Virtual Screening for Inhibitors of Anti-apoptotic Proteins: DCK, BCL-XL, MCL-1, MDMX, and MDM2

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Virtual Screening for Inhibitors of Anti-apoptotic Proteins:

DCK, BCL-XL, MCL-1, MDMX, and MDM2

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Dedication

To my late grand father who died of pancreatic cancer, a diagnosis that still means certain death.
Acknowledgments

I would like to acknowledge my wife, Ana, who has stuck by me through these at times difficult years in pursuit of my graduate degree. I would like to acknowledge firstly my parents who instilled a love and respect of math and science early in life and who have been supportive all these years. I would also like to acknowledge my in-laws Jed, Tony, and Pat for their support and for reading my dissertation at an early point. I would like to acknowledge all my friends who were showed curiosity and interest in my work and with whom I’ve had constructive conversations. I would like to thank my fellow lab mates who have shared in this journey.
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List of Abbreviations

BCL-2 = B Cell Lymphoma 2
BCL-XL = B Cell Lymphoma – Extra Large
DNA = Deoxyribonucleic acid
IC$_{50}$ = Inhibitory Concentration, Half Maximal
MCL-1 = Mantle Cell Lymphoma
MDM2 = Mouse Double Minute 2
MDM4 / MDM2 = Mouse Double Minute 4
P53 = Protein 53
RNA = Ribonucleic Acid
Abstract

Within this dissertation the topic of virtual screening is discussed with regard to three different cancer targets and also a brief introduction of the tools used in virtual screening. In Chapter 1, the reader will be introduced to virtual screening and the programs that are used in virtual screening. In Chapter 2, the first of three projects are discussed. This project consists of the work that was done to find inhibitors of the P53 binding domain of MDMX. In this project the mobility of residues within the binding site of MDMX are discussed and the ways in which we attempted to model how drugs would bind two adjacent pockets within MDMX. In Chapter 3, the virtual screening and modeling work done for RING domain of MDM2 and MDMX is discussed. This work was done in conjunction with Moffitt Cancer Center in order to solve the 60 year old mystery of the mechanism of how thalidomide and possibly its analog lenalidomide caused children to be born limbless. Current thinking is that Cereblon through an unknown teratogenic mechanism activates an increase in FGF8. We suggest a mechanism that may happen in parallel that involves stabilization of MDM2 and the reduction of P63 levels. Chapter 4, the work that was done against the BH3 binding domain of MCL-1 is discussed in conjunction with collaboration with the Manetsch lab. In order to complete this screening the validation of IC$_{50}$ values and then attempt to modify those products based upon the structure of MCL-1. Chapter 5 discusses the work done to find inhibitors of deoxycytidine kinase. All of these chapters taken together
provide a brief overview of the computational work done produce inhibitors of Protein-Protein Interaction against three major cancer targets.
Chapter 1: Virtual Screening in Drug Discovