MDM2-N\textsuperscript{18-111} construct was not further used in biophysical and structural studies. His-tag MDMX-N\textsuperscript{14-111} was purified but the protein aggregated when the concentration was above 0.5mg/ml. This construct had to be stored at low concentration to prevent aggregation. The His tag of MDMX-N\textsuperscript{14-111} did not interfere the binding site which has been shown in Figure 4.23 where the Kd value of fluorescently labelled p53 peptide obtained was in agreement with the publication (Section 4.8.1). MDMX-N\textsuperscript{23-109} construct was designed for crystallization trial but the protein was unfortunately not expressed in the expression trial and this construct was discontinued for further study.

MDM2-N\textsuperscript{11-130} and MDMX-N\textsuperscript{14-111} were used for the biophysical studies.
Chapter 3 Screening for hits against MDM2-N and MDMX-N

Over the past few decades high throughput screening (HTS) has been the dominant method in the drug discovery process (Bajorath, 2002, Shoichet, 2004). Millions of compounds were screened through HTS and many lead drugs were identified. Chemists have synthesized large numbers of compounds for HTS. However this is only a small proportion of the possible available compounds and the cost to screen through millions of compounds is high.

An alternative cost effective approach makes use of computer-aided methods. Virtual screening is a computational approach that is able to analyze millions of compounds stored in databases using high throughput computational docking. During the 1970s several investigations were carried out using molecular docking to study intermolecular interactions (Greer and Bush, 1978, Levinthal et al., 1975, Wodak and Janin, 1978, Salemme, 1976). In 1982, Kuntz et al. described a geometric approach to study the receptor-ligand interactions (Kuntz et al., 1982) and numerous computational algorithms were gradually developed and optimised (McInnes, 2007). Virtual screening is becoming an important computational technique for drug discovery.

Even though there are many successful cases in virtual screening there are still many problems which need to be solved. Firstly, the protein flexibility is only partially considered in virtual screening. Further developments are required to address this issue. The participation of water molecules in protein-ligand interaction is also not considered in the docking process. The molecular interactions are difficult to parameterise and the pose scoring and binding energy predictions are imprecise which still require more time to improve.

There are two approaches in virtual screening, one is the structure based approach that uses an X-ray crystal protein structure and a large number of compounds to identify an optimal compound based on docking into a defined binding site on the protein (Reddy et al., 2007, Ghosh et al., 2006). The other approach is the ligand based method that employs compounds or substrates structures which are known to bind to the protein binding site and identify other compounds with similar characteristics (Reddy et al., 2007). These techniques are applied in this chapter to screen for MDM2 ligands.
3.1 Background of database and virtual screening programs employed

In this project I have employed four virtual screening programs to explore compound databases and identify hits. Both structure based and ligand based programmes (Figure 3.1) were employed and background for these programs are described.

**Figure 3.1 Virtual screening programs applied.**
A diagram showing a list of virtual screening programs applied in the project to search for small molecule inhibitors.

#### 3.1.1 EDULISS database description

The compound library that we used for database mining is EDULISS which stands for **EDinburgh University LIgand Selection System** and was developed by Dr. Kun-Yi Hsin (Hsin et al., 2011). The library comprises over 5 millions compounds from 28 different suppliers. The library stores the 3D structure of the compounds for virtual screening and the pharmacophore, physicochemical and structural properties are also stored in the library.

There are over 3.9 millions compounds in the database that fit into the Lipinski’s rule of five (Hsin et al., 2011).

In the database there are over 220,000 compounds which fit the Astex rule of three (Hsin et al., 2011) that are suitable for fragment based design (Hsin et al., 2011). When
new compounds are added into the suppliers catalogue they will also be added into the EDULISS library and thus, the library is continuously expanding. The concept of drug-like or lead-like has been introduced into the drug design to characterise the properties for a drug to be successful. Three well known examples are Lipinski’s rule of five, Oprea lead-like criteria and Astex rule of three. These features have often been used in the screening libraries to keep compounds that have smaller molecular weight with higher solubility. However applying these criteria into the search libraries may exclude out some potential hits that don’t fit the criteria. One example is the natural products which have been explicitly excluded by the Lipinski’s rule but they have contributed to the development of many drugs (Beutler, 2001).

3.1.2 Structure based docking
Structure based docking can be split up into several stages which are illustrated in Figure 3.2. The first stage is to identify a protein that has therapeutical interest and can be purified for experimental validation. An X-ray crystal structure of the protein and ligand is required as input for the virtual screening. The results are validated using different biophysical assays.

![Figure 3.2 Stages involved in Structure based docking.](image)

The diagram represents the pipeline involved in structure based docking. The compounds selected from virtual screening have to be validated using biophysical assays.

3.1.3 LIDAEUS
The first high throughput docking program that we employed was the University of Edinburgh in-house virtual docking program LIDAEUS which stands for LIgand Design At Edinburgh UniverSity (Taylor et al., 2008). LIDAEUS is performed in several steps which are listed in Figure 3.3.
In the first stage the pdb structures of the protein and the natural ligand are obtained and used to generate an energy site map. The program MAPGEN is then used to generate the energy map around the binding site. There are three classifications of the site points generated; hydrogen bond donor (blue points), hydrogen acceptor (red) and hydrophobic (white). The site points generated can be manually inspected and modified using PyMOL (Delano, 2002). In POSE, LIDAEUS uses an exhaustive matching algorithm to fit molecules into the binding site by matching atoms with the site points in different positions to find the best poses. Then each compound fitted in the binding site will be scored according to the calculated energies of van der waals, hydrophobic and hydrogen bond interactions. However the desolvation force is not taken into account in LIDAEUS. Finally, the result will be sorted and the top 1000 compounds will be kept and ranked according to the score. In our work the top 50,000 compounds were kept for the next stage.

LIDAEUS is performed in a rigid body docking manner, which means the compounds are treated as rigid during the docking. Different conformations and flexibility in the compounds were not considered: the compounds were unable to change the conformation during docking. In order to partially overcome this problem a multi-conformer library of compounds was generated in which an average of 4 to 5 molecular conformations are stored for each flexible molecule. LIDAEUS is suitable for screening millions of compounds and provides a high throughput first step for virtual screening.
Figure 3.3 LIDAEUS pipeline of docking process.

LIDAEUS is a rigid body in-silicon screening program that docks the EDULISS library compounds into the protein binding site through this pipeline. The poses are ranked according to the score and the top 1000 compounds will be kept.

3.1.4 AutoDock & AutoDock Vina

AutoDock and AutoDock Vina (Vina) are both molecular docking tools for protein-ligand docking (Morris et al., 2009, Trott and Olson, 2010). Compared with LIDAEUS that docks the compounds in a single conformation, AutoDock and Vina are able to dock the compounds flexibly in multiple conformations.

AutoDock used the program Autogrid to create an interaction map containing grid points around the binding site of the target protein. Different atom types (hydrophobic, hydrogen bond donor and acceptor) are assigned in the map. These grid points are stored and used in the docking simulation. The ligand atom is sequentially placed in
the grid and the interaction energy between the ligand atom and the protein is calculated.

In AutoDock the algorithm used to predict the conformation is the Lamarckian genetic algorithm (LGA) combined with semi-empirical free energy force field scoring function. In ligand docking the particular arrangement of a ligand and a protein is referred to as a state variable which corresponds to the genotype. The atomic coordinates of the ligand state is referred as the phenotype.

There are five stages involved in the LGA: mapping and fitness evaluation, selection, crossover, mutation and elitist selection.

Mapping reads in the phenotype of each ligand in the population and evaluates the fitness of every ligand. Fitness evaluation involves the calculation of the total interaction energy between the ligand and the protein using a semi-empirical free energy force field. This is the sum of the intramolecular energies of the unbound state ligand and the intermolecular energies between the ligand and the protein. Crossover also occurs in which random pairs of individuals crossover to produce a new genotype. The new genotype will possess genes from both parents. This is followed by selecting the individuals to reproduce depending on the fitness. The conformation with the lowest binding energy is selected.

Vina also employs grid map to calculate the interaction energies between the ligand and protein. However, Vina is able to precalculate the grid map internally and thus decreases the run time (Trott and Olson, 2010).

There are many successful examples of using AutoDock and Vina which can be seen in these papers (Azam et al., 2014, Penta et al., 2014, Gallicchio et al., 2014, Zor et al., 2014).

### 3.1.5 Ligand based similarity search

Ultra Fast Shape Recognition Atom Type (UFSRAT) is a tool that performs similarity searches on the compound of interest against millions of compounds in the EDULISS library (Shave, 2010).

UFSRAT performs shape recognition first by creating four descriptors from the geometric distribution of the molecule. The four descriptors are defined as points 1 (P1) to 4 (P4) (Figure 3.4). P1 is the 3D coordinate of the centre of the volume...
occupied by the molecule. The Euclidean distances of all the atoms to P1 position were calculated and stored in a list. From the list, mean, variance and skew were generated. P2 is the atom that is closest to the geometric centre. P3 is the atom that is furthest away from P2 and P4 is the atom that is furthest away from P3. The Euclidean distances were generated between all the atoms and points 1 to 4. From the list of the Euclidean distances; mean, variance and skew were also generated for all distributions. These values allow UFSRAT to recognise the shape of the molecule. Three extra distributions were created to incorporate recognition and distributions of atom types. These are hydrophobic, hydrogen bond acceptor and hydrogen bond donor atom types similar to those used in LIDAEUS. This is used to classify the atoms in the molecule and enables UFSRAT to calculate 12 descriptors for each molecule.

The geometric distribution descriptors for the molecules in the library were previously generated. The geometric distribution descriptors for the query molecules were then compared with the molecules in the library and the similarity score between the two molecules was generated. Using the precalculated descriptors UFSRAT is able to compare the query compound with 1 million compounds within 3 seconds (Shave S. 2010).
The molecule atoms are considered as points and all the atom distances are calculated from the geometric centre (P1). From the list of the Euclidean distances; mean, variance and skew were also generated for all distributions.

### 3.1.6 Scoring algorithms

After the docking models were generated scoring algorithms were used to estimate the strength of the binding between the protein-ligand complex. Two different types of scoring algorithms, X-cscore and DrugscoreX, were employed in CODASS (as described in Section 3.1.7).

X-cscore is an empirical scoring function that estimates the binding affinity of protein-ligand complexes based on calculated energy terms (Huang et al., 2010). It combines three different scoring algorithms to form a consensus scoring strategy and estimates the binding affinity of a given complex. The total binding energy can be dissected into energy of van der waals interaction, hydrogen bonding, hydrophobic effects, desolvation effect and effective rotatable bonds.
Each scoring algorithm has its own advantages and disadvantages. Consensus scoring combines the advantages and reduced the disadvantages which improved the success rate over using a single algorithm (Wang et al., 2002).

DrugscoreX is a knowledge based scoring function which extracts the structural information from crystallographically determined protein-ligand complex data. It uses distance-dependent pair potentials to observe specific interactions between atoms of the protein and ligand.

The protein data bank (PDB) and Cambridge structural database (CSD) were used and the energies between the atoms of protein and ligand at a distance $1.0 \leq d \leq 6.0 \text{ Å}$ were observed. This builds up the knowledge based potential database for statistical observation of intermolecular contacts. Scoring of protein-ligand complexes is calculated as the sum of all occurring atom-atom interactions (Velec et al., 2005).

However, currently there are still some limitations for the scoring algorithms to simulate the binding between protein-ligand complexes (van Gunsteren et al., 2006). The free energy that drives the biomolecular processed is the summation of many atom pairs contributing to the non-bonded interaction. The accuracy decreases when deriving the summation of atom pairs in the large system (such as protein-ligand and protein-DNA etc.). Another problem is that different X-ray crystal structures were obtained using different conditions (such as different temperature, buffer solution or pH) which can affect the conformation of the proteins and the water molecule contribution (solvation and desolvation effect). This results in an entropic effect that plays an important role in the biomolecular systems and brings more difficulties for the computer to simulate the force field parameters.

### 3.1.7 Combining Docking and Similarity Search (CODASS)

If a protein structure with inhibitor or substrate bound is available, then structure based design may be performed and this involves multiple sequential phases. CODASS is an integrated virtual screening script developed by Dr. Douglas Houston that combines a multitude of docking algorithms, similarity algorithms and scoring algorithms to search through millions of commercially available compounds in the
EDULISS (Hsin et al., 2011) database. Through combining these virtual screening programmes the run time is minimised while the enrichment is maximised.

Figure 3.5 shows a work flow chart of CODASS. Initially, Oprea lead-like criteria (MW ≤ 460, \(-4 \leq \text{LogP} \leq 4.2\), rotatable bond ≤ 10, number of rings ≤ 4, Hydrogen bond donor ≤ 5 and Hydrogen acceptor ≤ 9) (Hann and Oprea, 2004) was used in order to extract smaller and more soluble compounds. This criteria was used as a filter in the EDULISS library which contains 4 million compounds and 20 million conformers. After this initial screen 1,162,000 compounds and around 4.8 million conformers were identified.

These compounds were fed into LIDAEUS (See section 3.1.3), a high throughput rigid body docking program that docked the compounds into the p53 binding site of the MDM2 structure. The results were ranked based on the LIDAEUS score and around 50,000 compounds were selected. These compounds were docked into MDM2 using Vina (Trott and Olson, 2010) which has an improved binding mode prediction and allows flexible docking of the compounds. The docked poses were ranked using Vina and the top 5000 compounds were selected. These 5000 hits were used with UFSRAT to identify compounds with similar structures that was not recognized by LIDAEUS. UFSRAT identify 24,000 compounds from the “Oprea” subset of EDULISS and redocked with Vina. The ranked poses were merged with the original Vina result and duplicates were removed. Ligand based method was used at this stage to pull out analogues to enrich the search results that were not previously identified from the docking programmes. These compounds were then docked using AutoDock. The binding poses predicted by AutoDock and Vina were compared via RMSD (root mean square deviation). AutoDock and Vina uses its own unique algorithm to dock the compound and combining the binding modes predicted by these programmes can significantly increase the success rate (Houston and Walkinshaw, 2013). This is an interesting technique which has proven to generate more reliable docked poses (Houston and Walkinshaw, 2013). Bind poses that were similar with RMSD ≤ 2Å were also scored with X-cscore and DrugscoreX. Final rank list was prepared according to the scores from Vina, AutoDock, X-cscore and DrugscoreX.
The compounds in the EDULISS library are docked into the protein through multiple virtual screening programs. Numbers were used in the flow chart to indicate the order in the process. Additional similarity search is performed using UFSRAT to enrich the search results. The docked poses predicted by AutoDock and Vina are ranked and scored using DrugscoreX and X-score. The top 1000 ranked poses are kept for visual analysis.

3.1.8 Fragment based drug discovery (FBDD)

FBDD focuses on identifying small molecules that are able to fit into binding grooves to achieve efficient binding (Barker et al., 2008). These small molecules tend to be quite soluble and provide good starting points for subsequent chemical modification. This contrasts strongly with the HTS approach in which HTS libraries contain many compounds of high molecular weight with a complex structure. These characteristics cause poor physiochemical properties such as poor solubility and low permeability (Gribbon and Andreas, 2005, Lipinski et al., 1997). Thus, this may result in the genuine hits identified from HTS to be unsuitable as a starting point for lead optimisation.
Fragment design is now well established in many pharmaceutical companies and many successful examples are now published (Hajduk and Greer, 2007, Wyatt et al., 2008). Fragments are low molecular weight molecules (< 250Da) with weak binding affinity ranging from μM – mM. These weak affinity fragments require high sensitive biophysical techniques for detection. Currently the most commonly used techniques for fragment screening are X-ray crystallography (Wyatt et al., 2008, Hartshorn et al., 2004), Nuclear Magnetic Resonance (NMR) (Lepre et al., 2004) and Surface Plasmon Resonance (SPR) (Schulz and Hubbard, 2009). These techniques are highly sensitive but they have constraints on the type of proteins that can be screened.

The rationale of choosing small molecular fragments as the lead-like compounds is that adding sub groups during the optimization stages will result in an increase in molecular mass, complexity and hydrophobicity of the compounds. Using a small molecular weight compound as the starting point is therefore important for drug development.

It is also possible to break down the structure of the known inhibitors into multiple substructures that interact in the binding site (Figure 3.6). These substructures can be used to identify higher quality interaction fragments that bind deeply into the binding grooves. Several papers have demonstrated this method to link high quality interaction fragments together to create more potent compounds (Scott et al., 2012).

Figure 3.6 Fragment based drug discovery.
Hit identified from screening may interact with multiple binding grooves. When compared with fragment it is more ligand efficient and can provide more optimised interaction. Taken from (Scott et al., 2012).
Ligand Efficiency – an effective index for drug discovery

Ligand efficiency (LE) is a measure of the average binding energy of each heavy atom in a compound to its binding target. Equation 3.1 (Hopkins et al., 2004) was used to estimate the efficiency of the compound and assess the binding affinity contribution per atom. It is the ratio of binding affinity to molecular size.

\[ LE = -\Delta G \frac{R \cdot \ln C_{50}}{HA} \]

Equation 3.1

\( \Delta G \) = Gibbs free energy
\( R \) = Gas constant
\( T \) = Temperature in Kelvin
\( HA \) = Number of heavy atoms

LE has often been used in lead assessment and during the fragment optimisation process. The values are calculated when analogues are synthesized to evaluate the binding efficiency on the added sub group. As the fragment molecular weight grows in optimisation the LE will decrease. A drug like compound should have a LE of ~0.3 (Schultes et al., 2010).

3.1.9 Lipinski’s Rule of 5 vs Astex Rule of 3

Lipinski’s rule of 5 is a rule that has been widely used by the pharmaceutical companies to evaluate the drug likeliness of a compound and its oral availability into the human body. The rule was proposed by Christopher Lipinski in 1997 and describes the molecular properties of a compound generally required for good pharmacokinetics in the human body (Lipinski et al., 1997). The four pharmacokinetic parameters are Absorption, Distribution, Metabolism and Excretion (ADME). Lipinski predicts compounds that do not obey the rule of five are more likely to have poor absorption and permeability (Lipinski et al., 1997). In Lipinski’s rule of five it states orally active drugs should match these criteria:
• Molecular weight ≤ 500Da.
• cLogP ≤ 5.
• Number of Hydrogen bond donors ≤ 5.
• Number of Hydrogen bond acceptors ≤ 10.

cLogP is the calculated LogP that stands for the octanol-water partition coefficient and is a value for measuring the compound hydrophilicity. Low hydrophilic compounds have high cLogP values which leads to a poor absorption and permeability (Lipinski et al., 1997).

Compounds that do not match the rule of five, with 10 or fewer rotatable bonds and polar surface <140 Å² are less likely to permeate across the membrane bilayer (Lipinski et al., 1997, Veber et al., 2002).

Most of the HTS compounds were selected based on the Lipinski’s rule of five. However the compounds that match this rule may be more than $10^{60}$ (Congreve et al., 2008) and these compounds are more difficult for optimisation. Therefore, an alternative approach has arisen. The Astex rule of three is a more strict criteria compared to the Lipinski’s rule of five and it is often used in the fragment selection process (Congreve et al., 2003):

• Molecular weight < 300
• Number of hydrogen bond donors ≤ 3
• Number of hydrogen acceptor ≤ 3
• cLogP ≤ 3

Using the rule of three the compounds in the library can be narrowed down to small molecule fragments that are more chemically diverse and less in number. There are around $10^7$ fragments which match the rule of three and this number of compounds is more feasible to be screened and provides a greater chance of finding more diverse hits.
3.2 Virtual screening for MDM2 ligands

3.2.1 Structure model selection

An X-ray protein crystal structure template is vital for computational docking when undertaking structure based virtual screening. An analysis was carried out to review the MDM2 crystal structures available in the PDB website. The structures 3JZK, 3LBK and 3LBL were found to contain chromenotriazolopyrimidine, WK23 and Mi63 analogue bound (Table 3.1). From these three structures 3LBL was selected as the template for virtual screening as it had been refined using the highest resolution data with the lowest R-factor.

<table>
<thead>
<tr>
<th>Structure PDB ID</th>
<th>Ligand name</th>
<th>Ligand structure</th>
<th>Resolution (Å)</th>
<th>R factors-work-free</th>
<th>MDM2 sequence</th>
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</thead>
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<td>0.30 0.32</td>
<td>17-111</td>
</tr>
<tr>
<td>3LBK</td>
<td>WK23</td>
<td><img src="image2" alt="Structure image" /></td>
<td>2.30</td>
<td>0.21 0.25</td>
<td>18-111</td>
</tr>
<tr>
<td>3LBL</td>
<td>Mi63 analogue</td>
<td><img src="image3" alt="Structure image" /></td>
<td>1.60</td>
<td>0.19 0.24</td>
<td>18-111</td>
</tr>
</tbody>
</table>

Table 3.1 Crystal structures of MDM2 with bound small molecules.

An alignment was performed on these three crystal structures to analyze possible side chain movement. Figure 3.7 shows that in the 3LBL structure the Tyr67 has rotated its side chain into the binding site to form a steeper wall. The Tyr67 – His73 loop in 3LBL
folds differently compared to the other two structures which may be caused by the rotation of Tyr67 side chain.

Figure 3.7 Alignment of multi MDM2 crystal structures.
The structure of 3LBL, 3LBK and 3JZK are coloured in green, blue and pink respectively. The Mi63 inhibitor in 3LBL is shown in the binding site and the Tyr67 side chain in 3LBL has rotated into the binding site.

A control docking experiment was carried out using AutoDock and Vina to demonstrate the accuracy of the docking. The control ligand used was Mi63 and the protein model used was the X-ray crystal structure 3LBL pdb file (Figure 3.9). Both AutoDock and Vina have successfully reproduced the crystallographic binding mode of Mi63 with an RMSD of <0.18 Å (Figure 3.8). AutoDock predicted the free energy of binding to be -9.38kcal/mol and Ki of 133nM while Vina predicted the free energy of binding to be -6.1kcal/mol. This is relatively close to the experimentally
measured Ki of 36nM (Popowicz et al., 2010) which was not be expected and may be just a relative trends.

**Figure 3.8 Control docking results of Mi63 in MDM2/p53 binding site.**
Crystallographic pose of Mi63 in yellow stick is compared with the AutoDock docking pose shown in red (Top) and Vina docking pose shown in light blue (Bottom). The MDM2 crystal structure used is the 3LBL pdb structure.
3.2.2 First CODASS run with MDM2

As described in section 3.1.7, 1.1 million compounds for MDM2-N were screened. The script CODASS was used as the tool for screening (Figure 3.5), the X-ray crystal structure from 3LBL pdb file (Popowicz et al., 2010) was used as the protein model and Mi63 inhibitor was used as the template for site points generation around the binding site (Figure 3.9).

Analyzing the MDM2/Mi63 interaction revealed that the three side groups of Mi63 were binding into the three hydrophobic pockets in MDM2 which mimic MDM2/p53 interaction. The 6-chlorooxindole group on Mi63 binds into the Trp23 hydrophobic pocket of MDM2 and the NH on the oxindole group is making hydrogen bond contact with Leu54 residue (yellow dashed line in Figure 3.9). The other two major groups 2-fluoro-3-chlorophenyl ring and neopentyl have filled into the Leu26 and Phe19 hydrophobic pockets in MDM2 respectively. The amide group on the Mi63 also forms hydrogen bond interaction with the His96 imidazole group.

After the CODASS run is complete a ranked list of top 266 compounds was generated. These compounds possess multiple hydrophobic rings due to the fact that the MDM2-N binding site is built up by hydrophobic side chain residues.
The docked poses predicted by AutoDock and Vina showed that these compounds use their hydrophobic rings to bind to the three hydrophobic grooves in MDM2-N. The calculated average AutoDock and Vina scoring energies of these 266 hits are between -7.10 to -10.30 and -7.50 to -10.50 respectively. Ligand efficiencies for these compounds were calculated using equation 3.1 and calculated LE values are between 0.24 to 0.34. These compounds are relatively drug like since the drug like compounds have LE ~0.3 (Schultes et al., 2010).

The 266 top scoring hits from this CODASS run were then screened “visually” by Dr. Simon Pettit (Chemist at Selcia) and 6 compounds were selected (Table 3.2) based on the following criteria:

- Good docking score by both methods.
- Drug like structure: no reactive groups (no acid chlorides, no alkyl halides).
- Not too lipophilic (LogP ≤ 4).
- When several closely related compounds were selected the final selection was made based on commercial availability and one compound was selected to represent the family.

For the 6 compounds in Table 3.2 the poses predicted by AutoDock and Vina (Table 3.2) showed highly similar docking poses with RMSDs within 2Å. The AutoDock and Vina free energy binding calculated for these compounds are between -7.4 to -8.4kcal/mol and -8.2 to -9.3kcal/mol respectively.

Many of these compound structures were composed of multiple hydrophobic rings that dock into the three hydrophobic grooves of MDM2’s binding site. This is relatively similar to the known MDM2 small molecule inhibitors listed in Table 3.1.

Compounds 3 and 4 use a pyrrole and imidazole ring as the core to link with three hydrophobic rings. Compound 6 uses a formaldehyde group and compound 2 uses a urea group to link with three hydrophobic rings. These six compounds shared different chemical properties and compounds 1 and 2 appeared more novel and possess little similarities compared to the known inhibitors (Table 3.1).

Subsequently these compounds were validated using FP and CE assays (Section 4.7.3, Section 5.4.1.4). The FP and CE assays results showed 5 out of 6 as active in the competitive binding assays (Discussed in Section 4.7.3 and Section 5.4.1.4).
<table>
<thead>
<tr>
<th>Docked poses from AutoDock and Vina</th>
<th>Chemical structure</th>
<th>Vina (kcal/mol)</th>
<th>AD (kcal/mol)</th>
<th>Xscore</th>
<th>Drugscore</th>
<th>% Inhibition using CE</th>
<th>% Inhibition using FP</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Chemical Structure" /></td>
<td>-9</td>
<td>-8.44</td>
<td>6.69</td>
<td>-423197</td>
<td>40% at 300µM</td>
<td>25% at 500µM</td>
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</tr>
<tr>
<td><img src="image2.png" alt="Chemical Structure" /></td>
<td>-9</td>
<td>-8.37</td>
<td>7.48</td>
<td>-427206</td>
<td>42% at 300µM</td>
<td>18% at 500µM</td>
<td></td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical Structure" /></td>
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<td>-7.79</td>
<td>7.61</td>
<td>-389617</td>
<td>40% at 300µM</td>
<td>37% at 500µM</td>
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</tr>
<tr>
<td><img src="image4.png" alt="Chemical Structure" /></td>
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<td>-7.42</td>
<td>7.48</td>
<td>-393773</td>
<td>13% at 50µM</td>
<td>0% at 50µM</td>
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</tbody>
</table>
Table 3.2 Ligands docking poses predicted by AutoDock and Vina are presented in the table.

Ligands coloured in purple represent docking by AutoDock and ligands coloured in light blue are docked by Vina. The chemical structure, Vina score, AutoDock score, X-score and Drugscore are also listed in the table. The compounds in the table are ranked based on the AutoDock score.

### 3.3 Second CODASS screening with MDM2 and MDMX

#### 3.3.1 Template structure selection:

With the success from the previous virtual screening run which identified 5 hits against MDM2, a new round of CODASS screening was performed on MDM2 and MDMX to explore the possibility of identifying dual inhibitors. In this virtual screening run a reduced complexity subset of EDULISS library was used in order to obtain compounds that are smaller and more soluble. The subset contains a new criteria (MW 200 – 300Da, \(-4 \leq \log P \leq 4\), \(-4 \leq \log S \leq 4\), Rotatable bond \(\leq 8\), Number of rings \(\leq 4\), Hydrogen bond donor \(\leq 4\), Hydrogen bond acceptor \(\leq 8\), Atoms in largest non-aromatic ring \(\leq 6\)) to filter the EDULISS library. After the screen around 518,000 compounds were identified. A simpler protocol to that described in section 3.6.2 was used where all compounds (518,000) were docked with Vina without the use of Combisearch.
The MDM2 template used in previous CODASS run (Section 3.7.2) was used in this run. The crystal structure 3LBJ (Popowicz et al., 2010) was selected as template for the MDMX run because it was the only crystal structure of MDMX with inhibitor bound (Figure 3.10).

WK298 inhibitor binds to MDMX in a similar position as the p53 peptide with a Ki of 11µM (Popowicz et al., 2010). The 6-chloroindole ring on WK298 is posed into the hydrophobic pocket allowing the NH group to form a hydrogen bond interaction with the carbonyl group of Met53. The other 4-chlorobenzyl ring and the phenyl ring on WK298 both fill into the other two hydrophobic pockets in MDMX.

![Figure 3.10 The binding of WK298 in the MDMX-N binding site.](image)

On the left is the crystal structure of WK298 binding to MDMX-N (3LBJ) and the chemical structure of WK298 is displayed on the right. WK298 interacts with the MDMX-N residues using two intermolecular hydrogen bonds as shown in yellow dashes.

In order to confirm the accuracy of the docking of AutoDock and Vina control docking experiments were performed. The control ligand used was WK298 and the protein model used was the X-ray crystal structure 3LBJ pdb file (Figure 3.10). Both AutoDock and Vina have successfully reproduced the crystallographic binding mode of WK298 (Figure 3.11). AutoDock predicted the free energy of binding to be -
8.33kcal/mol and Ki of 779.48nM while Vina predicted the free energy of binding to be -6.1kcal/mol. It has been noted that AutoDock has docked N,N-dimethylaminopropyl pyrrolidine close to Gln71 residue of MDMX-N for interaction (Figure 3.11 top) and Vina has docked 4-chlorobenzyl group out of Leu26 pocket and interact with Pro95 residue of MDMX-N (Figure 3.11 bottom).

Figure 3.11 Control docking of AutoDock and Vina for WK298 in MDMX-N
WK298 inhibitor is shown as stick, in yellow color is the X-ray crystallographic pose, AutoDock docking pose shown in red (top) and Vina docking pose shown in light blue (bottom).
3.3.2 Comparison of MDM2/MDMX ligand-amino acid interactions

The co-crystal structures of MDMX-WK298 (3LBJ) and MDM2-WK23 (3LBK) were compared. WK23 inhibitor is an analogue of WK298 but in the absence of N,N-dimethylpropylamine (Figure 3.12).

![Chemical structures of WK23 (Left) and WK298 (Right).](image)

WK23 binds to MDM2 in an identical mode as MDMX-WK298 interaction. WK23 and WK298 both occupy the three hydrophobic pockets using the three aromatic groups in the structure. The 6-chloroindole group on WK23 and WK298 forms hydrogen bonds with Leu54 on MDM2 and Met53 on MDMX respectively.

The two main differences between MDM2 and MDMX are the substitution of Leu53 to Met53 and His96 – Tyr100 helix to Pro95 – Tyr99 helix. These differences resulted in the reduced size in MDMX Leu26 binding pocket and possibly weakens the affinities for the inhibitors (Figure 3.13).
Figure 3.13 Comparison of MDMX-WK298 (3LBJ) structure with MDM2-WK23 (3LBK) structure.

WK298 inhibitor is coloured in salmon stick and WK23 inhibitor is coloured in yellow stick. MDM2 structure is shown as light blue ribbon and MDMX structure is shown as green ribbon. The WK298 and WK23 inhibitors bind to MDMX and MDM2 in an identical mode despite the structural differences between MDM2 and MDMX.

It has also been discovered that the Leu26 binding pocket of MDMX experiences induced fit changes upon binding to the compound (Popowicz et al., 2010) (Figure 3.14). When the p53 peptide bound MDMX (2Z5S) is superimposed with WK298 bound MDMX (3LBJ) there are several residues that retract to enable the 6-chlorobenzyl ring to enter (Figure 3.14). The residues Met53, Leu56, Leu98, Tyr99 and Leu102 have retracted to allow binding of WK298 in the binding pocket. However this makes the pocket more shallow and results in WK298 binding more weakly in MDMX (Ki = 11µM) than MDM2 (Ki = 109nM) (Popowicz et al., 2010).
MDMX-p53 peptide complex structure is coloured in green and MDMX-WK298 complex structure is coloured in pink. WK298 inhibitor is coloured in light blue stick. Met53, Leu56, Leu98, Tyr99 and Leu102 are retracted to allow the inhibitor to enter the pocket.

**3.3.3 Results from CODASS screen:**

When analysing the binding energies of the compounds between MDM2 and MDMX, the binding energies are generally weaker when binding to MDMX than MDM2. In MDM2 the AutoDock and Vina scoring energies are between -7.2 to -10.02kcal/mol and -8.0 to -9.7kcal/mol. In MDMX the AutoDock and Vina scoring energies are between -6.7 to -8.6kcal/mol and -6.8 to -8.2kcal/mol. There is ~2kcal/mol energy difference between MDM2 and MDMX indicating the compounds bind weaker to MDMX.
The result list contains 1000 compounds from various suppliers and initially these compounds were filtered based on the following two criteria:

- Present in Chembridge supplier catalogue.
- Able to bind to both MDM2 and MDMX binding site.

The reason for choosing compounds from only Chembridge is to reduce the cost and purchase time. However the downside is that this method may miss out some potential hits.

After the initial filter there are 140 compounds from Chembridge and 61 compounds bind to both MDM2 and MDMX. These compounds were then screened visually and 7 compounds were chosen using the same criteria listed in Section 3.2.2.

The docking poses of the ligands in MDM2 and MDMX binding sites were shown (Table 3.3) and the free binding energies and predicted Ki were also listed. The predicted Ki of these compounds are generally more potent in MDM2 than MDMX. Interestingly, almost all the compounds identified in this virtual screening do not contain the imidazoline core to link three hydrophobic rings. These compounds shared little similarity with the compounds in the first virtual screening (Section 3.2.2) as well as the known MDM2 inhibitors. These compounds were subsequently measured in the FP and CE assays to confirm the interaction (Section 4.9, Section 5.4.1.7 and Section 5.4.2.3). The assays have confirmed that 6 out of 7 as hits and in particular compound 2 which has been shown to bind to both MDM2 and MDMX in the assays (Discussed in Section 4.9, Section 5.4.1.7 and Section 5.4.2.3).
Table 3.3 CODASS screen against MDM2 and MDMX.

The listed compounds in the table are ranked based on the AutoDock score of MDM2. The compounds in purple coloured sticks are docked using AutoDock and in light blue coloured sticks are docked using Vina.
3.4 Discussion

This chapter describes the result of two different CODASS virtual screening runs. The first using Oprea’s lead like criteria (MW <450Da) contains 1.1million compounds. The second run used the reduced complexity (fragment-like) library of 518,000 compounds with MW <300Da. These two runs identified very different molecular scaffolds as the best scoring hits. The Oprea hits showed a number of multiple-ring compounds. Some of the most active (Table 3.2) have a 5-membered ring core motif which is similar to that found in the Nutlin family of MDM2 inhibitors. Of the top six experimental hits, four have a 5-membered heterocyclic core motif. There is a good correlation between the ranked poses for AutoDock/Vina and the experimental hits and the top 3 scoring hits have the best percentage inhibition values as measured by CE (Table 3.2).

For the second CODASS run, the reduced complexity hits showed a more flexible selection of compounds compared to the top hits from the first set and showed little similarity to the currently known Nutlin-like MDM2 inhibitors. In this second set the top hits are generally more active with a top activity of 72% inhibition at 50µM. However there is poorer correlation between the ranked poses for AutoDock/Vina and the experimental hits. The top scoring hit (-8.4kcal/mol) gives 58% inhibition as measured by CE while the best experimental value of 72% inhibition for 7 (Table 3.3) only has an AutoDock score of -7.3kcal/mol.

The AutoDock poses for series one has range of scores between -7.4 to -8.4kcal/mol. As only 6 compounds were selected for testing it is not possible to draw a firm conclusion correlating AutoDock score and measured binding affinity. For series two the range of scores for the best hits are -7 to -10kcal/mol which fits with the generally better measured inhibition values for this second series.

In each case (as part of the CODASS routine) there is an excellent agreement between the Vina and AutoDock poses for each compound (RMSD ≤2Å) as can be seen from the closely overlaid final poses from Vina and AutoDock (Table 3.2 and Table 3.3). The 3 hydrophobic pockets of MDM2-N (Phe19, Trp23 and Leu26 pockets) are to a greater degree filled by the compounds selected. In the first series, compound 1 and 3...
have a hydrogen bond interaction with Val93 residue. This additional hydrogen bond interaction may explain the higher inhibition shown experimentally for these compounds. For the second series, only compound 7 has a hydrogen bond interaction with Leu54 residue which again may explain the high inhibition found in the CE assay. In the second CODASS run the reduced complexity library was also screened against MDMX protein in parallel with MDM2 in order to identify dual action inhibitors. The AutoDock pose for binding to MDMX has range of scores between -6.7 to -8.6 kcal/mol which is relatively lower than the AutoDock scores for MDM2 interaction (-7 to -10 kcal/mol). The compounds selected for testing do not fill all the hydrophobic pockets in the MDMX template, in fact five out of seven compounds only interact with the Phe19 and Trp23 pockets (Table 3.3). This may be because the MDMX Leu26 pocket is more shallow compared to the MDM2 Leu26 pocket. This constrasts with the same compounds docked into the MDM2 template where in every case (except compound 7) the binding pose fills each of the available pockets. It has been noted that Compound 1, 3, 4 and 5 form hydrogen bond interactions with Gln71 residue in MDMX which is absent in MDM2. This difference in amino acid sequence at the p53 binding site may also explain observed experimental differences in ligand binding. It is however difficult to draw a broad conclusion about the prediction of MDM2 and MDMX binding specificity as the MDMX CE assay is not fully optimised and our test set of compounds are too small to provide reliable correlations between AutoDock/Vina ranked poses and experimental results.
Chapter 4 Development of a fluorescence polarization screening assay for MDM2 inhibitors

4.1 Introduction

The theory of Fluorescence Polarization was first described by Perrin in 1926 and it was first introduced in biological assays by Dandliker in 1970 (Dandliker and de Saussure, 1970, Perrin, 1926). The FP anisotropy assay is based on observing the polarized fluorescence of a fluorescently labelled small molecular weight fluorophore.

The fluorescence polarization (FP) assay is a homogeneous versatile solution based assay that allows rapid and quantitative analysis of the receptor/ligand interaction. The FP assay was chosen as the compound screening method because it offers numerous advantages in studying protein-ligand binding. It is cheap, easy to set up and it is able to screen compounds in a 384 well plate format at high throughput (Owicki, 2000). The FP assay has a lower limit of detection in the sub nanomolar range (Invitrogen, 2006). Binding assays can be carried out to obtain the Kd of labelled peptide and competition assays can also be applied to obtain IC\textsubscript{50} and Ki values of the compounds.

4.1.1 The principle of Fluorescence Polarization

When plane polarized light excites a “stationary” fluorescent molecule the light will emit in the same polarized plane. However if the molecule rotates out of the plane during excitation then the light will emit in a different plane. The emitted light can be monitored in vertical and horizontal planes. The difference in the emission intensity from the vertical and horizontal planes is dependent on the rotational speed of the fluorescent molecule.

Fluorescence polarization is therefore defined by the equation:

\[ P = \frac{I - I \perp}{I + I \perp} \]

I = Intensity with polarizer parallel
I\perp = Intensity with polarizer perpendicular
The schematic diagram in Figure 4.1 describes the principle of fluorescence polarization. When excited by polarized light the fluorophore will absorb the photon and undergo rapid tumbling motion and emit light. For low molecular weight compounds emitted light will be largely depolarized due to rapid rotation. When the fluorophore is bound to a high molecular weight protein the rotation speed will be reduced and polarized light will be emitted.

![Figure 4.1 Schematic depicting the FP differences between small molecule and large complex.](image)

Fluorophore undergoes rapid rotation when excited by the polarized light. The emitted light is largely depolarized during unbound state. Upon binding to large molecules the rotation speed of the fluorophore is reduced and polarized light is emitted.

**4.1.2 Competition FP assay**

Many papers and reviews have described the development of FP assays in detecting protein-ligand interactions (Burke et al., 2003, Parker et al., 2000). The competition assay depends on measuring the fluorescence polarization of a labelled substrate molecule in the presence and absence of an (unlabelled) inhibitor molecule. Figure 4.2 shows a schematic diagram of FP competition. When the fluorescently labelled peptide is bound to a protein it emits a significant degree of FP signal due to the large mass and slow tumbling rate of the protein ligand complex. Ligand interaction with the protein can be detected by measuring the decrease of FP signal when the ligand
competes the fluorescent peptide out from the protein. If there is no interaction then the FP signal from the fluorescent peptide bound to the protein will remain unchanged.

**Figure 4.2 Schematic diagram of competition FP assay.**
The tumbling rate of fluorescent peptide (p53-F) was monitored. Tumbling rate is reduced when interacting with high molecular weight protein MDM2-N.

### 4.2 Materials and Methods

In the FP assay the fluorescently labelled p53 peptide (p53-F) used was identical to that described in the paper from C. Brown (Brown et al., 2010). The p53-F peptide (I) was purchased from Biomatik Ltd. and fluorescein amidite (FAM) (II) was labelled at the N-terminus of the peptide.

**Fluorescently labelled p53 peptide (p53-F)** (I)
5-FAM-QETFSDLWKLLP-OH
The assay was originally carried out on a SpectraMax M5 multimode plate reader but was replaced with the LJL Biosystems Analyst AD multi-plate reader due to the poor sensitivity. The Biosystems Analyst multi-plate reader contains a dichromatic mirror for fluorescein filter and has the power to reduce the background noise.

The FP assay standard operation procedure (SOP) can be found in the Appendix A2

**Fluorescence anisotropy**

Polarization and anisotropy values are both derived from measured parallel and perpendicular intensities. The values can be interconverted.

$$A = \frac{I-I\perp}{I+2I\perp}$$  \hspace{1cm} \textbf{Equation 4.1}

$A$ = Fluorescence anisotropy (or “experimental anisotropy”)

$I$ = Intensity with polarizer parallel

$I\perp$ = Intensity with polarizer perpendicular
**Kd Equation**

At equilibrium, the law of mass action states:

\[
K_d = \frac{LF \times RF}{B} \quad \text{Equation 4.2}
\]

Where LF is the unbound ligand, RF is the unbound receptor and B is the Receptor: Ligand complex. The equation can be rewritten as:

\[
K_d = \frac{LF \times (RT-B)}{B} \quad \text{Equation 4.3}
\]

RT = The total receptor concentration  
RT – B = Free receptor concentration

In the experiment, the amount of bound ligand is only a small portion of the total ligand concentration. The total ligand concentration can therefore be used to calculate the free ligand concentration. The equation can be rearranged and substitute LF with (LT – B):

\[
B = \frac{RT \times (LT-B)}{Kd+(LT-B)} \quad \text{Equation 4.4}
\]

Using the quadratic to solve B, the equation is rearranged to:

\[
B = \frac{(LT+Kd+RT) - \sqrt{(RT+Kd+LT)^2 - 4LTxRT}}{2} \quad \text{Equation 4.5}
\]

To use the anisotropy data directly, equation 4.5 was used to substitute B:

\[
B = LT \times \frac{Af}{Ab-Af} \quad \text{Equation 4.6}
\]

LT = Total concentration of ligand  
Af = Anisotropy of free ligand  
Ab = Anisotropy of bound ligand
\[ A = A_0 + (A_b - A_0)(K_d + [L]_t + [P]_t) - \sqrt{\frac{(k_d+[L]_t+[P]_t)^2-4[L]_t[P]_t}{2[L]_t}} \]  

Equation 4.7

\( A_0 \) = anisotropy of free p53-F 

\( A_b \) = anisotropy of p53-F bound to protein 

\([L]_t\) = Total p53-F concentration 

\([P]_t\) = Total protein concentration 

Where \( K_d \) is defined as the concentration of protein at which 50% of the protein is occupied.

**IC\textsubscript{50} Equation**

The titration curve was fitted with log (inhibitor) vs. response – variable slope (four parameters) using GraphPad Prism 5 (GraphPad software, San Diego California USA, www.graphpad.com).

\[ Y = \frac{Bottom+(Top-Bottom)}{(1+10^{(\log IC_{50}-X)\cdot HillSlope})} \]  

Equation 4.8

\( X \) = Concentration of compound 

Bottom = anisotropy of free p53-F 

Top = anisotropy of p53-F bound to protein 

Hill Slope = Slope factor

As shown in Figure 4.3.

**Ki Equation**

The Cheng Prusoff relationship was applied to calculate \( Ki \) from the IC\textsubscript{50} (Lazareno and Birdsall, 1993).

\[ Ki = \frac{(IC_{50}-(\frac{[P]}{K_d}))}{(1+([L]/K_d))} \]  

Equation 4.9

\([P]\) = Protein concentration. 

\([L]\) = The concentration of p53-F. 

\( K_d \) = The dissociation constant of the p53-F from the protein.
4.3 Development of an FP binding assay for MDM2-N

A Fluorescence Polarization assay was set up for screening compounds on MDM2-N. In order to implement the assay a fluorescently p53 labelled peptide (p53-F) that binds to MDM2-N was selected. There are a number of references that use fluorescence polarization assays to monitor binding of ligand with MDM2-N (Lai et al., 2000, Brown et al., 2010, Zhang et al., 2004). In these references the labelled peptides (p53-F) bind to the N-terminal pocket in MDM2 with nanomolar affinity (Table 4.1); this is too tight to monitor weak fragment binding. Only the p53-F ligand in Brown et al. 2010 has a suitably low affinity of 1.1µM. This p53 derived peptide was labelled with fluorescein FAM dye at the N-terminal of the sequence (FAM)-QETFSDLWKLLP (Brown et al., 2010).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Kd</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Phe-Arg-Dpr-Ac6c-(6-Br)Trp-Glu-Glu-Leu-NH2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With FAM label</td>
<td>2nM</td>
<td></td>
</tr>
<tr>
<td>With Cy3B label</td>
<td>3nM</td>
<td></td>
</tr>
<tr>
<td>With Texas Red-X label</td>
<td>20nM</td>
<td></td>
</tr>
<tr>
<td>With Cy5 label</td>
<td>5nM</td>
<td></td>
</tr>
<tr>
<td>5-FAM-LTFEHYWAQLTS</td>
<td>5.6nM</td>
<td>Czarnar A. et al. 2009</td>
</tr>
</tbody>
</table>

Table 4.1 List of different labelled peptides synthesised for MDM2-N FP assay in different literatures.

The equilibrium binding of FAM labelled peptide with MDM2-N protein was carried out by adding increasing concentrations of MDM2-N protein to a fixed concentration of labelled peptide. The excitation/emission and cut-off used were 494/530nm and 515nm which were determined in the excitation and emission scan for FAM labelled peptide. These experiments were carried out using three different fixed concentrations of labelled peptide. 60nM, 120nM and 200nM of FAM labelled peptide were used to determine Kd values and to find the optimal concentration for FAM labelled peptide. Each of these three assays were repeated three times and the Kd values were then determined from experimental data (Figure 4.4). The Kd value calculated using three
concentrations of peptide were reasonably consistent. 60nM labelled peptide gave a Kd of $0.59\mu M \pm 0.25$, using 120nM gave a Kd of $0.7\mu M \pm 0.43$ and using 200nM gave a Kd of $0.68\mu M \pm 0.38$ (Figure 4.4).

In conclusion, different concentrations of p53-F yielded very similar Kd values and all lie within the experimental error range. Therefore 60nM concentration for FAM labelled peptide was used for further FP assay measurements.

Figure 4.4 60nM, 120nM and 200nM FAM labelled peptide binding curves. Excitation 494nm, Emission 530nm and cut off at 515nm. The assay was repeated three times and the Kd from the three repeats were averaged to be $0.59\mu M \pm 0.25$, $0.7\mu M \pm 0.43$ and $0.68\mu M \pm 0.38$. 
4.4 Development of an FP competition assay

Unlabelled p53 peptide titration provides a control for the competition assay:
The unlabelled p53 peptide can also bind to MDM2-N protein and was used for developing the competition binding assay to identify active compounds. The assay works by measuring the decrease of anisotropy caused by a ligand displacing the labelled peptide. Different concentrations of unlabelled p53 peptide were titrated into wells containing a fixed concentration of p53-F and MDM2-N. A solution of 60nM p53-F and 5µM MDM2-N in a total volume of 50µl was chosen for the FP competition assay. In the displacement curve the initial anisotropy value corresponds with fully bound p53-F, and as the unlabelled p53 peptide concentration increases the p53-F is displaced and anisotropy decreases. However the curve in Figure 4.5 shows there was a trend of increased anisotropy when the unlabelled p53 peptide was over 250µM. It is likely that this increase of anisotropy is caused by formation of oligomer or dimer of p53-F.

![Unlabelled p53 peptide titration](image)

Figure 4.5 Unlabelled p53 peptide titration.
The titration results in an increase in the anisotropy when the unlabelled p53 peptide was above 250µM.
A control was performed to confirm if there was any non-specific interaction. This was done by titrating unlabelled p53 peptide against a fixed concentration of p53-F. If the unlabelled p53 peptide has no effect on p53-F then there will be no change in anisotropy, however, when the unlabelled p53 peptide concentration increased, a decrease of anisotropy was found (Figure 4.6). The anisotropy decreases immediately when unlabelled p53 peptide concentration increases and the anisotropy started to increase again as unlabelled p53 peptide concentration increased. The unexpected and dramatic decrease in anisotropy at low concentration could possibly be explained by oligomerization or aggregation of labelled and unlabelled peptides. However subsequent experiments showed that the absorption of peptide onto the surface of the polystyrene well also affected the anisotropy measurement.

Figure 4.6 Control experiment of unlabelled p53 peptide with p53-F.
FP assay control was performed by titration of unlabelled p53 peptide against a 60nM solution of p53-F peptide.
4.4.1 Effect of detergent on the FP assay

The effect of Tween-20 on peptide solubility:

To address the problem of increased anisotropy at higher concentration of p53-U (as seen in Figure 4.6), detergent was added to help improve solubility and help prevent dimerization or aggregation. To determine the effect of Tween-20 on p53-F different concentrations of Tween-20 were added from 0% - 0.25% into wells containing 60nM of p53-F. On addition of Tween-20 a decrease in anisotropy can be seen. From the bar chart in Figure 4.7, in the presence of Tween-20 the anisotropy is lower than without Tween-20 suggesting Tween-20 is acting to disrupt the dimerization or oligomerization of the p53-F. The anisotropy decreases to a value of 0.041 at 0.0125% Tween-20.

![Figure 4.7 FP assay in addition of Tween-20.](image)

Different concentrations of Tween-20 were added into the assay to compare the change in anisotropy.

The critical micelle concentration for Tween-20 is 0.07%. For all subsequent assay measurements a 0.02% concentration of Tween-20 was included.

Repeating the original control experiment in the presence of Tween-20 (Figure 4.8) does not show the anomalous drop in anisotropy (Figure 4.6). Using the same conditions but in the presence of 0.02% Tween-20, the anisotropy stays constant at ~0.04. This suggests that Tween-20 has helped to solubilise the p53-F.
Figure 4.8 Control of FP assay in the presence of 0.02% Tween-20.

A control experiment was carried out on unlabelled p53 peptide with p53-F in the presence of 0.02% Tween-20. The plot showed no change in the anisotropy on titrating the unlabelled peptide when Tween-20 was present.

The effect of Tween-20 on the FP competition assay:

The unlabelled p53 peptide titration was repeated with addition of 0.02% Tween-20. Figure 4.9 shows the curve of the unlabelled peptide titration and, in contrast with Figure 4.5, no increase of anisotropy was observed as peptide concentration increased. The IC$_{50}$ and Ki of unlabelled peptide binding to MDM2-N was calculated using equation 4.8 and 4.9 respectively and IC$_{50}$ was 23.52µM and the Ki was 18µM. The published IC$_{50}$ of p53 peptide using a similar sequence was 2µM (Phan et al., 2010). The calculated IC$_{50}$ was ten times weaker than the published value but p53-U is one amino acid longer than the p53 peptide used by Phan et al.

<table>
<thead>
<tr>
<th>P53-U</th>
<th>ETFSDLWKLLPEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phan</td>
<td>ETFSDLWKLLPE</td>
</tr>
</tbody>
</table>
Figure 4.9 FP assay titration curve of unlabelled p53 peptide on addition of 0.02% Tween-20.
The concentrations of p53-F and MDM2-N used were 60nM & 5µM respectively. Tween-20 has solubilised the p53-F and there was no increase of anisotropy at higher concentration of unlabelled p53 peptide.

4.5 Optimising the FP assay using the LJL Biosystem Analyst Multi-plate reader

4.5.1 Determining the optimised labelled peptide concentration for the assay

To improve the sensitivity of the FP assay the protein concentration has to be decreased which means that the p53-F concentration also has to be decreased. The signal in the FP assay depends on the amount of p53-F present. Therefore the lowest concentration of p53-F that can be used has to be assessed.

In order to reduce the p53-F concentration another multi-plate reader was tested. The LJL Biosystem Analyst Multi-plate reader was used. The machine has an optimal signal range between 156,000 to 31,200,000 counts per second. 384 well plates were used for setting up the assay. The total volume in each well is 30µl, so lower volume samples were needed for setting up the assay. The read out from the machine is in counts per second (cps).
The fluorescence signals (ex 485/ex 530) from different p53-F concentrations were examined. A steady increase of signal can be seen when increasing the labelled peptide concentration (**Figure 4.10**). At 5nM concentration the signal has more than 2 million cps which is in the optimal signal range and can be used to test in the FP assay (**Figure 4.10**). If the signal is too low then background noise will interfere with the signal.

![Fluorescence signal at different labelled peptide concentrations](image)

**Figure 4.10** Plate reader output as a function of p53-F concentration.
Labelled peptide concentrations tested are from 5nM – 30nM. An increase of signal can be seen when concentration is increased. The signals from these concentrations were in the optimal signal range of the machine.

### 4.5.2 Determining the optimum MDM2-N concentration for the FP assay

To optimise the sensitivity of the FP assay it is generally desirable to use a p53-F concentration as low as possible, but in the range for the instrument detector. At 5nM p53-F there was background in the anisotropy readings. The noise was subsequently reduced when the p53-F concentration was increased to 8nM (**Figure 4.11**) and this concentration was selected for further assay work.

To determine the optimum MDM2-N concentration to use for the FP competition assay binding experiments were performed in the presence and absence of Nutlin-3. The
binding assay in the presence of Nutlin-3 shows the competition of Nutlin-3 and p53-F and decreases caused by competing out bound p53-F. When compared with the control binding curve without Nutlin-3 an optimal protein concentration was calculated by looking at the gap between the two curves. The curves fitted in Figure 4.12 show that the optimum protein concentration for seeing the displacement of the curve in the competition assay is 0.5µM.

Figure 4.11 FP binding assay of MDM2-N against 8nM p53-F probe

Each experiment point was measured in triplicate. Three independent curves are shown to provide a measure for the reproducibility of the assay.
Figure 4.12 FP binding assays of MDM2-N with ± Nutlin-3 (5µM).
The curves are measured in the presence (blue line) and absence (red line) of Nutlin-3. Optimal MDM2-N concentration to use for competition assay was determined as 0.5µM.

### 4.5.3 Comparison of His6-tag-MDM2-N and Untagged-MDM2-N

In the construct there is a His6-tag located at the N-terminal of MDM2-N which is close to the MDM2-N/p53 binding site. Therefore it is crucial to confirm if there is any effect on the MDM2-N binding. His-tag cleaved MDM2-N was tested in the FP binding assay with p53-F to compare the Kd obtained from with the His-tag MDM2-N. His6-tag cleaved MDM2-N was diluted into different concentrations and titrated into wells with 8nM p53-F. The assay was done in triplicate and the curve was fitted using equation 4.7. The average Kd for the p53-F with tag cleaved MDM2-N was calculated to be 0.25µM ± 0.02 (Figure 4.13A). The Kd for p53-F when running with tagged MDM2-N was 0.34µM ± 0.02 (Figure 4.13B).

Comparing the FP assay results of tag cleaved MDM2-N and un-cleaved MDM2-N shows that there is no significant difference between cleaved and un-cleaved MDM2-
N. This indicates the His-tag at the N-terminal of MDM2-N does not affect labelled peptide or small molecules binding to the binding site.

Figure 4.13 FP binding assays of p53-F against His-tag MDM2-N and untagged-MDM2-N.

The binding assays of untagged-MDM2-N (A) and His-tag MDM2-N (B) showed no significant difference in the Kd obtained.

4.5.4 Nutlin-3 titration against MDM2-N

Nutlin-3a is the first known inhibitor for MDM2 and has been commonly used in the biochemical and cell based studies. Nutlin-3a has an affinity of IC$_{50}$ = 90nM and a Ki = 36nM (Shangary et al., 2008, Vassilev et al., 2004) as measured by SPR and fluorescence polarization studies respectively. Initially in our studies we used relatively high concentrations of MDM2-N (5µM) and p53-F (60nM). With these high concentrations it was not possible to obtain a complete titration curve. The assay was optimised and the concentrations of both MDM2-N and p53-F could be significantly reduced to 500nM and 8nM respectively. The Nutlin-3 titration curve using these values gave a good sigmoidal curve (Figure 4.14) with an IC$_{50}$ value of 1.18µM and Ki = 0.91µM. However the affinity estimated from this titration was 25 times weaker than the published affinity (36nM). This may be due to the weak sensitivity of the FP assay and further optimisation was carried out in section 4.6.
One reason for the discrepancy is due to the fact that Nutlin-3 from Sigma Aldrich (Cat No. N6287) which was used in our assay is a racemic mixture. This means that both active Nutlin-3a and inactive Nutlin-3b are present in equimolar amounts. Interestingly, there was a published paper (Bernal et al., 2010) that also used racemic Nutlin-3 in an FP assay and the IC\textsubscript{50} obtained from this work was 2.11\,\mu M, a value very similar to that obtained in our work.

Another possible reason for the discrepancy between the low published IC\textsubscript{50} value of 90\,nM is the presence of the His\textsubscript{6}-tag located near the MDM2-N/p53 binding site. In order to examine the effect of this tag on Nutlin-3 binding the protein was treated with thrombin to provide a tag-free construct (as described in Chapter 2, section 2.2.1.4). The untagged MDM2-N resulted to an IC\textsubscript{50} of 1.8\,\mu M (Figure 4.15). This is nearly identical to the Nutlin-3’s affinity with His-tagged MDM2-N and suggests that the His-tag near the MDM2-N/p53 binding site has no effect on the small molecule binding. In all subsequent work the assays were performed using His-tagged-MDM2-N.
4.6 Optimising the FP assay using siliconised equipment

Comparison of two CE runs showed that when unsiliconised tips were used the limit of detection for p53-F peptide was 250nM. When siliconised tips were used the limit of detection was reduced to 500pM. This suggested that p53-F was sticking to the unsiliconised tips and the effective concentration was reduced by a factor of ~1000.

To confirm this effect an experiment was carried out to compare fluorescence after pipetting p53-F into a siliconised plate using a siliconised tip (Figure 4.16). For this experiment a non-binding surface plate (NBS) (Corning cat No: 3820) was used. Absorption of p53-F peptide onto unsiliconised plate and tips strongly reduces the fluorescent signal.
Figure 4.16 Fluorescence of p53-F was measured.
Serial dilution of p53-F with ± siliconised plate/tips and measured fluorescence in Analyst multiplate reader.

4.6.1 Siliconised tips using dimethyldichlorosilane solution
Siliconised tips were identified to be essential in diluting the p53-F. However when setting up the FP assay tips were always used in large quantities and it would have been too expensive to continuously purchase them from the supplier. We developed a way to siliconise tips in-house as described below.
Siliconising the pipette tips was performed in a chemical fume hood. Dimethyldichlorosilane solution was poured into glass bowl and pipette tips were immersed in the siliconising solution. Using the pipette the solution was pipetted into the tips so that the inner surfaces of the tips were fully covered with the siliconizing solution. The tips were taken out from the solution and washed with distilled water then allowed to dry overnight in the fume hood.
In order to compare different siliconised tubes fluorescence measurements on p53-F were carried out by diluting p53-F using commercial siliconised tips, home siliconised tips and normal tips.
From the results in Figure 4.17, it can be seen that there are no significant differences between the fluorescence readings of p53-F diluted using commercial siliconised tips and home siliconised tips. There was a 3 fold difference on the fluorescence of p53-F diluted using normal tips. This verifies that home siliconised tips were also able to prevent p53-F sticking. Therefore home siliconised tips were used for subsequent assay measurements.

**Figure 4.17 Fluorescence of p53-F diluted using different type of tips.**
p53-F was diluted using commercially siliconised, home siliconised and normal plastic tips. Fluorescence of p53-F was measured using NBS plate and expressed in counts per second (CPS).

### 4.6.2 Siliconised equipment allows FP assay using 2nM p53-F
In order to quantify the effect of the p53-F peptide absorption on the plate three binding assays were performed. A lower p53-F concentration of 2nM was used in these trials. The first binding assay in Figure 4.18A was set up by diluting the samples using siliconised tips/vials and measured in the NBS plate. A second binding assay was carried out in Figure 4.18B using siliconised tips/vials and measured in a black polystyrene plate. In the third a control was set up in Figure 4.18C with samples using unsiliconised tips/vials and measured in the polystyrene plate.
As shown in **Figure 4.18A**, the binding assay using siliconised tips/vials and measured in the NBS plate gave a reproducible result. Higher noise was found when setting up the binding assay using siliconised tips/vials in the normal plate (**Figure 4.18B**) and using non-siliconised tips/vials in the polystyrene plate (**Figure 4.18C**). These results show that p53-F binds to the unsiliconised plate. From the binding assay using 2nM p53-F (**Figure 4.18A**) the K_d calculated for p53-F was 0.32µM which was identical to the K_d obtained previously using 8nM p53-F (0.35µM).

This confirms that using siliconised tips with NBS plate can help improve the sensitivity of the FP assay and the p53-F concentration used in the assay can now be significantly reduced.
Figure 4.18 Three binding assays were carried out in different conditions.

A) The assay was performed using siliconised equipment and a non-binding surface plate.
B) The samples were diluted in the siliconised equipment and measured in a polystyrene plate.
C) The samples were diluted using non-siliconised equipment and measured in a polystyrene plate.

4.6.3 Effect of siliconised equipment on Nutlin-3 titration

To validate the effect of siliconised equipment the titration of Nutlin-3 was repeated. Competition assay was done on Nutlin-3 from 0-10µM using the condition of p53-F (2nM) and MDM2-N (500nM). Several publications have suggested that fluorescent compounds can cause interference to the fluorescence based assay leading to false positive or false negative results (Owicki, 2000). This interference was often resolved by using a higher concentration of labelled probe but this will reduce the sensitivity for detecting weak ligand interactions. When using low concentration of labelled probes the sensitivity can be increased but this will decrease the signal and make the measurements more sensitive to any fluorescent background. As a control the fluorescence of each compound is measured at the same concentration (but in the absence of peptide and protein) and this reading is subtracted from the assay measurement.

Nutlin-3 titration was repeated and, after compound fluorescence subtraction, the IC$_{50}$ of racemic Nutlin-3 was found to decrease to 266nM (Figure 4.19) with a Ki
calculated at 264nM. The sensitivity has been greatly improved compared to the Nutlin-3 titration that was carried out when using unsiliconised equipment (IC$_{50} = 1.18 \mu$M) (*Section 4.5.4*). However the IC$_{50}$ of enantiomeric Nutlin-3a from the literature is 90nM (Vassilev et al., 2004). The calculated IC$_{50}$ from the FP assay was $\sim$3 times larger than the literature value and this discrepancy is possibly explained by the fact that the Vassilev study used enantiomerally pure Nutlin-3a, although this would only explain 2 fold increase in IC$_{50}$. The other reason may be that the sensitivity of the FP assay was still not sensitive enough.

![Nutlin-3 in FP competition assay with MDM2-N (500nM)](image)

**Figure 4.19 Nutlin-3 titration with MDM2-N using siliconised equipment.**

The samples were prepared using siliconised tips and vials and titrated into a non-binding surface plate for measurement.

A detailed “SOP” for carrying out a 384 well format FP screen is given in *Appendix A2.*
4.7 FP assay compound screening results

4.7.1 Screening the Selcia fragment library

As described in Chapter 3 fragment based drug discovery is a relatively new approach in developing leads with good drug like properties (Carr et al., 2005, Neidle and Hubbard, 2006). Fragments are small molecular weight molecules and conform to the Rule of three: molecular weight ≤ 300, hydrogen bond donor ≤ 3, hydrogen bond acceptor ≤ 3 and calculated logP ≤ 3 (Congreve et al., 2003).

The Selcia fragment library contains approximately 1400 compounds that have been selected based on the Rule of three criteria. From the analogues screening, there were 14 fragments selected from the library for screening in the FP assay (Table 4.2).

Mi63 and Nutlin-3 inhibitors (Figure 4.20) were analysed and 14 fragments were selected based on the substructures of these inhibitors. For simplicity a name was given for each substructure group, MR1-4 and NR1-4 for Mi63 and Nutlin-3 respectively (Figure 4.20). Table 4.2 shows the structures of these fragments and many of these fragments selected were analogues of the MR3 substructure of Mi63 inhibitor (Figure 4.20). The SFL001328 fragment is an analogue of the MR3 group but without the chlorine atom at 6 position and the SFL001303 fragment has moved the chlorine atom from 6 position to 7 position. To assess if the imidazole ring on the MR3 group has any effect on binding we have replaced the imidazole ring with the pyridine ring on SFL00134 and SFL001397 fragments. In SFL001290, SFL001542, SFL001675 and SFL000829 fragments, we have substituted the chlorine atom on the MR3 group with different substructures. SFL000576 is an analogue of Nutlin-3 at NR3 (Figure 4.20) and this fragment was also selected to confirm the binding.

Figure 4.20 Known inhibitors Mi63 (left) and Nutlin-3 (Right) were used for fragment selection.
### Table 4.2 Fragments selected for FP screening

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Structure</th>
<th>Fragments</th>
<th>Structure</th>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<td>SFL001542</td>
<td><img src="image12.png" alt="Structure" /></td>
</tr>
</tbody>
</table>

### 4.7.2 FP studies on “Selcia fragments”

This initial screen of fragment compounds was carried out before the problem of peptide-absorption onto the plates had been resolved. This assay was therefore less sensitive than in the later work (as described in [Section 4.7.3](#)) as higher concentration of peptide was used.

The FP assay was set up to screen the fragments at 2mM concentration (as described in [Appendix A2](#)). SFL001290 and SFL001669 were insoluble so they had to be screened at lower concentrations. Nutlin-3 (5µM) was used as a positive control to
show full inhibition. Most of the fragments showed ~10% inhibition and SFL001669 showed the highest inhibition of 23% at 250µM (Figure 4.21). The fragments were also screened in CE at Selcia US which confirmed SFL001328 and SFL001669 to bind at 300µM concentration (Results not shown). Thus SFL001669 was the only confirmed hit from the FP assay; the other fragments screened did not show an appreciable drop in anisotropy. SFL001328 was not active in the FP assay when screening at 2mM concentration.

SFL001290 is possibly insoluble at 2mM and has a higher anisotropy than the anisotropy of MDM2-N on its own. This could be caused by the light scatters from particles of partially dissolved compound present in the solution. The relatively weak binding of low molecular weight fragments means that screening for binding needs to be carried out at high concentrations. Only SFL001669 was confirmed binding at 250µM. In the hope of identifying more weak binding fragments, further optimization trials are described in the next section to improve the sensitivity of the FP assay.
Figure 4.21 Screening of Selcia fragments in the competition FP assay.

The fragments were at 2mM concentration and 250µM for SFL001669 and screened in the presence of MDM2-N (0.5µM), p53-F (8nM) and 2% DMSO. Nutlin-3 (5µM) was used as a positive control.

4.7.3 Validating the virtual screening hits in the FP assay

As described in Chapter 3, the CODASS routine was used to rank 1000 hits from a library of 1.1 million compounds. The top 266 ranked hits were examined for substructures of known binding inhibitors. Six compounds (Table 4.3) were selected based on the criteria listed in Section 3.7.2.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>CODASS score/Binding energy</th>
<th>Compounds</th>
<th>CODASS score/Binding energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL101063</td>
<td>-7.42kcal/mol</td>
<td>SEL101067</td>
<td>-8.44kcal/mol</td>
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<tr>
<td>SEL101064</td>
<td>-7.79kcal/mol</td>
<td>SEL101068</td>
<td>-7.38kcal/mol</td>
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<td>SEL101066</td>
<td>-8.37kcal/mol</td>
<td>SEL101069</td>
<td>-7.38kcal/mol</td>
</tr>
<tr>
<td>Nutlin-3</td>
<td></td>
<td>SFL001502</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 The structures of the compounds selected from the CODASS ranked list.
The competition assay for these compounds is described in detail in Appendix 2. Siliconised equipment was used throughout. Nutlin-3 was used as a positive control and SFL001502 was used as a negative control. The virtual screening hits were initially screened at 500µM with 1% DMSO. SEL101063 and SEL101068 were found to be insoluble at 300µM when screening in the CE assay. Subsequently they were screened at 50µM.

The bar chart in Figure 4.22 shows the results on the compound screening. SEL101063 showed small (1-2%) increase in anisotropy which may be due to insoluble particles present in the solution. The poorly soluble compound SEL101068 showed only 3% inhibition which may be due to screening at a low concentration. All other virtual screening hits showed inhibition. From the FP assay screen SEL101064 gave the highest inhibition of 37% at 500µM concentration.

Figure 4.22 Result of FP competition assay on virtual screening hits.

SEL1063 gave an increase of anisotropy which may be due to compound particles present in the solution.
4.8 FP assay studies on MDMX-N

MDMX has also been identified to be a negative regulator of p53 and many studies have demonstrated that MDMX cooperates with MDM2 to ubiquinate p53. The X-ray crystal structure of MDM2 and MDMX showed that they are very similar in the p53 binding site at the N-terminal domain (Popowicz et al., 2010) (Figure 1.11 and Figure 1.20, Chapter 1).

4.8.1 FP binding assay of MDMX-N against p53-F

The MDMX-N protein was initially tested in the binding assay against p53-F to validate that p53-F can also bind to MDMX-N. Several literature studies demonstrated p53 peptide was able to bind to MDMX (Popowicz et al., 2007, Phan et al., 2010). The assay was optimised with siliconised plastic ware (Appendix A2). MDMX-N was titrated in the presence of p53-F (2nM). The results are shown in Figure 4.23. The Kd of the p53-F was calculated as 0.2µM. This result suggests that there is not a significant difference of affinity of p53-F binding to MDM2-N and MDMX-N. This result is in agreement with the results of Popowicz et al. 2007, for the p53 peptide with MDMX-N which gives a Kd value of 0.21µM.

![Figure 4.23 FP binding studies of MDMX-N14-111.](image)

Titration of MDMX-N into the p53-F (2nM). The binding assay was performed twice. Binding affinity of the p53-F to MDMX-N was ~0.22µM.
4.8.2 Nutlin-3 titration against MDM2-N and MDMX-N

Several studies have revealed that the known tight binding inhibitors of MDM2 bind weakly to the MDMX-N due to the differences in the binding site (Shangary et al., 2008, Popowicz et al., 2008). The side chains Met53 and Tyr99 are pointing into the binding site of MDMX-N thus making the p53 binding cleft smaller (See Chapter 1 and Figure 4.24).

Figure 4.24 The difference in the binding site of MDM2-N (top) and MDMX-N (bottom). Surface representations of MDM2-N (PDB: 3LBL) and MDMX-N (PDB: 3DAC). The residues Met53 and Tyr99 on MDMX-N and Leu54 and Tyr100 on MDM2-N are coloured in magentas. Leu26 pocket in MDMX-N is smaller than MDM2-N due to Met53 and Tyr99 side chains pointing into the binding site.
Titration of the racemic Nutlin-3 was performed to compare the affinity with MDM2 and MDMX. The Nutlin-3 titration against MDMX-N was done using the same conditions as MDM2-N (p53-F (2nM), MDMX-N (500nM), 1% DMSO) as described in section 4.6.4.

In this assay Nutlin-3 has a $K_i$ value of 266nM for binding to MDM2-N (Figure 4.25 Top) and a $K_i$ value of 2.8µM for binding to MDMX-N (Figure 4.25 Bottom). This is consistent with Popowicz who reported a $K_i$ of 70µM and $IC_{50}$ of 221µM for binding to MDMX-N (Popowicz et al., 2010). The other reported value is from Shangary et al. with a $K_i$ of 9µM (Shangary et al., 2008). The conclusion is therefore, that Nutlin-3 binds to MDMX-N with an over ten times weaker affinity than binding to MDM2-N.

Figure 4.25 Nutlin-3 titration against MDM2-N (Top) and MDMX-N (Bottom).

Competitive binding of Nutlin-3 to MDM2-N and MDMX-N was carried out to examine the affinities of Nutlin-3 in these two highly similar proteins.
4.9 Virtual screening against MDM2-N and MDMX-N FP assay

Given the success of the previous virtual screening, which resulted in the identification of 5 hits against MDM2, a new round of virtual screening was carried out. It would be therapeutically useful to find inhibitors against both MDM2 and MDMX. A reduced complexity library containing 518,000 compounds were therefore screened against both MDM2-N and MDMX-N. The reduced complexity criteria filters the library to identify compounds with smaller molecular weight and therefore improved solubility. The result of this docking score are summarised in Chapter 3 (Chapter 3). Only the compounds supplied by Chembridge were manually viewed in the PyMOL. The docking poses of these compounds in the MDM2-N and MDMX-N binding site were investigated. To maximise chemical diversity seven compounds were selected that contain different chemical scaffolds and chemical properties.
<table>
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<tr>
<th>Compounds</th>
<th>Structures</th>
<th>Predicted Ki against MDM2</th>
<th>Predicted Ki against MDMX</th>
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<td>1.10 µM</td>
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<td>SEL101881</td>
<td><img src="SEL101881.png" alt="Structure" /></td>
<td>4.41 µM</td>
<td>3.12 µM</td>
</tr>
</tbody>
</table>

Table 4.4 A list of compounds selected from virtual screening.
The compounds were initially screened at a relatively low concentration of 200µM to ensure the compounds did not precipitate in solution. SEL101875, SEL101876 and SEL101880 needed to be further diluted to 50µM due to observed precipitation. The assays were screened at MDM2-N (500nM), MDMX-N (500nM), p53-F (2nM) and 1% DMSO (as described in Section 4.6.4). In the assay Nutlin-3 (1µM) was used as a positive control and the results showed Nutlin-3 (1µM) gave 95% inhibition against MDM2-N whereas only 16% inhibition was found against MDMX-N (Figure 4.26).

The results of this screen showed that only compound SEL101876 gave a relatively high inhibition of 33% at 50µM while other compounds only showed 1% - 12% inhibition. In the screen against MDMX-N, SEL101876 was also identified as having the highest (84%) inhibition at 50µM.

These data were evaluated using the ANOVA statistical analysis (as described in Section 4.2) to determine if effect of the inhibition is significantly different from the control MDM2-N/MDMX-N values. Only SEL101876 showed a significant difference when screening in both MDM2-N and MDMX-N proteins. The other compounds showed no significant difference to the control. Another more sensitive assay using capillary electrophoresis is described in Chapter 5 and validates the SEL101876 hit.
### Dunnett's Multiple Comparison Test

<table>
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<th>Significant?</th>
<th>P &lt; 0.05?</th>
<th>Summary</th>
<th>95% CI of diff</th>
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<td>***</td>
<td>0.04</td>
<td>0.03 to 0.05</td>
</tr>
<tr>
<td>MDM2N vs 1μM Nul lin</td>
<td>0.04</td>
<td>22</td>
<td>Yes</td>
<td>***</td>
<td>0.04</td>
<td>0.03 to 0.04</td>
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<tr>
<td>MDM2N vs SEL101875</td>
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<td>ns</td>
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Figure 4.26 Virtual screening hits were screened against MDM2-N and MDMX-N. 
Top panel) Virtual screen hits were screened in the competition FP assay with MDM2-N and MDMX-N. Nutlin-3 (1µM) was used as a positive control. MDM2-N (0.5µM), MDMX-N (0.5µM) and p53-F (2nM) represents the anisotropy of fully bound and unbound. The compounds were screened at 200µM and 50µM depending on the solubility.
Lower panel) ANOVA statistic analysis was performed using one way ANOVA in GraphPad Prism. \( P < 0.05 \) was considered as significant.
4.10 Discussion

This chapter describes establishing FP as an orthogonal assay to screen weak binding small molecules as protein-protein interaction (PPI) inhibitors. The target of interest is the MDM2-N/p53 interaction and there have been several papers that use FP as a tool to screen for small molecule inhibitors against this interaction (Shangary et al., 2008, Popowicz et al., 2010, Reed et al., 2010). A summary of results generated in this work along with the most relevant published work is given below (Table 4.5). There are three known published peptide inhibitors that bind to both MDM2-N and MDMX-N with affinity range of 0.9nM – 216nM and 2.31nM – 229nM respectively (Bernal et al., 2010, Pazgier et al., 2009, Chang et al., 2013). The affinities of these peptide inhibitors were measured using SPR and FP (Table 4.5). Apart from peptide inhibitors, there are also numerous known published small molecules inhibitors discovered which inhibit MDM2-N and the most well-known inhibitors are listed in Table 4.5 (Vassilev et al., 2004, Shangary et al., 2008, Popowicz et al., 2010, Hardcastle et al., 2011, Allen et al., 2009, Grasberger et al., 2005). The first published MDM2-N small molecule inhibitor is Nutlin with an IC_{50} value of 90nM. Many of the MDM2-N inhibitors use the Nutlin-like concept to fill the three hydrophobic pockets in MDM2-N (Phe19, Trp23 and Leu26 pockets) and have affinities in the range of 3nM – 1.2μM (Table 4.5). Currently there are only two inhibitors that bind tightly to MDMX-N, namely SJ-172550 and RO-2443 with an EC_{50} value of 2.3μM and an IC_{50} value of 41nM respectively (Reed et al., 2010, Graves et al., 2012). SJ-172550 showed a weaker affinity when interacting to MDM2-N with an EC_{50} value of 26μM and only RO-2443 binds potently to both MDM2-N and MDMX-N (Table 4.5). In our work, the first CODASS virtual screening run identified several 5-membered ring compounds that are similar to the known Nutlin-like inhibitors and these compounds bind to MDM2-N with percentage inhibitions between 3% - 37% (Table 4.5).
<table>
<thead>
<tr>
<th>Structures/Sequences</th>
<th>Inhibitor name</th>
<th>Binding affinity with MDM2</th>
<th>Binding affinity with MDMX</th>
<th>Techniques used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nutlin-3a</td>
<td>IC50 = 90nM</td>
<td>Ki = 9µM</td>
<td>SPR</td>
<td>(Vassilev L. et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>MI63</td>
<td>Ki = 3nM</td>
<td>Ki = 55µM</td>
<td>FP assay</td>
<td>(Shangary S. et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>WW298</td>
<td>Ki = 109nM</td>
<td>Ki = 11µM</td>
<td>FP assay</td>
<td>(Popowicz G. M. et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Isoindolinone</td>
<td>IC50 = 170nM</td>
<td></td>
<td>NMR</td>
<td>(Hardcastle I. R. et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>Chromonoisioxyquinolines</td>
<td>IC50 = 1.2µM</td>
<td></td>
<td>FRET</td>
<td>(Allen J. et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>Bentodiazepinedione</td>
<td>Kd = 80nM</td>
<td></td>
<td>ThermoFluor</td>
<td>(Grasberger B.L. et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>SJ-172550</td>
<td>IC50 = 26µM</td>
<td>IC50 = 2.3µM</td>
<td>FP assay</td>
<td>(Reed D. et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>RO-2443</td>
<td>IC50 = 33nM</td>
<td>IC50 = 41nM</td>
<td>TR-FRET</td>
<td>(Graves et al. 2012)</td>
</tr>
<tr>
<td></td>
<td>TSFAEYWNLSP</td>
<td>Kd = 3.36nM</td>
<td>Kd = 4.15nM</td>
<td>SPR</td>
<td>(Pazgier et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>Ac-QSQTPNLWLL*QN-NH2</td>
<td>IC50 = 216nM</td>
<td>IC50 = 122nM</td>
<td>FP assay</td>
<td>(Bernal et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Ac-LTRREYWACNNSAA</td>
<td>Kd = 8.91nM</td>
<td>Kd = 2.31nM</td>
<td>SPR</td>
<td>(Chang et al. 2013)</td>
</tr>
<tr>
<td>Structures/Sequences</td>
<td>Inhibitor name</td>
<td>Binding affinity with MDM2</td>
<td>Binding affinity with MDMX</td>
<td>Assay used</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------</td>
<td>----------------------------</td>
<td>---------------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>SEL-101881</td>
<td>CE = 72% at 50µM</td>
<td>FP = 1% at 200µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>SEL-101879</td>
<td>CE = 59% at 100µM</td>
<td>FP = 0% at 200µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>SEL-101876</td>
<td>CE = 58% at 50µM</td>
<td>CE = 100% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>SEL-101877</td>
<td>CE = 18% at 50µM</td>
<td>FP = 25% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>SEL-101878</td>
<td>CE = 58% at 100µM</td>
<td>FP = 1% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>SEL-101875</td>
<td>CE = 40% at 50µM</td>
<td>FP = 0% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>SEL101066</td>
<td>CE = 42% at 300µM</td>
<td>FP = 18% at 500µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure 8" /></td>
<td>SEL101067</td>
<td>CE = 40% at 300µM</td>
<td>FP = 25% at 500µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image9.png" alt="Structure 9" /></td>
<td>SEL101064</td>
<td>CE = 40% at 300µM</td>
<td>FP = 37% at 500µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image10.png" alt="Structure 10" /></td>
<td>SEL101069</td>
<td>CE = 40% at 300µM</td>
<td>FP = 13% at 500µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure 11" /></td>
<td>SEL101877</td>
<td>CE = 18% at 50µM</td>
<td>FP = 25% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image12.png" alt="Structure 12" /></td>
<td>SEL101063</td>
<td>CE = 13% at 50µM</td>
<td>FP = 0% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image13.png" alt="Structure 13" /></td>
<td>SEL101068</td>
<td>CE = 0% at 50µM</td>
<td>FP = 3% at 50µM</td>
<td>CE assay</td>
<td></td>
</tr>
<tr>
<td><img src="image14.png" alt="Structure 14" /></td>
<td>SEL-101880</td>
<td>CE = 0% at 100µM</td>
<td>FP = 5% at 200µM</td>
<td>CE assay</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Summary table of known published MDM2 and MDMX inhibitors along with the compounds screened in this work.
Development of the FP assay: Numerous published papers use an FP assay for screening inhibitors of MDM2, therefore this assay becomes an ideal technique for validating the hits in this work (Shangary et al., 2008, Popowicz et al., 2010, Reed et al., 2010). Initially there was a problem with the low fluorescent signal when running the FP assay. This was found to be caused by p53-F sticking to the plastic tips and Eppendorf tubes which resulted in the use of high concentrations of p53-F and MDM2-N and thus greatly reduce the sensitivity of the assay. This problem was overcome by using siliconised tips/tubes in order to prevent absorption of p53-F to the tips/tubes. This change in plasticware allowed us to greatly reduce the p53-F concentration by a factor of ~1000 and hence significantly improved the sensitivity of the assays. This improved assay gave excellent and consistent results which matched the published values for standard compounds. For example the published values for Ki and IC₅₀ for Nutlin-3 using an FP competition range from 36nM to 2.11µM (Shangary et al., 2008, Bernal et al., 2010, Popowicz et al., 2010). This confers with a value of 266nM from our FP competition assay (using racemic Nutlin-3a and Nutlin-3b mixture). The binding data obtained from our FP is also found to be comparable with the Ki and IC₅₀ values determined using other techniques including SPR, ITC and FRET which gave values for Nutlin-3 between 23nM to 263nM (Vassilev et al., 2004, Pazgier et al., 2009, Allen et al., 2009).

Studies from several published papers have shown that MDMX is also a negative regulator of the tumour suppressor p53 protein (Marine and Jochemsen, 2005, Marine et al., 2007). There are a few peptides and small molecules in the literature which show dual activity against both MDM2 and MDMX (Bernal et al., 2010, Chang et al., 2013, Pazgier et al., 2009, Graves et al., 2012). The binding data presented in Table 4.5 shows significant differences in affinities for the known inhibitors when binding to MDM2 and MDMX. These Nutlin-like inhibitors bind more weakly to MDMX (affinities range of 9 - 55µM) compared with MDM2 (affinities range of 3nM – 1.2µM) which is an average reduction of ~99%. In order to identify small molecule inhibitors to inhibit both MDM2-N and MDMX-N an FP assay was developed for screening compounds on MDMX-N/p53 interaction. Initially, titrations of Nutlin-3 with MDM2-N and MDMX-N were performed to compare the affinity with MDM2-N and MDMX-N...
The Nutlin-3 titrations resulted in Ki values of 266nM and 2.8µM against MDM2-N and MDMX-N respectively. This indicates that Nutlin-3 binds to MDMX-N ten times more weakly than MDM2-N. The results are in agreement with the findings from Shangary S. et al. and Popowicz G. M. et al. where Ki values of 9.3µM and 70µM are determined for the interaction of Nutlin with MDMX-N (Shangary et al., 2008, Popowicz et al., 2010). As can be seen from Figure 4.24, MDM2-N contains a deep hydrophobic binding site whereas the binding site in MDMX-N is shallower. This may cause difficulty for the 4-chlorophenyl groups on Nutlin-3 to accommodate into the MDMX-N binding site.

In order to identify dual binding inhibitors that interact with both MDM2-N and MDMX-N, a reduced complexity subset EDULISS library was used to screen against MDM2-N and MDMX-N in CODASS (discussed in Chapter 3). The compound screening results for Table 4.5 showed that only SEL101876 gave a significant inhibition for both MDM2-N and MDMX-N with values of 33% and 84% at 50µM concentration respectively. There is a higher inhibition against MDMX-N which may be due to the flexible conformation of the compound that allows it to fit into the shallow binding site in MDMX-N. The identification of SEL101876 shared little similarity with the known published Nutlin-like inhibitors (Figure 4.20). The flexible conformation feature in the SEL101876 (Figure 4.27 left) is also present in the new published dual binding inhibitor RO-2443 (Figure 4.27 right) (Graves et al., 2012). This may support the idea that compounds with flexible conformations are more suitable for designing dual binding inhibitors. Further validation on these compounds will be carried out using a more sensitive capillary electrophoresis (CE) assay (Chapter 5).

![Figure 4.27 Chemical structure of SEL101876 (left) and RO-2443 (right).](image)
Chapter 5 Screening of compounds using capillary electrophoresis (CE)

5.1 Introduction to capillary electrophoresis (CE)

Capillary electrophoresis (CE) has been used as an analytical technique to study the interaction between biomacromolecules. CE was developed by Stellan Hjerten in 1967 and only really became popular in 1981 when James Jorgenson and Krynn Lukacs demonstrated CE using a capillary with internal diameter of 75µm (Jorgenson and Lukacs, 1981).

![A schematic diagram of CE machine](image)

A schematic diagram showing the main components of a CE machine which include the buffer vials, a capillary, electrodes, a detector and a high voltage supply. The capillary and electrodes are placed in the buffer vials. When voltage is applied the molecules will migrate through the capillary according to their charge to mass ratio.

A schematic diagram of CE can be seen in Figure 5.1. Two electrodes connected to high voltage supply are dipped into the solution with the ends of the capillary. When voltage is applied the electrodes induce an electric field to initiate the migration of the sample, either from cathode to anode or anode to cathode, through the capillary tube depending on the polarity. The capillary contains a 2mm window which is cut at the end of the capillary depending on the polarity, and this allows the laser induced fluorescence or UV light to pass through the window for fluorescence/UV detection.
The data from the CE is generated into an electropherogram as a plot of time from injection on the x-axis against the detector signal (relative fluorescence unit (RFU)/absorbance unit (AU)) on the y-axis.

CE has the ability to separate ions based on their electrophoretic mobility with the use of the applied voltage. Several factors affect the electrophoretic mobility of the molecule. These include the viscosity, the charge of the molecule and its molecular size.

**Equation 5.1** describes the electrophoretic mobility:

\[
\mu_e = \frac{q}{6\pi \eta r}
\]

This equation states that the electrophoretic mobility will be affected by the differences in the charge to size ratio of the analyte ion (Beckman 1994). Therefore it is possible to separate mixtures of various ions by mass and charge ratio under the same electric field strength (Figure 5.2).

Another force that can be present in CE is the electroosmotic flow (EOF) that drives the migration of neutral ions in an uncoated capillary (Coulter, 1994). The ionisable silanol group that is present on the surface of the capillary wall provides a negative charge to the wall surface. This negatively charged surface attracts positively charged ions from the buffer and creates a double layer composed of a rigid layer and a diffuse layer. When a voltage is applied the cations in the diffused layer move towards the cathode carrying positive, negative and neutral charged ions. In EOF positive charged ions moved the fastest and negative ions the slowest with neutral ions as intermediates (Coulter, 1994).

In a coated polyvinyl alcohol (PVA) capillary there is little EOF and migration of ions is dependent mainly on the electrophoretic separation. The PVA coating can prevent hydrophobic and electrostatic interaction between the solute and wall surface.
5.1.1 Affinity capillary electrophoresis (ACE)

In order to identify specific binding interaction we have used affinity capillary electrophoresis (ACE) to monitor the migration patterns of the interacting molecules in the capillary. The technique uses the electrophoretic mobility of the molecules to distinguish between the binding and dissociation of formed ligand-receptor complexes during electrophoresis (Pierceall et al., 2004, Espada and Molina-Martin, 2012). ACE has been used in different varieties of interaction studies including protein-ligand, protein-protein or protein-DNA (German et al., 1998, Pierceall et al., 2004, Austin et al., 2012). Using appropriate laser-induced fluorescence (LIF) or UV detection, a fluorescently labelled probe ligand can be monitored as it migrates across the capillary. LIF detection enables CE to be able to detect the probe ligand-target interaction at much lower concentrations (nM to pM) (Pierceall et al., 2004). This is one format of an ACE assay to enable detection of weak affinity ligand (Hughes et al., 2002).

Using the probe ligand (PL), ACE can measure the mobility shift and the peak area of the PL (trace 1 in Figure 5.3). In the presence of the protein target in the separation buffer the PL interacts with the protein target during its migration through the capillary resulting in a mobility shift and the peak shape change of the PL (trace 2 in Figure 5.3). Addition of an inhibitor in the separation buffer containing the protein target and injection of the probe ligand results in prevention of the PL binding to the target which results in a reduced target dependent mobility shift of the PL (trace 3 in Figure 5.3). It is possible to determine the binding affinity of the inhibitor depending on how much the mobility shift is reduced.

\[
\begin{array}{c|cc|c|c|c}
 & A^2+ & A^{1+} & A & A & A^{1+} & A^{2+} \\
\hline
\text{Anode} & & & & & & \\
\hline
\text{Electroosmotic Flow} & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow
\end{array}
\]

**Figure 5.2 Schematic of EOF.**

Separation of analytes in CE according to their electrophoretic and electroosmotic flow mobility.
Figure 5.3 Schematic diagram of affinity capillary electrophoresis (ACE)

An example of an ACE experiment involving monitoring of an injected probe ligand (PL) with the protein target and test inhibitor in the separation buffer. The electrophoretic mobility of the probe ligand varies depend on the binding to the protein target. Upon binding to the protein target the mobility of the PL peak will shift from trace 1 to trace 2. Addition of an inhibitor in the separation buffer with the target results in prevention of PL binding resulting in reduction of the target dependent mobility shift (trace 3).

There are several advantages of using a CE assay in compound screening. CE requires only small injection volumes and the analysis cost is low. CE is performed using a high voltage to produce a high resolution profile with a short migration time to separate numerous analytes. The assay provides a high sensitivity through the use of the laser induced fluorescence (LIF) detector. The CE enables the assay to be performed with a low labelled probe concentration which allows compounds to be screened at low concentrations.
5.1.2 Principle of competitive CE assay

A competitive CE assay was developed in this work to detect competition between compounds and fluorescently labelled p53 peptide (p53-F) in MDM2-N/p53 and MDMX-N/p53 binding site.

Figure 5.4 shows an example of how the mobility of p53-F peak shifts in the presence of MDM2-N with and without compounds. The migration times of p53-F peak and p53-F/MDM2-N complex peak have to be determined prior to screening the compounds. These migration times can be used to calculate inhibition (%) of an inhibitor disrupting the p53-F interaction with MDM2-N.

Initially the p53-F is injected into the capillary and the migration time of the p53-F can be monitored by the LIF detector (Figure 5.4 trace 1). Upon binding to the target protein MDM2-N the mass of p53-F increased due to MDM2-N/p53-F complex formation during the capillary run time. Hence the migration time increased (Figure 5.4 trace 2).

For screening of compounds the capillary is filled with running buffer containing MDM2-N and the compound. The compound will therefore be bound to MDM2-N before injection of the p53-F. When p53-F is injected into the capillary and a high voltage applied across the capillary, p53-F will move through the capillary and compete with MDM2-N/compound complex. Where there is a compound/MDM2-N interaction the compound prevents p53-F from binding to the binding site. Depending upon how strong the compound/MDM2-N interaction is and how much compound is present the p53-F peak will gradually shift back to its original position (Figure 5.4 trace 3). When there is no interaction present between the compound and the binding site the p53-F peak will remain at p53-F/MDM2-N peak position.

An optimised condition of MDM2-N/p53 competitive CE assay was developed by Dr. Dallas Hughes, Dr. Yuriy Dunayevskiy, Dr. Alexy Korneev and Dr. Leonid Rashkovetsky in Selcia US (see Appendix A3 for SOP). The plan was to reproduce the assay, validate with known ligands and use the conditions for subsequent compound screening work.
Figure 5.4 Example of CE traces.
LIF detection of injected PL (p53-F (500pM)) when it is alone (first trace), in the presence of MDM2-N (160nM) in the separation buffer with (third trace) and without (second trace) test compound (50µM or 300µM).

5.2 Materials and methods

5.2.1 Materials and methods for MDM2-N CE
A detailed CE standard operation procedure (SOP) can be found in the Appendix A3. In the CE the labelled probe used was a fluorescently labelled p53 peptide (p53-F) (Sequence is shown in section 5.3.3) which can be detected using a laser induced fluorescence (LIF) detector. The model of the LIF detector used was a Beckman Coulter 488nm laser module and the laser used had an argon ion laser source with excitation/ emission wavelengths of 488nm/520nm. The excitation/ emission wavelengths used in the CE were similar to the p53-F detection in the FP assay where 485nm/530nm were used.

When screening the compound in the CE the injection sample contained Carboxyfluorescein (internal standard (10pM)) and p53-F (500pM) in TAPS/Tris (1mM) pH 8.0, DTT (1mM).

The separation sample buffer contained MDM2-N (160nM), test compound (300µM depending on solubility), TAPS/Tris (200mM) pH 8.0, DMSO (1% f/c) and glycerol (0.25% f/c). In this work Nutlin-3 and unlabeled p53 peptide were used as test inhibitors to validate the assay.
5.2.2 Materials and methods for MDMX-N CE

In the MDMX-N CE assay the same fluorescently labelled p53 peptide (p53-F) was used. The SOP used for MDM2-N CE assay was also used in the MDMX-N CE assay (Appendix A3). The injection sample buffer contained Carboxyfluorescein (10pM) and p53-F (500pM) in TAPS/Tris (1mM) pH 8.0, DTT (1mM). The separation sample buffer contained MDMX-N (160nM (requires further optimization)), test compound (concentration depended on the compound solubility), TAPS/Tris (200mM) pH 8.0, DMSO (1% f/c) and glycerol (0.25% f/c).

5.2.3 Sequences of the MDM2-N and MDMX-N

The protein constructs used in the assays contained only N-terminal sequences because the p53 binding site is located at the N-terminal domain of MDM2 and MDMX. It should also be noted that the full length sequence structures of MDM2 and MDMX are highly unstable and prone to precipitation.

The MDM2-N and MDMX-N constructs that were used in the FP and CE assay were:

His6-tag-MDM2-N\textsuperscript{11-130}

\begin{verbatim}
  10  20  30  40  50  60
MGSSHHHHHH SSGLVPRGSH MDGAVTTSQI PASEQETLVR PKPLLKLLK SVQAQKDTYT
  70  80  90  100  110  120
MKEVLFLYLG YIMTKRLYDE KQQHIVYCSN DLLGDLFGVP SFSVKEHRKI YTMIIYNLV
  130
VNQOESSDSG TSVSENLE
\end{verbatim}

His6-tag-MDMX-N\textsuperscript{14-111}

\begin{verbatim}
  10  20  30  40  50  60
MGSSHHHHHH SSGLVPRGSH MDSASRISPG QINQVRPKLP LLKILHAAGA QGEMFTKEV
  70  80  90  100  110
MHYLGYIMV KQLYDQEQH MVYCGGDLG ELLGRQFSV KNPSPLYDML RKNLVTLAT
\end{verbatim}
Upon examining a list of different labelled p53 peptide of MDM2-N used in the literature (See Chapter 4) only the labelled p53 peptide (p53-F) from Brown C. et al. has a suitably low affinity for our competition experiment (Brown et al., 2010). The fluorescently labelled p53 derived peptide (p53-F) used in the CE assay was:

5-FAM-QETFSDLWKLLP-OH

Fluorescein amidite (FAM) is a fluorescein dye that can be detected by the LIF detector at 520nm emission wavelength.

5.2.4 Testing of compounds in the CE assay

Compounds were screened at an initial screening concentration of 300µM or 50µM depending on the compound solubility. Appendix A3 gives the method for the CE compound screening.

After injection of the sample a voltage of 10kV was applied and separation was carried out for 6.5min. The migration time of the p53-F peak was recorded and the inhibition (%) was calculated using the equation below.

\[ \% \text{ Inhibition} = 100 \left( \frac{T_H - T_L}{T_H - T_R} \right) \]

Where:  
- \( T_H \) is migration time of p53-F peak in the presence of MDM2-N.
- \( T_R \) is the migration time of p53-F peak.
- \( T_L \) is the migration time of p53-F peak in the presence of MDM2-N + compound.

Selected hits shown to be active in CE were titrated to confirm if the compound gave a dose response curve. Where possible an IC\textsubscript{50} was calculated.

5.2.5 IC\textsubscript{50} Calculation

Compounds were titrated using ~10 different concentrations and the percentage inhibition was calculated from the migration time. Calculated % inhibition of each concentration was input into GraphPad prism software. Compound concentration was
converted into Molar unit and transformed to log_{10}. A four parameter variable slope was used to fit the curve and the IC_{50} was calculated.

The curve was fitted using the equation:

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{((\text{LogIC}_{50} - X) \times \text{Hillslope})})} \]

Where: X is log of dose or concentration.

Y is response, decreasing as X increases.

Top and Bottom is Plateaus in same units as Y.

\text{logIC}_{50} is same log units as X.

Hillslope is Slope factor.
5.3 Results

Initial experiments using the Selcia US conditions were not reproducible (data not shown) and after discussion with Selcia US they suggested using siliconised tips/vials to prevent p53-F sticking to plastic. The optimised MDM2-N and p53-F condition developed by Selcia US is MDM2-N (160nM) and p53-F (500pM). A new experiment was therefore carried out to confirm the effectiveness of siliconised tips/vials.

5.3.1 Improving sensitivity using siliconised vials/tips

In this study normal pipette tips and PCR vials were replaced with siliconised tips/vials in order to minimise sticking of p53-F to vials and tips. Carboxyfluorescein (4nM) and p53-F (250nM) were first tested and repeated 3 times to confirm if the results were reproducible. It was found that the three repeats overlapped and in fact the p53-F (250nM) peak was saturated (Figure 5.5). These results showed that using siliconised tips/vials can help prevent the p53-F sticking and also improves the reproducibility of the assay.

Due to the p53-F peak being saturated it was possible to decrease the concentration of Carboxyfluorescein and p53-F to find the minimum concentration required. Serial dilution of Carboxyfluorescein and p53-F in Figure 5.6 showed the concentration could be lowered to the pM range and reproduction of the Selcia US conditions becomes possible.
Figure 5.5 Repeating runs of Carboxyfluorescein and p53-F.
Carboxyfluorescein (4nM) and p53-F (250nM) were first tested and repeated 3 times. The results became reproducible after using siliconised tips/vials. The peak was saturated when using p53-F (250nM) and there were other peaks present which may be contaminants that were not removed during the peptide synthesis.

Figure 5.6 A and B Serial dilution of p53-F and Carboxyfluorescein using siliconised tips/vials.
The area of p53-F (A) and Carboxyfluorescein (B) peaks were recorded in each dilution and plotted. With decreasing p53-F or Carboxyfluorescein concentration the peak area decreased.
5.3.2 Addition of MDM2-N in the CE assay

In order to carry out compound screening an optimum concentration of MDM2-N has to be determined. The optimum conditions given from Selcia US were p53-F (500pM) and MDM2-N (160nM) using siliconised tips/vials. Using these conditions it was possible to obtain an assay window of one minute between the p53-F peak and the MDM2-N/p53-F complex peak. Assay window is the difference between the minimal and the maximal signal and an adequate window ensures the detection of active inhibitor that disrupts p53-F/MDM2-N interaction. Figure 5.7 shows that with addition of MDM2-N (160nM) in the solution the p53-F peak shifted from 4.32min to 5.36min. This one minute assay window is sufficient for subsequent inhibitor screening.

![Figure 5.7 CE electropherogram of MDM2-N (160nM) run with Selcia US conditions.](image)

The electropherogram shows the migration time of p53-F (500pM) in black colour trace and the migration time of p53-F (500pM)/MDM2-N (160nM) complex in light blue colour trace. Carboxyfluorescein (25pM) was added as an internal standard.
5.3.3 Nutlin-3 titration

Nutlin-3, a known inhibitor of the MDM2-N/p53 interaction, was used in order to confirm if the p53-F/MDM2-N peak shifted back in the presence of an inhibitor of the MDM2-N/p53 interaction. From the literature Nutlin-3 showed IC$_{50}$ values of 90nM and 432nM determined using SPR and FP assay (Vassilev et al., 2004, Ding et al., 2006), while the IC$_{50}$ observed in our FP assay was 266nM (Chapter 4). It may be possible to see a full inhibition with the addition of twice the Ki concentration of Nutlin-3. Upon adding Nutlin-3 (100nM) the peak shifted back to the p53-F peak position with a change in peak shape indicating competition between p53-F and Nutlin-3 in the MDM2-N binding site (Figure 5.8 bottom trace).

![Negative control 500pM p53-F](image)

**Figure 5.8 Nutlin-3 titration.**

As the Nutlin-3 concentration increases the peak shifted back to the p53-F control peak position. In this titration the injection buffer used contains Carboxyfluorescein (internal standard (10pM)) and p53-F (500pM) in TAPS/Tris (1mM) pH 8.0, DTT (1mM). The top trace is the p53-F (500pM) alone and contains TAPS/Tris (200mM) pH 8.0, DMSO (1% f/c) and glycerol (0.25% f/c) in the separation buffer. This is followed by p53-F (500pM) running in the separation buffer containing MDM2-N (160nM), TAPS/Tris (200mM) pH 8.0, DMSO (1% f/c) and glycerol (0.25% f/c). Nutlin-3 was then added into the sample with increasing concentrations (separation buffer: Nutlin-3, MDM2-N (160nM), TAPS/Tris (200mM) pH 8.0, DMSO (1% f/c) and glycerol (0.25% f/c).
With additional titration the IC$_{50}$ of Nutlin-3 could be calculated. Nutlin-3 was titrated from 0.5nM to 400nM and % inhibition was calculated from the migration time of Nutlin-3 (Figure 5.9).

The % inhibition was fitted against Nutlin-3 concentration in Log scale using GraphPad Prism and IC$_{50}$ was calculated to be 37.3nM. The calculated IC$_{50}$ is similar to the literature value of 90nM (Vassilev et al., 2004). This confirms that the CE assay is able to identify inhibitor binding and calculate an accurate IC$_{50}$.

![Transform of nutlin titration](image)

**Figure 5.9** Graph showing inhibition (%) plotted against Nutlin-3 concentration.

From the data an IC$_{50}$ of 37.3nM was determined.

### 5.3.4 Unlabelled p53 peptide titration

To further evaluate the reliability of the CE assay an unlabelled p53 peptide (13amino acid sequence) that is known to bind to the MDM2-N binding site (Kussie et al., 1996) was tested in the assay. A titration of unlabelled p53 peptide was performed to obtain the IC$_{50}$ which can be used to compare with the IC$_{50}$ from the literature.

P53-U ETFSDLWKLLPEN

Phan ETFSDLWKLLPE

An IC$_{50}$ was performed over the range 0.025µM to 40µM. Increasing the concentration of unlabelled p53 peptide showed that the p53-F peak gradually shifted back to its
original position (Figure 5.10). This suggests that there was unlabelled p53 peptide/MDM2-N complex formation and p53-F was not able to bind to MDM2-N. A dose response curve was generated using the % inhibition calculated and the IC$_{50}$ was calculated to be 3.1µM (Figure 5.11). The IC$_{50}$ of unlabelled p53 peptide from the literature is 2µM (Phan et al., 2010) but only an IC$_{50}$ value of 23µM was observed in our FP assay. This suggest that FP assay is less sensitive and is unable to measure accurate IC$_{50}$. The IC$_{50}$ obtained from the CE was in good agreement with the IC$_{50}$ from the literature and implies that the CE assay is able to measure accurate IC$_{50}$.

Figure 5.10 Electropherogram of unlabelled p53 peptide titrated against p53-F (500pM).
The diagram shows some representative traces of p53-F peak gradually shifting back to its original position with increasing concentration of unlabelled p53 peptide.
5.3.5 Validation of virtual screening hits using CE

The six compounds purchased from the initial virtual screening CODASS (See Chapter 2) were selected as they had different chemical motifs. Unfortunately there was insufficient amount of the compound to perform solubility tests. Initially they were tested at one concentration (300µM), however SEL101063 and SEL101068 were tested at 50µM because it was discovered the compounds precipitated in the solution at 300µM. Subsequently they were tested at a final concentration of 50µM. Figure 5.13 – Figure 5.14 presents the CE electropherograms of the compounds. After each compound screen a control run with MDM2-N was carried out to ensure that the p53-F peak shifted back to the original MDM2-N peak position. Percentage inhibition was calculated as previously described and the results are summarised in Table 5.1. Of the six virtual screening hits only SEL101068 was found to be inactive. This may be due to screening at the low concentration of 50µM. All other virtual screening hits showed ~40% inhibition at 300µM concentration. SEL101063 was also shown to be active with 13% inhibition at 50µM; however it was not evaluated further due to poor...
solubility. The compounds were also shown to be active in the FP assay with only SEL101063 and SEL101068 inactive which may be due to the assay not being sensitive enough to detect weak binding at lower concentration (Table 5.1). From the CE screen SEL101066 was confirmed to be the strongest hit. Analogues of this compound were further explored to find the segment of structure that provides optimum inhibition (See Chapter 6).

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition from CE</th>
<th>% Inhibition from FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL101066</td>
<td>42.0% at 300µM</td>
<td>18% at 500µM</td>
</tr>
<tr>
<td>SEL101064</td>
<td>40.6% at 300µM</td>
<td>37% at 500µM</td>
</tr>
<tr>
<td>SEL101067</td>
<td>40.6% at 300µM</td>
<td>25% at 500µM</td>
</tr>
<tr>
<td>SEL101069</td>
<td>40.1% at 300µM</td>
<td>13% at 500µM</td>
</tr>
<tr>
<td>Compound</td>
<td>CE Inhibition at 300µM</td>
<td>CE Inhibition at 500µM</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>SFL001328</td>
<td>27.67%</td>
<td>8%</td>
</tr>
<tr>
<td>SEL101063</td>
<td>13.2%</td>
<td>0%</td>
</tr>
<tr>
<td>SEL101068</td>
<td>0%</td>
<td>3%</td>
</tr>
</tbody>
</table>

Table 5.1 Summary results of the CE and FP screen on virtual screening compounds

The compounds were ranked based on the % inhibition from CE.

5.3.6 CE screen on SFL001328 fragment

The Selcia fragment SFL001328 was also screened in CE at 300µM. The solubility of this compound was determined at Selcia to be 1mM with 3% f/c of DMSO as part of a solubility test of the Selcia fragment library.

SFL001328 is a fragment of the Spiro-oxindoles inhibitor Mi63 as shown in Figure 5.12. SEL101069 also contains a similar motif as SFL001328. SFL001328 showed a 27% inhibition in CE at 300µM concentration (Figure 5.13) which suggests that SFL001328 may be used as a starting point in the fragment optimisation process. Titration was done on SEL101069 and SFL001328 to obtain the IC₅₀ and also to confirm that the hits are not due to compound aggregation (See Section 6.7). If the inhibition was due to the presence of compound aggregation then a sudden increase in inhibition may occur with increasing compound concentration.
Figure 5.12 Chemical structure of Mi63 inhibitor and the oxindole analogues, SFL001328 and SEL101069.

Figure 5.13 Electropherogram of CE screen on virtual screening hits.

All compounds shifted the p53-F peak, which is indicative of an interaction taking place. The first trace is the control of p53-F (500pM). The second trace is the SFL001328 (300µM) fragment. The third and fifth traces are the controls of MDM2-N (160nM) with p53-F (500pM). The fourth trace is SEL101066 (300µM) and the sixth trace is the SEL101069 (300µM).
Figure 5.14 Electropherogram of CE screen on virtual screening hit compounds.
The diagram shows the change in migration time of the p53-F peak in the presence of compounds. The control run of p53-F (500pM) running in the separation buffer containing MDM2-N (160nM) was performed in between each compound screen. Only SEL101068 did not shift the p53-F peak which confirms no interaction taking place.

5.3.7 Screening the second set of CODASS hits against MDM2-N in the CE assay

Due to the success of the previous virtual screen a new CODASS run was performed to screen through a reduced complexity subset of EDULISS library containing 518,000 compounds to identify dual inhibitors against the MDM2-N and MDMX-N. The virtual screening results were explained in more detail in Chapter 2. From the 1000 hits ~100 compounds from Chembridge were pulled out. Seven compounds containing different chemical classes were purchased (Table 5.2).

Solubility tests were performed on the CODASS hits (Appendix A4). However it was only performed after screening in the FP assay and SEL101877 and SEL1881 were screened at the solubility limit in the FP assay. SEL101875 – SEL101877 and SEL101881 showed lower solubility so these compounds were screened at 50µM.
There is a similarity between these compounds in which they contain triazole piperidinyl motif in the structure (circled in red) which may be why they have lower solubility. SEL101878 – SEL101880 had higher solubility and were screened at 100µM.

Table 5.2 showed the summary results of the CODASS hits screened against MDM2-N in the CE and FP assays. Percentage inhibition calculation was described as in the methods (Section 5.2.4). Of the eight compounds only SEL101880 was found to be inactive in both assays. SEL101875, SEL101876, SEL101878, SEL101879 and SEL101881 were found to be active with inhibition over 40% at either 50µM or 100µM in the CE assay. SEL101877 was also active in the CE assay but with a weaker inhibition of 15%. These compounds however did not show similar inhibition in the FP assay. A possible reason is that SEL101875 and SEL101878 were screened at 50µM in the FP assay which may be at the limit of detection, whereas SEL101879 and SEL101881 were screened at 200µM in the FP assay which is at the solubility limit and the compounds may be aggregated in the solution.

From the screening several compounds were identified with inhibition >50%. The compounds SEL101876, SEL101878, SEL101879 and SEL101881 were selected for titration to obtain the IC50 of these hits. These compounds were titrated based upon the solubility (Appendix A4) and the % inhibition values obtained (Table 5.2). SEL101876 was titrated from 3.1µM to 100µM, SEL101878 and SEL101879 were titrated from 25µM to 200µM and finally SEL101881 was titrated from 3.1µM to 50µM. The titration curves can be found in the Appendix A5.

The IC50 values calculated for these compounds are listed in Table 5.2. The result showed SEL101881 was found to have the highest inhibition against MDM2-N with an IC50 of 17.6µM.

These compounds showed little similarity with the known MDM2-N inhibitors and have more rotatable bonds, therefore these flexible structure compounds may have a higher chance on binding to the MDMX-N binding site. These compounds were screened in the FP assay (Chapter 4) prior to screening in the CE assay and from the FP assay results the compound SEL101876 was binding tightly to MDMX-N with 86% inhibition at 50µM. CE assay for MDMX-N was then set up in order to verify these virtual screening hits against MDMX-N.
<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition from CE assay</th>
<th>% inhibition from FP assay</th>
<th>IC\textsubscript{50} determined using CE assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL101875</td>
<td>41% At 50µM</td>
<td>1% At 50µM</td>
<td></td>
</tr>
<tr>
<td>SEL101876</td>
<td>58% At 50µM</td>
<td>33% At 50µM</td>
<td>31.7µM</td>
</tr>
<tr>
<td>SEL101877</td>
<td>16% At 50µM</td>
<td>11% At 200µM</td>
<td></td>
</tr>
<tr>
<td>SEL101878</td>
<td>58% At 100µM</td>
<td>1% At 50µM</td>
<td>69µM</td>
</tr>
<tr>
<td>SEL101879</td>
<td>59% At 100µM</td>
<td>12% At 200µM</td>
<td>71µM</td>
</tr>
</tbody>
</table>
Table 5.2 Summary results on the CE and FP assays on second CODASS hits against MDM2-N

The compounds were screened at different concentrations in the CE and FP assays because the solubility test was not performed before running the FP assay. SEL101875, SEL101877 and SEL101881 contained a similar motif in the structure as highlighted by the red circles.

5.3.8 Developing the CE assay for MDMX-N

The CODASS hits were also screened against MDMX-N\textsuperscript{14-111} using CE. The materials and methods for MDMX-N CE assay can be found in section 5.3.2. MDMX-N was run in the same buffer conditions, using 200mM TAPS/Tris pH 8.0, as that for MDM2-N. MDMX-N has a pI of 9.2 and is consequently positively charged at pH 8.0, whilst p53-F was negatively charged at pH 8.0. Whereas MDM2-N is negatively charged at pH 8.0 and migrates from the negative end to the positive end in the capillary. During the separation MDMX-N will migrate from the positive end to the negative end and p53-F will migrate from the negative to the positive. This migration direction is the opposite of MDM2-N which suggests that there will be more interaction between the MDMX-N and the p53-F.

When the MDMX-N protein concentration was re-measured by Bradford assay before running in the CE assay the concentration was found to be three times less than originally measured. The laser protein concentration in the solution which may be due
to aggregation after storing in the -80°C freezer for one month. A new measured concentration was then used in the assays.

A protein titration was performed to analyse the binding of p53-F with MDMX-N. The titration was performed from 100nM to 1000nM and the p53-F gradually shifted further with increasing concentration of MDMX-N (Figure 5.15). From the protein titration 240nM concentration was found to provide the best assay condition with a 1min window for screening compounds. Higher concentration of MDMX-N may provide an even bigger window, however this will lose the sensitivity on detecting ligand binding.

![Figure 5.15 MDMX-N titration in the CE.](image)

The titration of MDMX-N was performed from 100nM to 1000nM concentrations. The first trace is the control experiment with p53-F on its own followed by increasing concentration of MDMX-N. The migration time of MDMX-N/p53-F peak gradually increased as the MDMX-N concentration increased.

Nutlin-3 was added into the separation buffer with MDMX-N (240nM) in order to confirm if the p53-F/MDMX-N complex peak will shift back to the p53-F alone peak position.

From the literature (Popowicz et al., 2010) and the previous FP assay result (Section 4.8.2), Nutlin-3 binds weakly to MDMX-N with an IC$_{50}$ >3µM. Therefore a higher concentration of Nutlin-3 had to be added into the CE to get full inhibition. Nutlin-3
(10µM) was added and 100% inhibition was seen (Figure 5.16). However when running the MDMX-N run after the Nutlin-3 run the migration time of the MDMX-N/p53-F peak did not shift back to its original position. This indicates that the Nutlin-3 may not have been fully washed away from the capillary. Intense washing was applied to try and remove the Nutlin-3 from the capillary. After the separation run, a 2 min running buffer rinse was added and the H2O rinse was increased from 0.5 min to 2 min followed by a 3 min BSA (1mg/ml) rinse. After the intense rinse the capillary was regenerated and the migration time of p53-F/MDMX-N (240nM) complex peak was restored to 5.8min (Second peak in Figure 5.17).

![Figure 5.16 CE traces of p53-F peak in the presence of Nutlin-3 (10µM).](image)

**Figure 5.16 CE traces of p53-F peak in the presence of Nutlin-3 (10µM).**

The third trace is the competition experiment of Nutlin-3 (10µM) run after the MDMX-N (240nM) control. The p53-F peak has shifted back in the presence of Nutlin-3 (10µM). Control experiments of p53-F and MDMX-N (240nM) were repeated after the competition experiment but the MDMX-N/p53-F complex peak did not shift as far as the first MDMX-N/p53-F run due to the Nutlin-3 remaining in the capillary.

However, when repeat adding Nutlin-3 (10µM) it was discovered that the p53-F peak did not shift back to the original position (third trace from the top in Figure 5.17). It is possible that at 10µM Nutlin-3 the concentration may be above the limit of its solubility and the aggregated Nutlin-3 damaged the coating on the capillary wall. After the Nutlin-3 (10µM) run the migration time of the MDMX-N/p53-F peak was not able to migrate back to its original position (Fourth trace in Figure 5.17). The migration
time of the MDMX-N/p53-F complex peak was reduced from 1.2min to ~0.65min. It is possible that Nutlin-3 was still not washed away from the capillary even with the additional rinsing steps. To try and remove all the particles and contaminants the capillary was rinsed with 10mM phosphoric acid for 3min followed by H_2O rinse for 5min. Phosphoric acid is used as a rinsing reagent to remove proteins adsorbed on the coated capillary wall. Following the rinses with phosphoric acid the migration time with MDMX-N was partially but not fully recovered (5 and 6th traces from the top in Figure 5.17). The migration time of MDMX-N (240nM)/p53-F peak only shifted by 0.65min from the p53-F peak. The migration time of MDMX-N/p53-F peak reduced by half compared to the beginning when Nutlin-3 was not added.

Figure 5.17 CE traces of MDMX-N/p53-F peak.
The MDMX-N/p53-F peak did not shift back to 5.9min after the Nutlin-3 (10µM) run. The capillary was washed and MDMX-N (240nM) run was repeated several times but the migration time of MDMX-N/p53-F peak was not fully recovered.

The migration time of MDMX-N peak was not reproducible which may have been caused by the Nutlin-3 particles damaging the coating on the capillary wall. Therefore a new capillary was inserted into the cartridge and conditioned with the running buffer and H_2O. The internal standard Carboxyfluorescein (20pM) and p53-F (500pM) control were run followed by a MDMX-N (240nM) run. The MDMX-N (240nM) run was repeated twice and p53-F (500pM) was run in between to ensure that the p53-F
peak would migrate back to its original position. As can be seen in Figure 5.18, the first MDMX-N (240nM) run (blue trace), the MDMX-N/p53-F peak migrated to 5.85min and the p53-F peak returned to its original position on the next run. However on the second MDMX-N (240nM) run the MDMX-N/p53-F peak required a longer time to migrate through the capillary (teal trace, 4th trace in Figure 5.18). The migration time has increased to over 6.5min with the assay window extended to over 1min.

It may be possible to repeat the CE run with MDMX-N (160nM) concentration and monitor if the migration time of MDMX-N/p53-F complex will change.

When running MDMX-N (160nM) the migration time was ~5.3min which was similar to the migration time in Figure 5.15. However the migration time of MDMX-N/p53-F peak increased when repeating the run (fourth and sixth in Figure 5.19) which may be caused by adsorption of MDMX-N to the capillary. More MDMX-N in the capillary will result in more MDMX-N/p53-F interaction and this increases the p53-F run time.
If the adhered MDMX-N was not washed away then it could interact with the p53-F and increase its migration time during the p53-F control run. However this was not observed in the p53-F run when running in between the MDMX-N run (Third and Fifth traces in Figure 5.19).

As discussed in section 5.3.8 there was aggregated MDMX-N found in the frozen stock. This could suggest that the aggregates adhered in the capillary and interact with MDMX-N to form large aggregates. The aggregates formation in the capillary further increase the migration time of the MDMX-N/p53-F complex. The migration time of p53-F was not affected when running p53-F control run possibly because the aggregates only interact with MDMX-N but not p53-F. To remove these aggregated proteins intense rinse steps were applied by rinsing with H2O for 5min, running buffer for 10min and H2O for 2min. Unfortunately, even when applying the intense rinse steps at the end of every MDMX-N run, the aggregated proteins were not completely removed in the capillary and the migration time of MDMX-N/p53-F still fluctuated in every run.

![Figure 5.19 Control runs of MDMX-N and p53-F.](image)

MDMX-N (160nM) and p53-F runs were repeated one after the other. The migration time of MDMX-N/p53-F peak shifted further when run several times. The migration time of the p53-F peak was not affected when running in between the MDMX-N (160nM) runs.
5.3.9 CODASS hits screening against MDMX-N^{14-111}

Due to the problem with the inconsistent migration time of MDMX-N/p53-F peak compound screening was difficult. It was a challenge to determine how much inhibition the compound had caused. However it was still possible to screen the compounds by monitoring the p53-F peak and observe if the compounds were able to shift the p53-F peak back to its original position. This method could show the displacement of p53-F by the compound but the % inhibition cannot be reliably determined. Further work on assay optimisation is required to confirm the results.

The compounds identified from the CODASS virtual screening were already measured by the FP assay (section 4.9) and showed SEL101876 to have a relatively high inhibition against MDMX-N. Therefore it will be interesting to verify SEL101876 in the CE to confirm the inhibition.

SEL101876 (50µM) was screened after the p53-F and MDMX-N (160nM) control runs. When SEL101876 (50µM) was added the p53-F peak shifted back to its original position. This suggested that p53-F was fully competed off from MDMX-N by SEL101876 (Figure 5.20). Screening SEL101878 and SEL101881 did not shift the p53-F back so these two compounds were ranked as non-inhibitors (Results not shown).

A summary of results for the CODASS hits screened using the FP and CE assays is presented in Table 5.3. From the FP and CE assays SEL101876 gave a relatively high inhibition at 50µM concentration. SEL101876 gave even higher inhibition against MDMX-N than against MDM2-N. However SEL101876 titration was not performed against MDMX-N due to the unstable migration time of MDMX-N/p53-F. The MDMX-N CE condition has to be further optimized in order to perform the titration.
Figure 5.20 CE traces of SEL101876 (50µM) screening against MDMX-N.

The trace in aqua is the sample with p53-F on its own. The black trace is the MDMX-N/p53-F complex peak. The pink trace is the competition experiment containing SEL101876, MDMX-N and p53-F in the solution. The p53-F peak shifted back to its original position when SEL101876 (50µM) was present.
<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition from CE assay</th>
<th>% inhibition from FP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL101875</td>
<td>Not tested</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>SEL101876</td>
<td>100%</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td>At 50µM</td>
<td>At 50µM</td>
</tr>
<tr>
<td>SEL101877</td>
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<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At 200µM</td>
</tr>
<tr>
<td>SEL101878</td>
<td>0%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>At 100µM</td>
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<tr>
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<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At 200µM</td>
</tr>
<tr>
<td>Compound</td>
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<td>FP Assay</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>----------</td>
</tr>
</tbody>
</table>
| SEL101880      | Not tested | 25%  
At 50µM |
| SEL101881      | 0%  
At 50µM | 1%  
At 200µM |

**Table 5.3 Summary table of CODASS hits screened against MDMX-N in the FP and CE assays.**

The table shows the % inhibition of the compounds against MDMX-N obtained in CE and FP assays. The concentrations above the bars are the concentrations used in the assays. Only SEL101876, SEL101878 and SEL101881 were screened in the CE assay.
5.4 Discussion

In this study we have employed capillary electrophoresis as an orthogonal assay to confirm the virtual screening results and verify with the FP assay results. The MDM2-N CE assay condition was initially optimised by the scientists in Selcia US and the assay was then reproduced in this work. In the CE assay for MDM2/p53 the p53-F peak migration time increased and the peak shape broadened upon binding to MDM2-N in the separation buffer. This was due to the slower migration of the MDM2-N/p53-F complex while migrating through the LIF detector at the capillary window.

The assay was validated using Nutlin-3 and unlabelled p53 peptide as controls, thus demonstrating that the CE assay is capable of detecting ligand-protein and protein-peptide interactions with high accuracy. The IC$_{50}$ determined by the CE assay showed tighter affinities than determined by the FP assay which gave values for Nutlin-3 and unlabelled p53 peptide of 266nM and 23µM.

5.4.1 CODASS hits verification using the CE assay

The initial virtual screening run using CODASS (Chapter 2) identified six hits and these were subsequently screened in the FP and CE assays. The screening results from the CE and FP assays were compared to ensure that the hits identified were active in both assays (FP assay is described in Chapter 4). This will confirm whether the hits are genuine and if the results are not comparable then this may suggest that the hits are false positive. Table 5.1 showed the % inhibition from the screenings and SEL101064, SEL101066, SEL101067 and SEL101069 were identified as active in both assays. SEL101068 was however inactive in both assays at 50µM. However we cannot necessarily conclude the compound does not bind. It may have a weak affinity and require a higher concentration to show signs of inhibition. SEL101063 was active in the CE assay but inactive in the FP assay and this was possibly due to screening at too low concentration for detection in the FP assay.

Overall in the comparison (Table 5.1) the results from the CE assay and FP assay show a positive correlation and confirmed the hits to be genuine. However the compounds show a weaker inhibition in the FP assay compared with the CE assay. This was also observed in the affinities of Nutlin-3 and unlabelled p53 peptide where FP assay gave weaker affinities than the CE assay. This could suggest that the FP assay is less sensitive than the CE assay and therefore requires a higher concentration of compound.
to detect binding. This was also shown in the Austin C. et al. paper where some fragment hits identified by the CE assay were not detected by FP assay against a different target Hsp90 (Austin et al., 2012).

**5.4.2 Hits of second CODASS screen were screened against MDM2-N using the CE assay**

With the success from the first virtual screening a new virtual screening run was performed using a reduced complexity EDULISS library to screen against MDM2-N and MDMX-N (Chapter 2). The structures in these compounds showed a more flexible scaffold than the first virtual screening hits. These compounds were pre-screened in the FP assay prior to screening in the CE assay and the results showed that only SEL101880 was inactive in the CE assay (Table 5.2). When comparing the CE assay result with the FP assay result, SEL101880 was confirmed inactive in both assays while SEL101876 and SEL101877 were found active in both CE and FP assays. However when examining SEL101875 and SEL101878, these compounds showed high inhibitions in the CE assay but no inhibition in the FP assay. These compounds were apparently screened at 50µM in the FP assay and this concentration is probably too low for the FP assay to detect the binding. This problem was already seen in the previous virtual screening hits screening where nearly two times of concentrations were required to detect the binding in the FP assay (section 5.4.1). For the SEL101881 compound a strong inhibition in the CE assay was identified with an IC₅₀ of 17µM. However there was only a weak inhibition in the FP assay. This may be because SEL101881 was screened at 200µM in the FP assay which was at the limit of its solubility and the compound may have aggregated in the assay, hence low inhibition in the FP assay.

These compounds showed higher binding affinities toward MDM2-N compared to the hits from the first virtual screening (section 5.3.5). One possible reason for higher binding affinities may be due to the flexible structures in these new virtual screening hits which enables a stronger interaction into the three hydrophobic grooves in the MDM2-N binding site. These compounds showed little similarity with the known MDM2-N inhibitors and do not contain an imidazoline group as the centre to link multiple aromatic rings.
5.4.3 Screening fragments using the CE assay
In this study the CE assay has also been used to study the weak affinity fragment-protein interaction. Through the use of the known inhibitor structures this work has identified an oxindole fragment (SFL001328) that can be used to test to see whether smaller molecule compounds would inhibit the p53-MDM2 interaction. From the CE assay screening SFL001328 was shown to be active (Table 5.1) and this demonstrated that the CE assay is capable of detecting weak fragment interaction and inhibition of the p53-MDM2 interaction with fragments is feasible. The ligand efficiency (LE) for SFL001328 was determined to be 0.5kcal mol\(^{-1}\) per non hydrogen atom (Chapter 6) which is an acceptable value for drug candidates (Schultes et al., 2010). Further fragment exploration will be carried out to identify more fragments with improved binding efficiency (see Chapter 6).

5.4.4 Using the CE assay to identify dual action inhibitors against MDM2 and MDMX
The negative role of MDMX in the regulation of the tumour suppressor p53 has gradually emerged from multiple studies and it has been shown that overexpression of MDMX allows the cancer cell to resist the MDM2 antagonist (Marine and Jochemsen, 2005, Wade and Wahl, 2009). Both MDM2 and MDMX use the N-terminal binding site to bind to the p53 N-terminal transactivation domain. In order to identify a dual binding inhibitor to inhibit both MDM2 and MDMX the CE assay for MDMX-N has to be developed.

In the initial MDMX-N CE assay development it was found that the MDMX-N/p53-F peak was not reproducible and the migration time continuously increased. One possible explanation could be that there was aggregation in the frozen stock after storing in the -80°C freezer for one month. The concentration measured by Bradford assay was three times less than originally measured using a Nanodrop spectrophotometer. In order to reproduce the MDMX-N/p53-F peak fresh MDMX-N protein should be purified and run in the CE assay immediately to prevent aggregation. MDMX-N is a highly unstable protein therefore it is possible that the protein will gradually aggregate after few days of storage. DMSO (100%) wash should also be
added into the cleaning step at the end of each compound screen in order to wash away compound particles that may be present in the capillary.

Even though the assay was not fully optimised it was still possible to screen the compounds by monitoring if the p53-F peak would shift back to its original position in the presence of inhibitors. In this study SEL101876, SEL101878 and SEL101881 were screened in the CE assay against MDMX-N and SEL101876 fully shifted the p53-F peak back to its original position at 50µM concentration. This is consistent with the results obtained from the FP assay (Table 5.3) and may signify that SEL101876 is a dual inhibitor with a low µM affinity against MDM2-N and MDMX-N. However further optimisation on the CE assay condition is required in order to validate this result and confirm the binding affinity. As discussed in Chapter 4 the structures of these compounds identified from the second CODASS run shared little similarity with the known published inhibitors. However the flexible conformation feature in SEL101876 is also present in the published dual binding inhibitor RO-2443 (see section 4.10). Thus this new chemical scaffold allows further optimisation to develop a more potent MDM2/X-N dual inhibitor.

In this study we have demonstrated that CE is a promising tool for characterising protein-ligand, protein-peptide and protein-fragment interactions. The results presented in this work provided the first example of using FP and CE as orthogonal assays for MDM2/X-p53 ligand screening and illustrate the importance of validation with different techniques.
Chapter 6 Identification and validation of small molecule families of MDM2 inhibitors

6.1 Introduction

The CE and FP assays described in Chapters 4 and 5 confirmed active compounds identified using virtual screening. However these virtual screening compounds have a high molecular weight and low solubility. Therefore, finding analogues of the virtual screening hits could help to identify small molecular weight fragments with higher solubility. Ideally we are looking for small molecule fragments which are able to interact in the binding site efficiently and which have good ligand efficiency. In current published literature the known inhibitors identified are complex structures with multiple hydrophobic groups and thus have poor solubility and ligand efficiency.

6.2 Identification of diphenylamine analogue fragments

Analogues of the virtual screening hit SEL101066 were further explored because the structure was novel and different from known published inhibitors. The virtual screening studies (Table 3.2 in Section 3.2.2) showed that the diphenylamine group was present in a number of the hits (Compound 3, 5 and 7) which suggests that the diphenylamine group may be responsible for the interaction with the MDM2 binding site. Hence, SEL101066 was further explored by Simon Pettit using the Selcia chemical store substructure search tool in the Selcia compound library to identify compounds containing the diphenylamine motif.

Structure of SEL101066 (left), Diphenylamine (right)
Fluspirilen, phenoxazine, carbamazepine, 2-chlorophenothiazine, phenothiazine, phentolamine HCl, 2-phenylamino-benzoic acid, N-phenyl-p-phenylenediamine, 4-hydroxydiphenylamine, 3-hydroxydiphenylamine and diphenylamine contained a similar motif (shown as red in Figure 6.1) and were tested by performing the CE assay. The analogues were validated dependent upon the % inhibition observed.

Figure 6.1 Chemical structures of compounds selected with diphenylamine motif.
The diphenylamine motif present in the structures is highlighted in red.

6.3 Analogue screen in CE

6.3.1 CE screen on analogues of SEL101066
Solubility tests of the compounds can be found in the Appendix A4. Due to low solubility, fluspirilen was screened at 100µM and phenoxazine was screened at 200µM. All others were screened at 300µM. 2-chlorophenothiazine and phenothiazine were ranked as having low solubility (Appendix A4) and for this reason not further evaluated by CE assay.

Figure 6.2 shows an electropherogram of the analogues screened using CE assay. In the assay the injection sample contains carboxyfluorescein (10pM) and p53-F (500pM) in TAPS/Tris (1mM) pH 8.0, DTT (1mM). The separation sample contains MDM2-N...
Initially p53-F (500pM) was run on its own as a control to record the migration time of p53-F (Trace 1 in Figure 6.2). This was then followed by running p53-F in the presence of MDM2-N (160nM) and the migration time of p53-F/MDM2-N was recorded (Trace 3 in Figure 6.2). The compounds were then screened and the MDM2-N control was run in between each compound screen. A % inhibition for every compound was calculated using the equation:

$$\% \text{ Inhibition} = 100 \left( \frac{T_H - T_L}{T_H - T_R} \right)$$

$T_H$ is migration time of p53-F peak in the presence of MDM2-N.
$T_R$ is the migration time of p53-F peak.
$T_L$ is the migration time of p53-F peak in the presence of MDM2-N + compound.

The results are summarised in Table 6.1 with calculated % inhibition. Phentolamine HCl (300µM) showed no inhibition whereas fluspirilene (100µM) showed a high inhibition of ~60%. However, fluspirilene contained a complex structure (see Table 6.1) with a low solubility so no further evaluation was carried out on this compound.

Both phenoxazine and carbazepine contained the similar motif of three rings linked together. Carbazepine only gave a 10% inhibition, whereas phenoxazine gave ~40% inhibition. It may be possible that the oxygen atom present in the phenoxazine ring gives rise to a hydrogen bond interaction in the binding site, indicative of the higher inhibition found for phenoxazine.

3-hydroxydiphenylamine, 4-hydroxydiphenylamine and diphenylamine were smaller fragments with high solubility and these fragments gave 40% - 60% inhibition at 300µM.

3-Hydroxydiphenylamine was found to have the highest inhibition of 66%, which was repeatable. This suggests the inhibition was improved when an OH group was in the 3-position. 3-Hydroxydiphenylamine was also a fragment of phentolamine, however phentolamine was found to be inactive in CE (Table 6.1). The results suggest that the
extra diazole group present in phentolamine HCl may cause the inactivity of the compound.

Phenothiazine type compounds are rigid in structure and not as flexible when compared to diphenylamine. Therefore the docking positions of phenothiazine type compounds are assumed to be limited. In contrast, the diphenylamine type compounds can freely rotate to fit tighter into the binding site.

Both 3-hydroxydiphenylamine and 4-hydroxydiphenylamine have good solubility as found in the solubility test (Appendix A4). Therefore titration was performed on these fragments and IC$_{50}$ determined (Section 6.3.2).

**Figure 6.2 Electropherograms of the analogues screening in CE.**

The first trace is the control with p53-F alone followed by the individual compound at the curves indicated. Control runs with 160nM MDM2-N were carried out between each compound screen.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration tested</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; repeat</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbazepine</td>
<td>300µM</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Fluspirilene</td>
<td>100µM</td>
<td>60%</td>
<td>53%</td>
</tr>
<tr>
<td>Phentolamine HCl</td>
<td>300µM</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>4-Hydroxy-diphenylamine</td>
<td>300µM</td>
<td>40%</td>
<td>65%</td>
</tr>
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<td>Diphenylamine</td>
<td>300µM</td>
<td>42%</td>
<td>31%</td>
</tr>
<tr>
<td>Phenoxazine</td>
<td>200µM</td>
<td>43%</td>
<td>25%</td>
</tr>
<tr>
<td>Concentration tested</td>
<td>% Inhibition</td>
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<td>2&lt;sup&gt;nd&lt;/sup&gt; repeat</td>
</tr>
<tr>
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<td>--------------</td>
<td>------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>2-Chlorophenothiazine</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenothiazine</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phenylamino-benzoic acid</td>
<td>300µM</td>
<td>58%</td>
<td>28%</td>
</tr>
<tr>
<td>3-Hydroxy-diphenylamine</td>
<td>300µM</td>
<td>66%</td>
<td>67%</td>
</tr>
<tr>
<td>N-Phenyl-p-phenylenediamine</td>
<td>300µM</td>
<td>30%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1 Summary of results of the CE screen of analogues binding to MDM2
6.3.2 Titration of 3-Hydroxydiphenylamine (SEL101266), 4-Hydroxydiphenylamine (SEL101267)

Titrations of 3-hydroxydiphenylamine (SEL101266) and 4-hydroxydiphenylamine (SEL101267) were carried out. They were titrated from 1 – 1000µM and 0.1 – 900µM respectively. From the titration curve the % inhibition was above 90% for both compounds (Figure 6.3). This reveals it is possible for both fragments to shift the p53-F peak back to its original position. The diphenylamine fragments contain a rotatable bond, suggesting the off rate may be slower than the rigid structures of SEL101069 and SFL001328 (Section 6.7).

From the dose response curve the IC\textsubscript{50} was calculated to be 362µM for SEL101266 and 116µM for SEL101267 (Figure 6.3). The affinity of SEL101267 was found to be higher than SEL101266 and when looking at the docking by AutoDock (Figure 6.3), SEL101266 occupied the Leu26 pocket and interacts with Val93 and His96 through hydrogen bonding. Whereas, SEL101267 fragment interacts with MDM2-N by occupying both Leu26 and Trp23 pockets. The models suggest a strong affinity may be obtained by binding deep into the hydrophobic pockets (Figure 6.4 bottom).

Figure 6.3 Titration of SEL101266 (Left) and SEL101267 (Right).
The fragments were titrated in the CE using MDM2-N (500nM) and p53-F (500pM). The % inhibitions were calculated from the CE traces and plotted into curve.
Figure 6.4 Docking models of SEL101266 and SEL101267.

3-Hydroxydiphenylamine (top) and 4-Hydroxydiphenylamine (bottom) are docked using AutoDock software into the MDM2-N binding site. MDM2-N protein structures are displayed in surface and the residues that can interact with the fragments are shown in stick. The interactions involved in fragment binding are indicated by dashed lines in black.
6.4 Analogue validation in FP assay

The diphenylamine fragments measured in the CE assay showed a high affinity and this was mimicked in the FP assay. The sensitivity of the FP assay was previously optimised, which enables the assay to be used in screening weak binding fragments. However, compared to the CE assay, the FP assay required a higher concentration of fragments in order to detect the binding.

The FP assay was performed using the method from the FP assay SOP (See section 4.6.4). SEL101267 showed the highest inhibition of 60% and SEL101266 showed an inhibition of 31%. The fragments; SEL101268, SEL101269 and SEL101270 were also validated as active using the FP assay. The data was analysed using ANOVA statistical analysis, which confirmed the fragments were significantly different from the MDM2-N control value (Figure 6.5 Bottom). The summary table in Table 6.2 showed the FP results correlates with the results obtained from the CE.

Ligand efficiency is the binding energy per atom for a ligand and it is a more useful concept to use for examining the druggability of the target (Hopkins et al., 2004). Using the equation 3.1 from Section 3.1.8, we are able to calculate the ligand efficiency for SEL101266 ($IC_{50} = 362\mu M$) and SEL101267 ($IC_{50} = 116\mu M$). The ligand efficiencies calculated for SEL101266 and SEL101267 are 0.34kcal/mol/atom and 0.38kcal/mol/atom respectively. These are the typical values for the contribution of a fragment like atom (Abad-Zapatero, 2007).

Despite these apparently encouraging results presented in Sections 6.3 and 6.4 using orthogonal assays of FP and CE as described in Section 6.6, it was shown using isothermal titration calorimetry and dynamic light scattering (in the following section) that the inhibitions seen in diphenylamine analogues are actually artefacts due to protein aggregation.
Figure 6.5 FP competition assay to screen diphenylamine fragments with MDM2-N.

Diphenylamine fragments were screened at 700µM in the presence of 500nM MDM2-N and 2nM p53-F. % inhibition was calculated using the equation in the FP assay SOP (Appendix). Bottom table shows the ANOVA analysis and $P < 0.05$ was considered as significant.
### 6.5 Analogue validation in ITC

Even though the CE and FP assay results were consistent, there were doubts about the sensitivity of the FP assay and a further biophysical technique was used to try and validate the binding results.

Isothermal Titration Calorimetry (ITC) is a technique that can study the binding interaction and the thermodynamic properties generated from an interaction between two or more molecules. ITC has the ability to ascertain the association equilibrium constant ($K_A$) of an interaction at different temperatures and the Gibbs free energy ($\Delta G_A$) correlates with the binding constant. Therefore, from the binding constant obtained, the other relevant thermodynamic parameters can also be determined by the following equations (Okhrimenko and Jelesarov, 2008):

1. \[ \Delta G_A = -RT\ln K_A \]

2. \[ \Delta G_A = \Delta H_A - T\Delta S_A \]

<table>
<thead>
<tr>
<th>Analogue</th>
<th>CE results</th>
<th>FP results</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL101266</td>
<td>66% at 300uM</td>
<td>31% at 700uM</td>
</tr>
<tr>
<td>SEL101267</td>
<td>52% at 300uM</td>
<td>60% at 700uM</td>
</tr>
<tr>
<td>SEL101268</td>
<td>36% at 300uM</td>
<td>33% at 700uM</td>
</tr>
<tr>
<td>SEL101269</td>
<td>30% at 300uM</td>
<td>8% at 700uM</td>
</tr>
<tr>
<td>SEL101270</td>
<td>43% at 300uM</td>
<td>27% at 700uM</td>
</tr>
</tbody>
</table>

Table 6.2 Overview of screening results from CE and FP assays.

The diphenylamine fragments were screened in both assays and were summarised in the table with the % inhibition and concentration used.
Where $\Delta G_A$ is Gibbs free energy, $R$ is the gas constant, $T$ is the absolute temperature in Kelvin, $K_A$ is the association equilibrium constant, $\Delta H_A$ is the change in enthalpy and $\Delta S_A$ is the change in entropy.

Heat is often generated or absorbed when molecules interact and this can be directly measured by ITC. The enthalpy change ($\Delta H$) is the relationship of the bonds created to bonds broken. If the energy produced by bond formation is greater than the energy required to break the bonds, then this will indicate a negative value of enthalpy change which favours the binding.

**6.5.1 Principle of ITC**

ITC has become a widely used biophysical technique to measure interaction in solution. The ITC instrument consists of a sample cell and a reference cell which are connected by a thermopile (Figure 6.6). During the ITC experiment the temperature in the cells was held constant and the reference cell was used to compare the change in heat occurring in the sample cell through a thermopile.

In the majority of the experiments ligand is injected into the protein and heat effects are observed by the heat of binding, the heat of mixing and the dilution of the protein and ligand.

The temperature differences between two cells are constantly monitored by the cell feedback network. A constant power is applied to the reference cell and this triggers the feedback network to apply the power to the sample cell to maintain the temperature difference caused by the reactions.
Figure 6.6 Schematic diagram of ITC equipment.

ITC contains the reference cell and sample cell where the temperatures between the cells are maintained by the cell feedback network. The syringe injects the ligand into the cell and the temperature difference between these two cells is constantly monitored.
6.5.2 Materials & Methods for ITC

The instrument model used in the ITC experiment was Auto-ITC HT Microcalorimeter (GE Healthcare). One vial of distilled H₂O was degassed and sonicated and carefully filled into the reference cell prior to the start of the experiment. Freshly prepared 2.5% Decon, degassed H₂O and methanol were added to the appropriate containers. The robot arms and the sample cell were initially washed with H₂O twice followed by Decon (2.5%), water and methanol. The samples were stored in 96 well 2ml round bottom plates in the cold cabinet at 4°C. The buffer solution used in the cell and the syringe were matched to prevent signal to noise problems. Organic solvent can lead to a large heat effect, therefore the DMSO concentration (1%) in both protein and ligand solution was matched. ITC experiments were performed using the parameters in Table 6.3.

<table>
<thead>
<tr>
<th>Cell temp.</th>
<th>No. Inject</th>
<th>Inject Volume</th>
<th>Inject Duration</th>
<th>Inject Spacing</th>
<th>Stirring (RPM)</th>
<th>Reference Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>19</td>
<td>14.83µl</td>
<td>29.7</td>
<td>200</td>
<td>250</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 6.3 Details of parameters selected for ITC experiment.

6.5.3 Nutlin-3 binding affinity determined by ITC

The interaction of MDM2-N with Nutlin-3 was measured by ITC (Figure 6.7). The interaction was performed at 298K. The interaction was initially performed using 15µM of Nutlin-3 with 1.5µM MDM2-N in the cell (Left panel in Figure 6.7). The data shown in Figure 6.7 (left panel) demonstrates decent signal levels using this condition (-5kcal/mole(injectant)). However the isotherm gave an incomplete sigmoidal curve with increasing molar ratio, which may be due to the low Nutlin-3 concentration used in the ITC experiment. Therefore, the ITC experiment was repeated using a more concentrated Nutlin-3 (25µM). The isotherm shown in Figure 6.7 (Right panel) for Nutlin-3 (25µM) gave a complete sigmoidal curve with increasing molar ratio. The determined value of the Kd is calculated based on the midpoint of the curve. The interaction of the Nutlin-3 gave a Kd of 52nM (Figure 6.7 Right panel). This is very
similar to the IC$_{50}$ (37.3nM) obtained by the CE assay (Section 5.4.1.2) and the Ki value (36nM) from published literature (Shangary et al., 2008).

From the isotherm the enthalpy of the interaction between MDM2-N and the racemic mixture Nutlin-3 is -5.1kcal/mol (Figure 6.7 Right panel). The enthalpy change is a negative value which indicates the enthalpy of the interaction favours the binding. The entropy of interaction between MDM2-N and Nutlin-3 is 16.3cal/mol/degree. The free energy of binding calculated using equation 6.2 gives -9.9kcal/mol at 298K in which the reaction is driven by both enthalpy and entropy.

In the Michelsen K. et al. paper, they have performed ITC experiments on the Nutlin-3a (Michelsen et al., 2012). Their results showed the interaction of the Nutlin-3a gave a Kd of 24nM which is half of the Kd that we have obtained for the racemic mixture Nutlin-3 (Kd = 52nM). The enthalpy of the Nutlin-3a interaction with MDM2-N is -10.2kcal/mol and entropy is 0.67cal/mol/degree. The free energy of binding is -10.4kcal/mol which suggests that the reaction is driven by enthalpy.

A higher entropy value in the ITC experiment of racemic mixture Nutlin-3 is caused by the inactive Nutlin-3 being present in the system. As discussed in section 1.5, there is a lid present at the MDM2/p53 binding site that is responsible for controlling the stability of the MDM2/p53 complex. In our ITC studies, the lid at the N-terminal domain of MDM2 may be in an open state which resulted in an increase of entropy to the system. The Nutlin-3a on the other hand may be stabilizing the lid by allowing the lid to interact with the side of the hydrophobic cleft which had been suggested by Showalter et al. paper and thereby reducing the entropy of the system (Showalter et al., 2008).
Figure 6.7 The ITC isotherm of MDM2-N/Nutlin-3 interaction.
The ITC experiments of Nutlin-3 titration were performed in two concentrations (15µM and 25µM). Top panel shows the energy data of the baseline sample against time. The bottom panel shows the ITC injection fit and plotted in kcal/mol against Molar ratio of protein and Nutlin-3.

6.5.4 Fragments screening by ITC
The ITC method developed for MDM2-N was then used to verify the interaction with the diphenylamine analogues. Figure 6.8 shows the raw data of the diphenylamine analogues screened in ITC. The fragments were injected at 1mM concentrations, with no significant heat signal present with increasing molar ratio. The heat signal level in the control of injecting buffer into buffer (Figure 6.8 bottom trace) is equivalent to heat signals seen in fragment screening.
One possibility for the lack of heat signal may be caused by the low fragment concentrations used, since these fragments are of weak affinity. Therefore, a much higher concentration of fragments will be required in order to detect the heat of binding. However it is not possible to further increase the fragment concentration in ITC due to the limit of solubility. An alternative competition assay was developed in order to identify the fragment/protein interaction at a lower concentration.

![Figure 6.8 ITC analysis on diphenylamine fragments interaction with MDM2-N.](image)

The image shows the energy data of the diphenylamine fragments (1mM) interaction with MDM2-N. Bottom trace shows the energy data of the control buffer into buffer. The energy data was plotted in sample against time.

**6.5.5 ITC competition assay to detect fragment interaction**

When using ITC for direct measurement of MDM2-N and fragment binding the data indicates no heat change in the cell. This is caused by the low concentration of the fragments used, which could not be increased due to the solubility issue of the fragments. The ideal conditions to determine low affinity ligand sometimes cannot be met. Low affinity binding often requires a high concentration of protein to obtain a detectable heat change, however this will result in a high ligand concentration.
Here, we describe the use of ITC to perform the competition experiments to study the weak binding affinity fragments. This involves injecting a known inhibitor in the presence of a fixed concentration of weak binding fragment. The fragment is incubated with the protein to pre-form the complex. The presence of known inhibitor will displace the fragment from the complex with protein. The difference between the thermodynamic of known ligand and the thermodynamic coupling of two ligands can be used to obtain the thermodynamic of the weak binding fragment. The fragment binding can be determined by the apparent binding constant obtained.

In this experiment the syringe containing Nutlin-3 (25µM) and SEL101267 (350µM) was injected into the cell with MDM2-N (1.5µM) and SEL101267 (350µM). The raw data in Figure 6.9 top shows that injecting SEL101267 with Nutlin-3 gave no signal. The Figure 6.9 bottom shows the control experiment of Nutlin-3 titration. In the presence of SEL101267 (350µM), there was no heat signal present upon the first injection and with increasing molar ratio. However the heat signal was present in the control experiment of Nutlin-3 titration when SEL101267 (350µM) is absent (Figure 6.9 bottom). The Kd and ΔH obtained from the control experiment showed no significant difference (Figure 6.10) compared with the data obtained from the previous ITC titration (Figure 6.7). From this result we can confirm that MDM2-N is still active and has not aggregated. The surprising result that showed no binding for the SEL101267/Nutlin-3 suggests that the fragment may have aggregated the protein during the incubation in the 96 well plate.
Figure 6.9 Raw data of (top) SEL101267 (350µM) carried out in ITC competition assay. Green trace shows the competition ITC assay with Nutlin-3 injected into the cell containing MDM2-N and SEL101267 (350µM). Purple trace is the energy data of the ITC control experiment of Nutlin-3 interaction with MDM2-N.
Figure 6.10 ITC binding isotherm of the control experiment with Nutlin-3.

**Top panel**) Shows the energy data of the baseline sample against time.

**Bottom panel**) The ITC injection fit plotted in kcal/mol against Molar ratio of MDM2-N and Nutlin-3.

To monitor if the aggregation may be caused by the high concentration of the fragments, the concentration of SEL101267 was reduced from 350µM to 100µM for the ITC competition experiment. A clear heat signal can be seen in the presence of Nutlin-3 (25µM) and SEL101267 (100µM) in Figure 6.11. There is a clear difference in the heat signals between the ITC titration in the presence and absence of SEL101267 (100µM). The isotherm shows that when SEL101267 (100µM) is present the heat signals reach saturation at a higher rate with lower molar ratio. However it is quite surprising that the fragment is able to displace the tight binding inhibitor Nutlin-3 with only 100µM concentration. The most likely explanation for this shift in the saturation
curve (Figure 6.11) is again that the protein is partially aggregated by SEL101267 and
the available concentration of MDM2 has been reduced. The protein aggregation rate
will increase with increasing fragment concentrations (Figure 6.9).

The same effect was also seen when assessing the related ligand SEL101266 (100µM)
in the ITC competition assay. In Figure 6.12 the saturation binding curves in the
presence of SEL101266 (100µM) could also be explained by aggregation of MDM2-
N.

**Figure 6.11 ITC isotherm of the MDM2-N/SEL101267 (100µM) interaction in
competition form.**

Black trace is the ITC competition experiment. Nutlin-3 was titrated into the cell containing
SEL101267 (100µM) and MDM2-N.

Green trace is the ITC control experiment of Nutlin-3 titration in the absence of SEL101267
(100µM).
Figure 6.12 ITC isotherm of the MDM2-N/SEL101266 (100µM) interaction in competition form.

Black trace is the ITC competition experiment. Nutlin-3 was titrated into the cell containing SEL101266 (100µM) and MDM2-N.

Green trace is the ITC control experiment of Nutlin-3 titration in the absence of SEL101266 (100µM).
6.6 Study of MDM2-N aggregation caused by diphenylamine fragments in the DLS

In order to examine protein aggregation caused by the fragments, Dynamic Light Scattering (DLS) can be used. DLS is a technique that can determine the hydrodynamic size of macromolecules in solution. The phenomenon of protein aggregation caused by diphenylamine analogues was studied in the DLS. SEL101266 and SEL101267 were incubated with MDM2-N (64µM) in protein buffer (50mM HEPES pH 7.5, 100mM NaCl, 1mM DTT) at various concentrations for 30 minutes on ice. Control experiments were also set up using the negative control SFL001328 and the published inhibitor Nutlin-3 as a positive control.

If we first look at the control experiments in Figure 6.13, the DLS profile of size distribution shows a peak at 5.3nm hydrodynamic radius representing the dominant species. A radius of 5.3nm is consistent with the hydrodynamic radius of monomer MDM2-N. In the profile, the hydrodynamic radii of the peaks for these samples was also shown to be 5.3nm when SFL001328 and Nutlin-3 were incubated with MDM2-N.

MDM2-N is stable when incubated with various concentrations of SFL001328 (Figure 6.13 top) or Nutlin-3 (Figure 6.13 bottom). There is no change in the hydrodynamic size of the protein, however an increase of size is seen when 10µM Nutlin-3 was added which suggests aggregation of Nutlin-3 at 10µM concentration.
Figure 6.13 DLS size distribution by mass profile of MDM2-N incubated with various concentrations of SFL1328 fragment (Top) and Nutlin-3 (Bottom).

The profile shows the hydrodynamic size of the molecules present in the solutions. MDM2-N is displayed as monomer in the solutions with 5.3nm size after incubating with SFL001328 (Top) and Nutlin-3 (Bottom).
Figure 6.14 top and bottom shows the size distribution by mass for MDM2-N incubated with SEL101266 and SEL101267 in the protein buffer on ice. In the absence of the fragments the hydrodynamic radii of MDM2-N were ~5.3nm. However when the diphenylamine fragments were present, large molecules are formed in the solution with hydrodynamic radii over 50nm. The molecules with radii of over 50nm indicate that large molecules are present in the solution. There were only small molecules with 0.1 – 0.3nm hydrodynamic radii present when running SEL101267 on its own in DLS (Figure 6.15). This means that the diphenylamine analogues alone do not form large molecules. These results have validated the hypothesis of MDM2-N forming large molecules by the diphenylamine analogues in the solution.
Figure 6.14 DLS size distribution by volume profile of MDM2-N incubated with various concentrations of SEL101266 (Top) and SEL101267 (Bottom) fragments. The profiles are shown in volume (%) against hydrodynamic size (d nm). Large hydrodynamic size molecules are formed after incubating with SEL101266 and SEL101267.
Figure 6.15 DLS mass distribution by volume profile of SEL101267 fragment.
The profile shows the hydrodynamic radius of molecules present in the solution. Only small size molecules are present when dissolving SEL101267 at concentrations of 400µM to 800µM.

6.7 Identification and characterisation of oxindole analogues

There are different potent inhibitors available for MDM2-N with structures having common features. The structures are mainly composed of multiple hydrophobic rings that occupy the three hydrophobic pockets in the MDM2-N binding site. In Mi63 inhibitor there is an oxindole present in the structures and it is also present in the isoindolinone and WW298 inhibitors (Figure 6.16) (Shangary et al., 2008, Hardcastle et al., 2011, Popowicz et al., 2010).
From the previous FP and CE assays (Section 5.4.1.5), we have identified two oxindole analogue fragments that showed an interaction with MDM2-N (Figure 6.17). These fragments showed 40% inhibition (SEL101069 (300µM)) and 27% inhibition (SFL001328 (300µM)) in the CE assay (Chapter 5). Additionally, the DLS data shows SFL001328 did not aggregate MDM2-N (Figure 6.13). Therefore, we can confirm the oxindole analogues as genuine hits.
Additional CE experiments were performed to further evaluate the affinity of these fragments. SEL101069 was titrated from 0.01µM – 1000µM and SFL001328 was titrated from 25µM - 750µM. The results of both Nutlin-3 and unlabelled p53 peptide titrations showed that the p53-F peak shifted all the way back to its original position and gave an inhibition of ~90% (Chapter 5 Figure 5.8 & Figure 5.11). However, in both SFL001328 and SEL101069 titration curves the maximum inhibition found was ~40% (Figure 6.18 top and bottom). The curve reached a plateau at ~40% inhibition and the inhibition did not increase any further when the compound concentration was further increased.

Two factors may contribute to this partial inhibition:

1. The compounds may aggregate at higher concentration. The aggregated compound may have sequestered around the binding site to cause inhibition. Alternatively the compounds may simply aggregate in solution at higher concentrations.

2. Every compound has a different binding kinetic when binding to the binding site. The molecular properties of compound may affect the kinetics of the compound (Miller et al., 2012). SEL101069 and SFL001328 are low molecular weight compounds with no rotatable bonds so these compounds may have a fast off rate.

The IC_{50} was calculated to be 352µM for SEL101069, whereas SFL001328 was calculated to be 242µM. SFL001328 is a fragment of SEL101069 but the affinity of SFL001328 was higher than SEL101069. It is possible that the nitrogen atom in the oxindole is required to be positioned at the 2-indolinone position to give tighter binding. The nitrogen in SEL101069 is at the 2-indolinone position which could be a reason for it being less potent than SFL001328.

The Hill slope in the SEL101069 titration curve was found to be steep with a value of 9.8. This indicates a rapid increase in inhibition which can be indicative of aggregating compounds (Feng and Shoichet, 2006). Aggregation can be inhibited using detergents. Therefore further controls need to be run to confirm if the inhibition is due to colloidal aggregation. A trial experiment was carried out by adding 0.005% Tween-20 into the separation solution with MDM2-N plus compound. However no
change in the dose response curve was observed (data not shown). An experiment was also carried out by adding Nutlin-3 (40nM) along with SFL001328 (700µM) and MDM2-N (160nM) and a further increase of inhibition was found (Results not shown). This increase in inhibition suggests the SFL001328 did not fully occupy the protein binding site and there was still unbound protein present in the solution.

Figure 6.18 Dose response curve of SFL001328 (top) and SEL101069 (bottom). The titration curves of SFL001328 and SEL101069 were fitted using GraphPad prism and IC50s were calculated from the titration curves.

In order to analyse whether these fragments are worth developing into leads, the ligand efficiency (LE) was calculated using the equation 3.1 from Chapter 3 and the IC50 was calculated from Figure 6.18. SEL101069 contains 17 non-hydrogen atoms and SFL001328 contains 10 non-hydrogen atoms. LE calculated for SEL101069 and
SFL001328 are 0.28kcal mol$^{-1}$ per non-hydrogen atom and 0.5kcal mol$^{-1}$ per non-hydrogen atom respectively. There is no improved ligand efficiency with the additional phenyl ring in the SEL101069. Further analogue exploration on the oxindole fragment will require further chemistry to identify analogues with improved ligand efficiency.

### 6.8 Oxindole analogue exploration

Further analogue exploration was carried out to identify more analogues of the oxindole fragment. Oxindole analogues were searched using eMolecules ([http://www.emolecules.com/](http://www.emolecules.com/)) and there were many compounds identified containing a similar motif.

Some analogues found are listed in **Table 6.4**, these analogues contain different heavy atoms on the side chain. Compounds 1 and 6 contains an extra methyl group at the 7 and 2 positions, compound 2 contains fluorine at 5 position, compounds 3 and 5 contain nitrate positions at 5 and 6, compound 4 has introduced a nitro group at 5 position, compound 7 contains a OH group at 5 position and compound 8 contains a chlorine atom at 6 position. Different heavy atoms were selected at different positions on the oxindole group to assist in understanding the change in the affinity and help in identifying a higher affinity oxindole analogue.

Only compound 8, 6-chloro-2-oxindole, was available in the Selcia Compound Library and could be used for analysis in the CE and FP assays immediately (Selcia number SEL101884). It should be noted that SEL101884 contains the same structure as the substructure in the published Mi63 inhibitor which has an Ki of 3nM (**Figure 6.16**) (Shangary et al., 2008).
Table 6.4 Analogues of SFL001328 identified using eMolecules.

Analogue of SFL001328 was explored using online similarity search tool eMolecules (http://www.emolecules.com). The structures of the SFL001328 analogues were listed in the table with the similarity score.
The solubility of SEL101884 was examined using a turbidimetric test and a solubility > 500µM was confirmed (Appendix A4). Initially the fragment was tested in the FP assay at 200µM and showed an 8% inhibition. When screening the fragment in the CE assay 24% inhibition was found at 100µM concentration (Table 6.5). Compared with SFL001328, which was tested at 300µM, SEL101884 achieved a similar inhibition with a concentration of 100µM (Table 6.5). A similar result was also discovered when screening the fragments in the FP assay (Table 6.5). This suggests the efficiency of interaction has been improved by three fold with the addition of a chlorine atom at 6 position.

<table>
<thead>
<tr>
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<th>% Inhibition</th>
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<tr>
<td></td>
<td>CE assay</td>
<td>FP assay</td>
<td></td>
</tr>
<tr>
<td>SFL001328</td>
<td>27.7% at 300µM</td>
<td>8% at 500µM</td>
<td></td>
</tr>
<tr>
<td>SEL101884</td>
<td>24% at 100µM</td>
<td>8% at 200µM</td>
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Table 6.5 Results of SEL101884 in CE and FP assays.
6.9 Discussion

This chapter describes the use of fluorescence polarization (FP), capillary electrophoresis (CE) and isothermal titration calorimetry (ITC) to validate the interaction of fragments with MDM2-N. Fragments possess weak binding affinities and hence require multiple orthogonal techniques for validation. Initial studies on the diphenylamine series fragments in the FP and CE assays showed convincing results. However, when studying the diphenylamine fragments in the DLS and ITC, the results showed that the diphenylamine compounds appear to aggregate the MDM2-N protein in solution (section 6.6). This fragment induced protein aggregation can be difficult to detect by the competition studies in both the CE and FP assays.

In further tests to examine this aggregation effect isothermal titration calorimetry (ITC) and dynamic light scattering (DLS) were used. However it should be noted that there is a difference in the protein concentration used in ITC and DLS. In ITC the MDM2-N concentration used is 1.5µM, however the concentration used in the DLS is 64µM which is 42 times higher. The higher protein concentration used in the DLS may result in an increase of aggregation. The percentage of aggregation present in the DLS profile is unclear because if there are large aggregates present in the solution then this can mask the signals of the small molecules.

Currently, how these diphenylamine analogues aggregate the MDM2-N protein is still unclear. One hypothesis could be that the diphenylamine fragments first interact with the MDM2-N binding site and then the phenyl ring of the fragment stacks with phenyl rings of other fragments molecules, thereby creating an aggregate. The phenyl rings on the diphenylamine fragment can adopt different conformations and therefore may be able to interact with each other at different angles leading to protein aggregation (Baell and Holloway, 2010).

It was however possible to identify genuine fragment inhibitors which did not show this protein aggregation effect. When examining different potent published (mainly Nutlin-like) inhibitors for MDM2-N a similar motif in the structures was identified. One of the features is that the MI series, isoindolinone and WW298 inhibitors (Figure 6.16) all contain an oxindole group in the structure to mimic the Trp23 side chain on
p53 peptide. Consequently the oxindole analogues SEL101069 and SFL001328 were tested in the FP and CE assays and it was confirmed that they interacted with MDM2-N (Chapter 4 and 5). Further titrations of SEL101069 and SFL001328 were performed and IC₅₀ values of 352µM and 242µM respectively were obtained. SEL101069 and SFL001328 were docked into the MDM2-N binding site using AutoDock. The docking poses of SEL101069 and SFL001328 are shown in Figure 6.19 and Figure 6.20 respectively. The best docked pose for SEL101069 shows the phenyl ring inside the Trp23 pocket forming hydrophobic interactions with Phe86, while the oxindole ring sits in the Phe19 pocket and forms van der Waals interaction with Ile61. There are two oxygen atoms on SEL101069 that also forms van der Waals interactions with Leu54 and Val93.

The best docked poses for SFL001328 with MDM2-N (Figure 6.20) shows the oxindole docked into the Trp23 pocket, forming a hydrophobic interaction with Phe86. The NH atom on the SFL001328 also forms a hydrogen bond interaction with Phe54. This additional hydrogen bond may explain why SFL001328 binds more tightly in the Trp23 pocket than SEL101069. From the published structure of MDM2-N with Mi63 (Figure 6.21), the oxindole substructure also fits in the Trp23 pocket and interacts in the same position as predicted by AutoDock.
Figure 6.19 Docking poses of SEL101069 in MDM2-N.

SEL101069 was docked into MDM2-N binding site using AutoDock. The arrows indicate the name for each hydrophobic groove. The black dashed lines show the distance between the compound and the residues.
Figure 6.20 Docking poses of SFL001328 in MDM2-N.
SFL001328 (pink stick) is docked into Trp23 pocket in MDM2-N binding site. The distance between the fragment and the residues are shown in black dashed lines.

Figure 6.21 Interaction of Mi63 with MDM2-N
The diagram shows the X-ray crystal structure of MDM2-N with Mi63 inhibitor bound (3LBL; (Popowicz et al., 2010)).
Further analysis of 6-chloro-2-oxindole showed the binding efficiency has been improved by three fold and this suggests that the addition of chlorine atom at the 6 position allows the fragment to bind deeply into the Trp23 pocket as shown in Figure 6.22.

These are encouraging results and provide a useful starting point for the design and synthesis of tighter binding analogues.

**Figure 6.22 Interaction of 6-chloro-2-oxindole with MDM2**

The diagram shows the 6-chloro-2-oxindole bind into the MDM2-N Trp23 pocket and the chlorine atom binds deeply into the pocket.
Chapter 7 Conclusion and forward look

7.1 Overall conclusions of the work

The original aim of this work was to look into the feasibility of identifying small molecule inhibitors to inhibit protein-protein interactions (PPI). Currently as described in Chapter 1 there are a handful of examples demonstrating the design of PPI inhibitors. However the PPI inhibitors designed are often large complex structures and designing small molecules to bind to PPI sites holds many challenges. The PPI sites are often large and shallow which make them difficult to target by small molecules. This leaves an open question on whether low molecular weight fragments are too small to inhibit protein-protein interactions and whether fragment based drug design is possible for designing PPI inhibitors.

The PPI system selected for study is the MDM2/p53 interaction. In several cancer models MDM2 has been discovered to be overexpressed and inhibits the tumour suppressor activity in p53. Huge efforts have been put into restoring the p53 activities by the use of small molecules to inhibit MDM2 from binding. There are numerous inhibitors identified to target MDM2/p53 interaction and high resolution crystal structures of MDM2 with inhibitors bound are available (Zhao et al., 2013). These structures provided useful information that allowed us to select potential fragments to test whether smaller molecule compounds would inhibit the p53-MDM2 interaction.

A series of orthogonal assays were designed. In order to validate the interaction of small molecule inhibitors with the PPI of interest a series of assays had to be established. It has been known that fragment hits need to be validated by more than one method, thus different methods had to be set up to validate hits. In this work the assays which were applied for ligand studies are fluorescence polarization (FP), capillary electrophoresis (CE) and isothermal calorimetry (ITC). There are only a few techniques that are capable of screening fragments against PPIs and CE is one of the few techniques that offers fragment based drug discovery on PPIs (Austin et al., 2012). CE is a solution based technique that uses low sample consumptions to detect weak affinity interactions and the technique is emerging in the small molecule drug discovery (Austin et al., 2012, Espada and Molina-Martin, 2012, Rauch et al., 2013). This work is one of the few published studies that have used a CE assay for the
identification of small molecular ligand-receptor interactions. As far as we know this is the first reported study using CE assay on MDM2/MDMX drug discovery. When screening the small molecules against MDM2-N/p53 in the FP and CE assays it was shown that the FP assay requires an approximately 2 fold higher concentration of compound in order to detect the inhibition (see section 5.4.1). This indicates that the FP assay is less sensitive than the CE assay in detecting weak ligand binding which has also been shown in Austin et al. paper where the FP assay was not able to pick up weak affinity interactions (Austin et al., 2012). The IC50 of Nutlin-3 calculated from the CE titration is in agreement with the published value, demonstrating its high accuracy. The high sensitivity and accurate results obtained using the CE assay indicates that it is a very promising tool for ligand discovery. The results presented in the thesis provided the first example of combining the FP and CE assays for p53-MDM2 and p53-MDMX ligand screening and demonstrate the importance of validation with different techniques.

**Problems with assay sensitivity and low signal.** When developing the assays there were several issues that needed to be tackled. The fluorescently labelled p53 peptide (p53-F) showed a low fluorescent signal when running in the FP and CE assays. This required the use of high concentrations of p53-F and MDM2-N and greatly reduced the sensitivity of the assays. It was eventually discovered that the main reason for low signal was that the p53-F was sticking to the pipette tips and Eppendorf tubes. In order to improve the fluorescent signal of p53-F siliconised vials/tips were used which prevent absorption of p53-F onto the plastic. This improved the effective concentration by a factor of ~1000 and hence significantly improved the sensitivity of the assays.

A related problem that often arises during ligand screening is compound interference that can mask genuine hits as well as result in false positives especially when screening at high concentration of weak fragments. Compound interference can arise from intrinsic fluorescence of the compound, especially when a low concentration of fluorophore was used in the screen (e.g. 2nM p53-F) with a high concentration of compound (e.g. 500µM). In this work it was particularly important to test and account for any compound fluorescence. Another problem that can arise during ligand screening is compound induced protein aggregation which can result in false positive
hits. Above certain concentrations some compounds appear to have the ability to and aggregate protein through an unknown mechanism (McGovern et al., 2003).

**Select fragment based on known inhibitors.** There are different potent inhibitors available for MDM2-N with structures having common features. In Mi63 inhibitor there is an oxindole motif present in the structure and it is also present in the WW298 and isoindolinone inhibitors (Shangary et al., 2008, Popowicz et al., 2010, Hardecastle et al., 2011). Using the CE and FP assays the oxindole fragment (SFL001328) was identified with 28% (300µM) and 8% (500µM) inhibitions respectively and using the CE assay an IC\textsubscript{50} of 242µM was determined. Docking model of SFL001328 showed the fragment buried deeply into the Trp23 pocket in MDM2-N and forms a hydrogen bond interaction with the Leu54 (Figure 7.1). This binding interaction mimics the binding mode of the oxindole subgroup in the Mi63 and WW298 inhibitors.

![Figure 7.1 Binding conformation of oxindole fragment (SFL001328)](image)

Oxindole fragment was docked into the MDM2-N binding site using AutoDock (Morris et al., 2009). MDM2-N binding site is represented using surface form and the name for the three hydrophobic grooves are pointed by the arrows. The oxindole fragment is shown as pink stick and the hydrogen bond interaction is displayed using black dash.
Further fragment studies using 6-chloro-2-oxindole (SEL101884) showed that the addition of a chlorine atom at 6-position improves the efficiency of interaction by three fold.

**Virtual screening to identify small molecule inhibitors.** In these studies we have employed virtual screening as an approach for ligand discovery and the hits identified were further evaluated by fragment identification. Virtual screening is a computational approach that is able to analyse millions of compounds stored in databases using high throughput computational docking. As part of the project an integrated virtual screening script CODASS, that combines multiple virtual screening programs to reduce the run time and maximises the enrichment, was used. Initially 1.1 million compounds in the EDULISS library were screened against MDM2-N (PDB: 3LBL) and the top 266 compounds from the rank were kept. These top scoring hits were then screened “visually” by Dr. Simon Pettit (Chemist at Selcia) and 6 compounds were selected based on the criteria listed in section 3.2.2. However this is a biased approach and a better approach would be to screen top hits blind but there is a cost implication with screening too many compounds.

Subsequent screening of these compounds in MDM2-N/p53 FP and CE assays showed 5 out of 6 compounds were active in both assays indicating the high hit rate of CODASS. The inactive compound was screened at 50µM only, therefore we cannot conclude that the compound does not bind. It may have weak interaction and requires a higher concentration to detect. This MDM2 study therefore provides an excellent example of the power of virtual screening using the CODASS routine. In this case showing a hit rate of 80%. This is well above the expected “industry standard” (Houston and Walkinshaw, 2013). The docking models were generated by AutoDock to provide the insight into the binding conformation of these hits. The compounds shared a common property of using a core imidazoline group as centre to stretch three hydrophobic rings into the Phe19, Trp23 and Leu26 pockets (Figure 7.2). This is generally caused by the hydrophobic pocket of MDM2-N and results in compounds being less soluble which is also evident in the well-known MDM2 inhibitors such as Nutlin-3 and MI219. The virtual screening hits showed no hydrogen bond interaction with the MDM2-N and the interactions are mainly driven by hydrophobic bonds.
Figure 7.2 Virtual screening hits showed similar binding conformation with the well-known inhibitors.

This is one of the virtual screening hits that showed similar binding conformation with the well-known inhibitors. MDM2-N is represented in surface form and the virtual screening compound is shown in red stick.

**Fragments exploration identified false positive hits.** Protein forming large molecules was found to be a particular problem with the family of diphenylamines described in **Chapter 6**. In both the FP and CE assays convincing dose-response curves were obtained. However it was not until dynamic light scattering (DLS) and isothermal titration calorimetry (ITC) studies were carried out that it was apparent that the compounds were causing protein to form large molecules and the apparent low FP signal was due to large molecules and not because of specific protein binding. Similarly the shift of the p53 peak in the CE assay was caused by release of p53-F upon protein multimerization. It is likely that the diphenyl fragment binds non-specifically with MDM2-N and intermolecular aromatic stacking between diphenyl molecules causes large molecules formation. Protein aggregation caused by compounds is often inevitable and incurs wasted resources when followed up in lead design. This problem often arises during high throughput screening (HTS) and fragment based drug discovery (FBDD) and efforts have been put in to minimise the
false positives. There are filters developed which filter out functional groups that are reactive or prone to aggregate (Rishton, 1997, Baell and Holloway, 2010). During compound screening it is always important to run DLS on protein with compound as a control to rule out compounds that cause protein aggregation.

**MDMX as a target to design a dual inhibitor.** It has been confirmed that MDM2 and MDMX are both negative regulators of the tumour suppressor p53 (Marine and Jochemsen, 2004). These proteins use their highly similar N-terminal domain to bind to the N-terminal transactivation domain of p53 and inhibit p53 transcriptional activity. In order to identify a dual binding inhibitor to inhibit both MDM2 and MDMX, we used a reduced complexity subset EDULISS library comprising of 518,000 compounds to screen against MDM2-N and MDMX-N in the CODASS. The chemical structures of the top 1000 ranked hits showed they do not possess the chemical scaffold that was seen in the published MDM2 inhibitors. In fact these compounds are more flexible and do not contain the imidazoline centre to link multiple hydrophobic rings. It was also noted that most of these compounds do not bind into MDMX-N Leu26 pocket where the discrepancy between MDM2 and MDMX is. Using a specific set of criteria discussed in section 3.2.2, there were 7 compounds selected from the top 1000 ranked hits to validate the binding against MDM2-N and MDMX-N. Using the FP and CE assays SEL101876 showed a relatively high inhibition against MDM2-N and MDMX-N. This result still needs to be repeated using a fully optimised MDMX-N CE assay condition. Unfortunately there was not enough time to optimise the assay and test all the compounds. The identification of the novel dual action inhibitor SEL101876 shared little similarity with Nutlin-3 and MI-63 inhibitors. The binding model showed the piperidinyl morpholine group in SEL101876 occupies the Leu26 pocket in the MDM2-N binding site to provide stacking interaction with the His96 ring (Figure 7.3 left), while the two phenyl rings bind into Phe19 and Trp23 pockets. However a different binding approach of SEL101876 was found when binding into the MDMX-N binding site (Figure 7.3 right). The phenyl rings in SEL101876 bind into the Trp23 pocket and interact with MDMX-N Met53 residue. The piperidinyl morpholine group does not interact in the MDMX-N Leu26 pocket. Instead it interacts with the MDMX-N Phe19 pocket to form hydrogen bond interaction with Gln71 residue. This is absent in MDM2-N which may explain the reason of stronger
inhibition seen in the assays. The data presented in this work suggests that evading the interaction with Leu26 pocket and providing hydrogen bond interaction between the compound and MDMX-N are required for developing a higher affinity dual inhibitor. Besides the peptide inhibitor found by Chang et al. there are only two examples of dual MDM2/X small molecule inhibitors in the literature (Graves et al., 2012, Popowicz et al., 2010, Chang et al., 2013). This new chemical scaffold allows further optimisation to develop a more potent MDM2/X-N inhibitor.

![Figure 7.3 Binding conformation of SEL101876 in MDM2 (left) and MDMX (right).](image)

The docking models of SEL101876 with MDM2-N (PDB structure 3LBL) and MDMX-N (PDB structure 3LBJ) showed that SEL101876 does not bind to the Leu26 pocket when it interacts with MDMX-N. There is a hydrogen bond formation (black dash line) on the piperidinyl morpholine group with Gln71-MDMX-N.

### 7.2 Forward look

In this work a dual action inhibitor SEL101876 was identified. In order to understand how SEL101876 interacts in the MDM2-N and MDMX-N binding site, X-ray co-crystal structures should be obtained. This was originally planned as part of the study, however there was not enough time to complete. As described in Chapter 2 the MDM2-N\textsuperscript{11-130} construct used in the assays contains an unstructured tail and also extra residues at the N-terminal that causes crystallisation to be impossible. Sequence alignment of MDMX-N\textsuperscript{14-111} with the published crystal structures’ sequences (Figure
2.2) showed that MDMX-N\textsuperscript{14-111} contains extra residues at the N-terminal that may affect the crystallisation.

Attempts on expression and purification of truncated MDM2-N\textsuperscript{18-111} and MDMX-N\textsuperscript{23-109} constructs were unsuccessful. In the MDM2-N\textsuperscript{18-111} construct the N-terminal His-tag linked by the TEV cleavage site was discovered to be difficult to cleave. It is possible that the His-tag is buried into the binding site and hides the cleavage site. If the His-tag on the protein is not cleavable then this will cause difficulty in crystallising the protein. Therefore untagged or C-terminal His-tag constructs should be designed for crystallisation trials.

The dual action inhibitor SEL101876 identified using the CE and FP assays showed that this compound is novel and different from known published inhibitors. Quantitative structure-activity relationship (QSAR) should be carried out to characterise the activities in every subgroup. This will assist in the identification of the subgroup in SEL101876 required for MDM2/X-N interaction and more potent fragments can be developed. In addition, with the combination of X-ray crystallography it is possible to add in hydrophilic subgroup into the fragment to improve the solubility of the compound. In the MDM2-N crystal structure there is a hydrophilic region between Gln72 and Lys94. Therefore extending to this region will provide additional interaction as well as improving the solubility and water accessibility.

MDM2/p53 is a typical PPI example representing a deep hydrophobic binding site. In this work the results have proven that virtual screening in combination with CE and FP assays can be used as a tool for fragment development and the small fragments can be active against PPI.
References


COULTER, B. 1994. Introduction to Capillary to Electrophoresis.


Appendix

Appendix A1. Protparam details

A1.1 His-tag MDM2-N^{11-130}

Number of amino acids: 138
Molecular weight: 15601.7
Theoretical pI: 7.13

Extinction coefficients:
Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.
Ext. coefficient 10430
Abs 0.1% (=1 g/l) 0.669, assuming all pairs of Cys residues form cystines

A1.2 MDM2-N^{11-130}

Number of amino acids: 121
Molecular weight: 13719.6
Theoretical pI: 6.33

Extinction coefficients:
Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.
Ext. coefficient 10430
Abs 0.1% (=1 g/l) 0.760, assuming all pairs of Cys residues form cystines
**A1.3 His-tag MDM2-N^{18-111}**

Number of amino acids: 119

Molecular weight: 14153.3

Theoretical pI: 7.88

Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

Ext. coefficient: 16390

Abs 0.1% (=1 g/l): 1.158, assuming all pairs of Cys residues form cystines

**A1.4 MDM2-N^{18-111}**

Number of amino acids: 96

Molecular weight: 11185.1

Theoretical pI: 9.24

Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

Ext. coefficient: 10430

Abs 0.1% (=1 g/l): 0.932, assuming all pairs of Cys residues form cystines
### A1.5 His-tag MDMX-N\(^{14-111}\)

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<tr>
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<td>QINQVRPKLP</td>
<td>LLKILHAAGA</td>
<td>QGEMFTVKEV</td>
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<td>MHYLQYIMV</td>
<td>KQLYDQQE</td>
<td>MVYCGGDL</td>
<td>ELLGRQSFSV</td>
<td>KNPSPLYDM</td>
<td>RKNLVTAT</td>
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</tbody>
</table>

**Number of amino acids:** 119  
**Molecular weight:** 13313.3  
**Theoretical pI:** 9.17  
**Extinction coefficients:**

Extinction coefficients are in units of \(\text{M}^{-1} \text{ cm}^{-1}\), at 280 nm measured in water.  
Ext. coefficient \(7450\)

Abs 0.1\% (=1 g/l) \(0.560\), assuming all pairs of Cys residues form cystines

### A1.6 MDMX-N\(^{14-111}\)

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<td>KEVMHYLGQY</td>
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<td>FSVKNPSPLY</td>
<td>DMLRKNLVTAT</td>
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</tbody>
</table>

**Number of amino acids:** 102  
**Molecular weight:** 11431.3  
**Theoretical pI:** 8.81  
**Extinction coefficients:**

Extinction coefficients are in units of \(\text{M}^{-1} \text{ cm}^{-1}\), at 280 nm measured in water.  
Ext. coefficient \(7450\)

Abs 0.1\% (=1 g/l) \(0.652\), assuming all pairs of Cys residues form cystines
**A1.7 His-tag MDMX-N^{23-109}**

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</table>

**Number of amino acids:** 111  
**Molecular weight:** 13001.9  
**Theoretical pI:** 6.48  
**Extinction coefficients:**
Extinction coefficients are in units of M−1 cm−1, at 280 nm measured in water.  
Ext. coefficient 13410  
Abs 0.1% (=1 g/l) 1.031, assuming all pairs of Cys residues form cystines

**A1.8 MDMX-N^{23-109}**

<table>
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<td></td>
<td></td>
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</table>

**Number of amino acids:** 88  
**Molecular weight:** 10033.7  
**Theoretical pI:** 8.07  
**Extinction coefficients:**
Extinction coefficients are in units of M−1 cm−1, at 280 nm measured in water.  
Ext. coefficient 7450  
Abs 0.1% (=1 g/l) 0.742, assuming all pairs of Cys residues form cystines

---

**Appendix A2. SOP of optimised FP assay**

**A2.1 Equipment**

LJL Biosystems Analyst AD

**A2.2 Materials**

* Siliconised polypropylene microcentrifuge tubes volume 0.65ml: Sigma-Aldrich T3281-500EA.  
* HEPES: Sigma H4034
* Sodium Chloride: Fisher Scientific S/3160/63
* DTT: Melford MB1015
* 384 well, low volume, flat bottom, non-binding surface, black polystyrene plate: Corning cat No: 3820
* Nutlin-3: Tocris 3984
MW = 590.5
* Fluorescently labelled p53 derived peptide (p53-F)
5-FAM-QETFSDLWKLLP-OH
MW = 1835.03
Custom synthesis by Biomatiks.

A2.3 Preparation of stock solutions
*Always use siliconised pipette tips and siliconised vials when working with p53-F and MDM2-N*

1M DTT
1M stock of DTT was prepared by dissolving 5g of DTT into 32.4ml of H2O. Aliquots of 1ml were prepared and frozen in -20°C freezer.

Running Buffer (50mM HEPES pH 7.5, 100mM NaCl, 1mM DTT)
Weigh 5.95g HEPES, 2.92g NaCl and dissolve in 490ml of distilled H2O and adjust pH using 5M NaOH. Adjust final volume to 500ml with distilled H2O and degas/ filter using 0.2µm filter membrane and filter on a degas station. Add 500µl of DTT (1M) into the solution prior to the experiment.

Preparation of p53-F stock
Use siliconised pipette tips and siliconised vials when working with p53-F.
Weigh 2mg of p53-F powder and dissolve in 150µl of running buffer (Final concentration p53-F = 7.3mM). 70µl of p53-F (7.3mM) was diluted in 70µl running buffer to obtain 3.65mM stock. Wrap the vials with tinfoil and store in the -20°C freezer.
Preparation of p53-F (10µM) stock
Dilute p53-F stock (2.7µl, 3.65mM) with running buffer (997µl). Store aliquots of 5µl in siliconised tubes in the dark -80°C freezer.

Analyst multi-plate reader setting
Excitation/Emission filter: Fluorescein 485nm/530nm
G factor: 0.94
Z height: Middle of well
Detector counting: Smart read
Sensitivity setting: 2

Parameters for Analyst FP assay setting

A2.4 Sample preparation and plate set up for FP MDM2-N/small molecules screening
Initially MDM2-N, p53-F and compounds were diluted into the siliconised vials using the following steps:

**Dilution for p53-F**

Using siliconised tube and tips, add 1µl of 10µM stock into 9µl assay buffer (f/c = 1µM). Dilute 6µl of 1µM p53-F into 1494µl assay buffer to get f/c = 4nM.

**Dilution for MDM2-N**

Use siliconised pipette tips and siliconised vials when working with MDM2-N. Thaw frozen 1mg/ml MDM2-N (64µM) and dilute 12µl MDM2-N (64µM) with 738µl assay buffer (f/c = 1.02µM).

**Compounds stock**

Make 100mM compound DMSO stock from ~1mg dry powder. Add 5µl of 100mM stock into 5µl DMSO (f/c compound = 50mM in 100% DMSO). Screen the fragments at 500µM as the initial screening concentration depends upon the compound solubility.

**Assay plate set up**

For screening compounds against MDM2-N the samples were set up in the 384 wells plate using the following protocol:

A1 – Background buffer (50mM HEPES, 100mM NaCl, 1mM DTT, 1% DMSO). Add 0.5µl DMSO (100%) into 49.5µl assay buffer. Use a pipette to mix the mixture.

B1 – Background buffer (50mM HEPES, 100mM NaCl, 1mM DTT, 1% DMSO). Add 0.5µl DMSO (100%) into 49.5µl assay buffer. Use a pipette to mix the mixture.
C1 – Background buffer (50mM HEPES, 100mM NaCl, 1mM DTT, 1% DMSO, 1µM Nutlin-3). Add 0.5µl Nutlin-3 (100µM) into 49.5µl assay buffer. Use a pipette to mix the mixture.

D1 – Background buffer (50mM HEPES, 100mM NaCl, 1mM DTT, 1% DMSO, 700µM compound). Add 0.5µl compound (70mM) into 49.5µl assay buffer. Use a pipette to mix the mixture.

A2:A4 – p53-F only (50mM HEPES, 100mM NaCl, 1mM DTT, 2nM p53-F, 1% DMSO). 24.5µl assay buffer was first added into the well then followed by 0.5µl DMSO (100%). 25µl p53-F (4nM) was added last into the well and mix the mixture using a pipette.

B2:B4 – MDM2-N + p53-F (50mM HEPES, 100mM NaCl, 1mM DTT, 2nM p53-F, 500nM MDM2-N, 1% DMSO). 24.5µl MDM2-N (1.02µM) was added into the well then followed by 0.5µl DMSO (100%). 25µl p53-F (4nM) was added last into the well and mix the mixture using a pipette.

C2:C4 – MDM2-N + p53-F + Nutlin-3 (50mM HEPES, 100mM NaCl, 1mM DTT, 2nM p53-F, 500nM MDM2-N, 1µM Nutlin-3, 1% DMSO). 24.5µl MDM2-N (1.02µM) was added into the well then followed by 0.5µl Nutlin-3 (100µM). 25µl p53-F (4nM) was added last into the well and mix the mixture using a pipette.

D2:D4 – MDM2-N + p53-F + fragments (50mM HEPES, 100mM NaCl, 1mM DTT, 2nM p53-F, 500nM MDM2-N, 700µM fragment, 1% DMSO). 24.5µl MDM2-N (1.02µM) was added into the well then followed by 0.5µl fragment (70mM). 25µl p53-F (4nM) was added last into the well and mix the mixture using a pipette.

A2.5 Results interpretation

Background subtraction

Background was subtracted manually by subtracting parallel and perpendicular values with the background buffer values.

Background subtraction:
Parallel - background reading
Perpendicular - background reading
% Inhibition equation

Percentage of inhibition will be calculated for each compound screen. The equation used is:

\[
\% \text{ Inhibition} = 100 \frac{(A_F - A_U)}{(A_F - A_I)}
\]

- \( A_F \) is the anisotropy value of p53-F in the presence of MDM2-N.
- \( A_U \) is the anisotropy value of p53-F.
- \( A_I \) is the anisotropy value of p53-F in the presence of MDM2-N + compound.

---

**Appendix A3. MDM2-N CE SOP**

**A3.1 Equipment**
P/ACE\textsuperscript{TM} MDQ Capillary Electrophoresis System/ Beckman Coulter
Beckman Coulter 488nm laser module

**A3.2 Materials**

- * Siliconised 200\(\mu\)l pipette tips: VWR Europe Cat No. 613-0279
- * Siliconised 10\(\mu\)l pipette tips: VWR Europe Cat No. 613-0337
- * Siliconised polypropylene microcentrifuge tubes volume 0.65ml: Sigma-Aldrich T3281-500EA.
- * 0.2\(\mu\)M Single use syringe membrane filter: Satorius stedim ref: 17597
- * Bovine Serum Albumin (BSA): Sigma A2153
- * TAPS: Sigma T5130
- * Tris base: Sigma T1503
- * DTT: Melford MB1015
- * Nutlin-3: Tocris 3984

MW = 590.5

- * Fluorescently labelled p53 derived peptide (p53-F)

5-FAM-QETFSDLKLLP-OH

MW = 1835.03

Custom synthesis by Biomatiks.

- * Carboxy-fluorescein (Sigma Aldrich 447293)

MW = 376.33
**MDM2-N**

MW = 15600

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</table>

<table>
<thead>
<tr>
<th>120</th>
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</thead>
</table>

Sequence of His-tag with thrombin cleavage site at N-terminus of MDM2-N:

MGSSHHHHHHSSGLVPRGS

His-tag MDM2-N was purified in University of Edinburgh. It was expressed from E. coli BL21 STAR strain. MDM2-N was purified using AKTA purifier. MDM2-N was loaded onto a 5ml Ni\(^{2+}\) HisTrap column followed by a polishing step through a S200 16/60 Gel filtration column.

### A3.3 Preparation of stock solutions

*Always use siliconised pipette tips and siliconised vials when working with p53-F and MDM2-N*

1mg/ml BSA

Dissolve 1.5mg of BSA in 1.5ml H\(_2\)O. Make up 1mg/ml BSA fresh every day.

100mM DTT

1M stock of DTT was prepared in Edinburgh by dissolving 5g of DTT into 32.4ml of H\(_2\)O. Aliquots of 1ml were prepared and frozen in -20°C freezer. Thaw 1ml of 1M stock of DTT and dilute into 9ml of H\(_2\)O (DTT f/c = 100mM concentration). Prepare aliquots of 8μl (~300 aliquots) and 105μl (~80 aliquots) and frozen in -80°C freezer. 105μl aliquot will be used for adding into Running Buffer and 8μl aliquot will be used for adding into injection sample.
Working Buffer (200mM TAPS/Tris pH 8.0)
Weigh 4.87g TAPS and dissolve in 90ml UHQ H₂O and adjust pH to 8.0 by adding dry Tris base. Adjust final volume to 100ml with UHQ H₂O and filter using a 0.2μm syringe filter and store at room temperature. Working buffer can be stored for ~3 weeks after which a new working buffer should be made up.

Running Buffer (200mM TAPS/Tris pH 8.0, 1mM DTT)
Mix 9.9ml of working buffer with 100μl of 100mM DTT and store at room temperature.

Tris Buffer (200mM Tris pH 8.0) for diluting Carboxyfluorescein and peptide
Add 2.42g Tris base into 100ml UHQ H₂O and adjust pH to 8.0 using HCl. Filter buffer solution through 0.2μm syringe filter and stored at room temperature. Fresh Tris buffer should be re-made after ~4 weeks.

Preparation of p53-F stock
Use siliconised pipette tips and siliconised vials when working with p53-F.
Dissolve 1.5mg of p53-F in 400μl of 200mM Tris pH 8.0 (final concentration p53-F= 2.04mM). Store aliquots of 20μl in the dark at –80˚C freezer in siliconised vials.

Preparation of 150μM stock of p53-F
Dilute p53-F stock (20μl, 2.04mM) with buffer (252μl, 200mM Tris pH 8.0). Store aliquots of 10μl in siliconised tubes in the dark at –80˚C freezer. Prepare new aliquots from the stock (2.04mM) every 3-4 weeks.

Internal standard Carboxy-fluorescein (CarboxyF)
Dissolve 1mg of Carboxy-fluorescein in 1ml of DMSO (f/c 1mg/ml). 1mg/ml = 2.6mM. Dilute 2.6mM CarboxyF to 30μM by adding 4.6μl of 2.6mM stock to 395μl DMSO. Put 2.6mM and 30μM CarboxyF stock into a box and keep in the dark in the drawer at room temperature. New stock was made up after 4 weeks.
His tag-MDM2-N

Use siliconised pipette tips and siliconised vials when working with MDM2-N.

Thaw frozen 1mg/ml MDM2-N (64μM) and dilute 1:1 (v/v) with 80% glycerol (f/c = 32μM). Final buffer solution contains 25mM HEPES pH 7.5, 50mM NaCl, 0.5mM DTT and 40% glycerol. Put aliquots (10μl) into siliconised tubes and keep in –80°C freezer.

A3.4 CE setting:

The capillary was prepared using the following protocol:

Capillary: Polyvinyl alcohol (PVA) coated capillary as per Cetek method with 30cm total length, 50µm internal diameter. A capillary window (2mm) was cut at 10cm from the outlet end to the detection window. Set temperature of the coolant at 15˚C.

Inject: Outlet injection 0.5psi for 5sec.

Separation: Normal polarity (+ to -), 10kV for 6.50min.

Detection: Laser Induced Fluorescence (LIF) detector.

Excitation/emission: 488nm/520nm

Filter: Normal.

Dynamic range: 10 RFU.

Temperature: Sample tray 4°C.

Inlet and Outlet trays 20-24°C.
Initial condition set up

LIF Detection condition set up

A3.5 Coated capillary condition Run setup
Method and buffer tray set up for coated capillary condition method
BI = Buffer Inlet tray
BO = Buffer Outlet tray
BO: A2 – Waste vial (empty glass vial, red cap).
BO: A6 – Home position (Add 1.5ml filtered water into 2ml glass vial with red cap).
BI: A2 – Water rinse vial (Add 1.5ml filtered water into 2ml glass vial with red cap).
BI: A3 – Running buffer rinse vial (Add 1.5ml running buffer into 2ml glass vial with red cap).

BI: A6 – Home position (Add 1.5ml filtered water into 2ml glass vial with red cap).

Table of Inlet and Outlet Buffer Trays layout

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<th>Home</th>
<th></th>
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<tr>
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<tr>
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<td>H₂O</td>
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<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
</tbody>
</table>

Inlet Tray

Instrument set up for the coated capillary condition run

1. Rinse with H₂O for 5min at 20psi pressure.
2. Rinse with running buffer for 10min at 20psi pressure.
3. Rinse with H₂O for 2min at 20psi pressure.

A3.6 Method and buffer tray set up for coated capillary p53-F control run method

Dilution for CarboxyF

Add 4μl of 30μM stock into 396μl of TB (200mM Tris pH 8.0) (f/c CarboxyF = 300nM). Dilute 10μl of 10nM stock into 290μl of TB (f/c = 10nM). Add 10μl of 10nM stock into 190μl TB (f/c = 500pM).
Dilution for p53-F

Using siliconised vials and tips, add 10μl of 150μM stock into 65μl Tris Buffer (f/c p53-F = 20μM). Add 10μl of 20μM into 390μl TB (f/c p53-F = 500nM). Add 4μl of 500nM stock into 396μl TB to get f/c = 5nM.

Buffer tray set up for p53-F control run

SO = Sample Outlet tray

SO: A2 – Injection sample (1mM TAPS/Tris pH 8.0, 1mM DTT, 10pM CarboxyF, 500pM p53-F). Cut the siliconised vials at 0.4ml mark position to be able to fit into plastic vial with spring. Add 1μl of Working Buffer, 2μl of 100mM DTT, 4μl of 500pM CarboxyF, 20μl of 5nM p53-F and make volume to 200μl by adding 173μl of H2O.

BO: A6 – Home position (Add 1.5ml filtered water into 2ml glass vial with red cap).

BO: A3 – BSA rinse vial (Add 1.5ml of 1mg/ml BSA into 2ml glass vial with red cap).

BO: A1 – Water rinse vial (Add 1.5ml filtered water into 2ml glass vial with red cap).

BO: B1 – Running buffer rinse (Add 200μl running buffer into PCR vial and place in plastic vial with spring and grey cap).

BO: C2 – Separation buffer (200mM TAPS/Tris pH 8.0, 160nM MDM2-N, 1% DMSO, 0.2% Glycerol). Add 197μl of Working Buffer, 1μl of 32μM MDM2-N and 2μl of DMSO into siliconised vial and place in plastic vial with spring and plastic cap. Then transfer 100μl of this solution to vial in BI: C2 position as balance.

BI: C2 – Separation Buffer (200mM TAPS/Tris pH 8.0, 160nM MDM2-N, 1% DMSO, 0.2% Glycerol).

BI: E2 – Running Buffer balance (Add 200μl running buffer into PCR vial and place in glass vial with spring and grey cap).

BI: F2 – Waste vial (Add 0.75ml filtered water into 2ml glass vial with red cap).

Table of Inlet and Outlet Buffer Trays layout

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<th>Home</th>
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</thead>
<tbody>
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<td>Separation Sample</td>
</tr>
<tr>
<td>RB</td>
<td>Waste</td>
</tr>
<tr>
<td>H2O</td>
<td>RB</td>
</tr>
</tbody>
</table>
Instrument set up for p53-F control run

1. Rinse with running buffer for 2min at 20psi pressure in reverse direction from BO:B1 vial to BI:F2 vial.
2. Rinse with separation sample for 1min at 20psi pressure to equilibrate capillary in reverse direction from BO:C2 vial to BI:F2 vial.
3. Inject sample from injection sample vial using pressure inject at 0.5psi for 5sec in reverse direction from SO:A2 vial to BI:E2 vial.
4. Separate sample at 10kV for 6.5min using normal polarity from BO:C2 vial to BI:C2 vial.
5. Rinse with H₂O for 0.5min with 20psi pressure in reverse direction from BO:A1 vial to BI:F2 vial.
6. Rinse with 1mg/ml BSA for 3min with 20psi pressure in reverse direction from BO:A3 vial to BO:F2 vial.

Coated capillary p53-F control run setup
LIF Detector set up

A3.7 Method and buffer tray set up for coated capillary MDM2-N/fragment run method

Compounds stock
Make 100mM DMSO stock from ~1mg dry powder. Add 3μl of 100mM stock into 7μl DMSO (f/c compound = 300μM in 100% DMSO). Screen the fragments at 300μM as the initial screening concentration depends upon the compound solubility.

Buffer tray set up for MDM2-N/compound screening
SO: A2 – Injection sample (1mM TAPS/Tris pH 8.0, 1mM DTT, 10pM CarboxyF, 500pM p53-F). Cut the siliconised vials at 0.4ml mark position to be able to fit into the plastic vial with spring. Add 1μl of Working Buffer, 2μl of 100mM DTT, 4μl of 500pM CarboxyF, 20μl of 5nM p53-F and make volume to 200μl by adding 173μl of H2O.
BO: A6 – Home position (Add 1.5ml filtered water into 2ml glass vial with red cap).
BO: A3 – BSA rinse vial (Add 1.5ml of 1mg/ml BSA into 2ml glass vial with red cap).
BO: A1 – Water rinse vial (Add 1.5ml filtered water into 2ml glass vial with red cap).
BO: B1 – Running buffer rinse (Add 200μl running buffer into PCR vial and place in plastic vial with spring and grey cap).
BO: C1 – Separation buffer (200mM TAPS/Tris pH 8.0, 160nM MDM2-N, 300μM compound, 1% DMSO, 0.2% Glycerol). Add 197μl of Working Buffer, 1μl of 32uM MDM2-N and 2μl of 30mM compound into siliconised vial and place in plastic vial with spring and grey cap. Transfer 100μl of this solution to a siliconised vial in BI: C2 position as balance.
BI: C2 – Separation Buffer (200mM TAPS/Tris pH 8.0, 160nM MDM2-N, 300μM compound, 1% DMSO, 0.2% Glycerol).
BI: E2 – Running Buffer balance (Add 200μl running buffer into PCR vial and place in plastic vial with spring and grey cap).
BI: F2 – Waste vial (Add 0.75ml filtered water into 2ml glass vial with red cap).

Table of Inlet and Outlet Buffer Trays layout

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<thead>
<tr>
<th>Home</th>
<th>Home</th>
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<tbody>
<tr>
<td></td>
<td>BSA</td>
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<td></td>
<td>H2O</td>
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<tr>
<td>Buffer</td>
<td>Buffer</td>
</tr>
<tr>
<td>Waste</td>
<td>Separation</td>
</tr>
<tr>
<td>Sample</td>
<td>Sample</td>
</tr>
</tbody>
</table>

Coated capillary MDM2-N/fragment run set up
Laser on method

Laser off method

A3.8 Results
Calculation of % Inhibition
Percentage of inhibition will be calculated for each compound screen.
The equation used is:

\[ \% \text{ Inhibition} = 100 \frac{(T_H - T_L)}{(T_H - T_R)} \]

\( T_H \) is migration time of p53-F peak in the presence of MDM2-N.
\( T_R \) is the migration time of p53-F peak.
\( T_L \) is the migration time of p53-F peak in the presence of MDM2-N + compound.

For example:
SEL101066 at 300\( \mu \)M has a migration time of 4.97min (\( T_L \)). The MDM2-N control peak migration time is 5.34min (\( T_H \)) and p53-F peak migration time is 4.37min (\( T_R \)).
To calculate % Inhibition of SEL101066:
\[ \% \text{ Inhibition} = 100 \frac{(5.34 - 4.97)}{(5.34 - 4.37)} = 38.1\% \]
Criteria

<table>
<thead>
<tr>
<th>Inhibition at 300μM</th>
<th>Classification</th>
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<tr>
<td>&lt;20%</td>
<td>Weak hits</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>Strong hits</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>Very strong hits</td>
</tr>
</tbody>
</table>

Weak hits will not be evaluated further.

Titrations will be performed on the strong and very strong hits and the IC\textsubscript{50} determined.

Quality control for MDM2-N CE runs

- Fresh buffer and filtered UHQ H\textsubscript{2}O will be added into the vials every morning.
- A condition run should be carried out at the beginning of each day to wash the capillary and wash away anything left in the capillary.
- The laser should be switched on and left for 30min to allow the laser to warm up before starting the control run. This can be performed automatically by setting laser on method before operator arrives.
- Before starting to screen the fragments, CarboxyF/p53-F control run should be run in triplicate to make sure that the migration time, peak height and area are reproducible.
- MDM2-N control run should be run after confirming the CarboxyF and p53-F peaks were reproducible. The migration time of p53-F/MDM2-N peak, p53-F peak and CarboxyF peak will vary by ~0.2min. The migration time will also vary if changing capillary.
- After screening each compound, MDM2-N control should be run to make sure that the peak shifts back to the MDM2-N migration time.
- If screening a large number of compounds then additional control runs will be run at the start of a compound screen after every 4-5 compounds and at the end of compound screen to confirm that the peak shifts back to the MDM2-N migration time.
- At the end of the day, a condition run should be carried out to wash the capillary.
- Laser should be switched off at the end of the day using the laser off method.

A3.9 Troubleshooting

Indication of broken capillary/ reduced coating

Smeary peak present

This indicates the PVA coating in the capillary has probably deteriorated. Try a condition run followed by 10min rinse with BSA and 10min rinse with UHQ filtered H\textsubscript{2}O. If this does not improve peak shape then a new capillary has to be installed.
Example of smeared peak

Check the current during control run.
Usually current error or a sudden drop in current if a capillary is broken. Check capillary window for a crack or sign of coolant leak.

What if the p53-F/ MDM2-N peak did not shift back?
During the compound screen, if the control p53-F/ MDM2-N peak did not shift back then extensive washing is required to remove any compound left in the capillary. Rinse capillary with 1mg/ml BSA at 20psi pressure for 5min followed by 5min rinse with UHQ filtered H2O at 20psi pressure. Then rinse with running buffer at 20psi pressure for 2min.

What happens if the peak area is smaller than normal while running p53-F control run?
If the peak area is smaller than normal in the control run then repeat the control run continuously. It is possible the laser is still warming up and requires several control runs to regain the normal peak area. If necessary, repeat the run with fresh sample.
If this fail then try switch off the laser module and let the laser module to cool down for ~5-10min and switch on again and run the control to see if peak area becomes normal. The laser should be switched on before the operator arrives using automated method to turn laser on.
Appendix A4. Turbidimetric test

Turbidimetric solubility standard assay was carried out by Sarah to confirm the solubility of the compounds from 1mM to 0.0625mM.

Equiment: V-bottomed PP 96 well plate (Costar)
- 400ul flat bottomed clear 96 well plate (Matrix)
- Heated plate shaker (Grant-Bio PHMP, Glas-Col LLC or equivalent)
- SpectraMax M5
- 100mM DMSO stocks of test compounds (200ul)
- 100mM DMSO stocks pyrene & aspirin (200ul)
- DMSO
- Assay buffer
- Tecan liquid handling system (optional)

1. Perform appropriate 100x f/c dilution series compounds in DMSO as per Table 2.1
   Add DMSO to row A and B for use as blanks.

Table 2.1 Dilution series of compounds in DMSO

<table>
<thead>
<tr>
<th>Stock (mM)</th>
<th>Volume (ul)</th>
<th>DMSO (ul)</th>
<th>Stock final conc (mM)</th>
<th>Checksum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>15</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>15</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>E</td>
<td>100</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>15</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>G</td>
<td>25</td>
<td>15</td>
<td>15</td>
<td>12.5</td>
</tr>
<tr>
<td>H</td>
<td>12.5</td>
<td>15</td>
<td>15</td>
<td>6.25</td>
</tr>
</tbody>
</table>

2. Add 247.5ul filtered assay buffer to all required wells.
3. Transfer 2.5ul 100x DMSO dilution series in triplicate into assay plates, note locations.
4. Seal plates with adhesive plate seal and ensure no gaps for evaporative loss. Shake the plate at 250rpm for ~18 hours overnight.

5. After shaking, remove plate seal and check for any bubbles and remove if present.

6. Put the plate into SpectraMax M5 with 5 sec pre-shake and read plate at OD\textsubscript{650}.

7. Results were export to excel spreadsheet for analysis.

### A4.1 Turbidimetric test result

<table>
<thead>
<tr>
<th>Solubility cut-off</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFL001290 0.75mM&lt;x&lt;1mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001675 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL000577 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001303 0.75mM&lt;x&lt;1mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL000829 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL000134 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL000576 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL000866 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001502 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001397 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001328 &gt; 2mM</td>
<td>High</td>
</tr>
<tr>
<td>SFL001542 &gt; 2mM</td>
<td>High</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solubility cutoff (mM)</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Chlorophenothiazine</td>
<td>&lt;0.0625 Low</td>
</tr>
<tr>
<td>Phenothiazine</td>
<td>0.0625&lt;x&lt;0.125 Medium</td>
</tr>
<tr>
<td>N-Phenyl-p-phenylenediamine</td>
<td>0.0625&lt;x&lt;0.125 Medium</td>
</tr>
<tr>
<td>2-Phenylamino-benzoic acid</td>
<td>&gt;1 High</td>
</tr>
<tr>
<td>3-Hydroxydiphenylamine</td>
<td>0.25&lt;x&lt;0.5 High</td>
</tr>
<tr>
<td>Compound</td>
<td>Solubility cutoff (mM)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>SEL101069</td>
<td>&gt;1</td>
</tr>
<tr>
<td>4-Hydroxydiphenylamine</td>
<td>0.25&lt;x&lt;0.5</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td>0.75&lt;x&lt;1</td>
</tr>
<tr>
<td>Phenoxazine</td>
<td>0.0625&lt;x&lt;0.125</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>#</th>
<th>Compound</th>
<th>Solubility cut-off:</th>
<th>Rank</th>
<th>Project</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyrene</td>
<td>&lt;0.0625mM</td>
<td>Medium</td>
<td>Positive Control</td>
</tr>
<tr>
<td>2</td>
<td>Aspirin</td>
<td>&gt;1mM</td>
<td>High</td>
<td>Negative Control</td>
</tr>
<tr>
<td>3</td>
<td>SEL101875</td>
<td>0.125mM&lt;x&lt;0.25mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SEL101876</td>
<td>0.125mM&lt;x&lt;0.25mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>SEL101877</td>
<td>0.125mM&lt;x&lt;0.25mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SEL101878</td>
<td>0.25mM&lt;x&lt;0.5mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>SEL101879</td>
<td>0.25mM&lt;x&lt;0.5mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>SEL101880</td>
<td>&gt;0.5mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SEL101881</td>
<td>0.125mM&lt;x&lt;0.25mM</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>SEL101884</td>
<td>&gt;0.5mM</td>
<td>High</td>
<td></td>
</tr>
</tbody>
</table>
Appendix A5. CODASS hits titration against MDM2-N in the CE

Titration was performed on four CODASS hits against MDM2-N using CE assay. The % inhibitions were fitted into the curve against compound concentrations in log scale. IC\textsubscript{50} was determined for each hits.
Appendix A6. Crystal plates set up for MDM2-N^{11-130}

The crystal plates were set up using hanging drop method and SEL101266 and SEL101267 were co-crystallised with MDM2-N^{11-130}. The final concentration in the drop for MDM2-N^{11-130} and fragments are 5mg/ml and 1.6mM respectively.

The crystallisation conditions used are as follow:

<table>
<thead>
<tr>
<th>PEG200</th>
<th>AS 60%</th>
<th>58%</th>
<th>56%</th>
<th>54%</th>
<th>52%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

54% saturated ammonium sulfate, 100 mM Tris, pH 7.8, 2.5 % PEG200, 50 mM glucose and 5 mM DTT.

<table>
<thead>
<tr>
<th>AS 1%</th>
<th>1.5%</th>
<th>2%</th>
<th>2.5%</th>
<th>3%</th>
<th>3.5%</th>
</tr>
</thead>
</table>

2-2.2% ammonium sulfate, 100mM Bicine pH 9.5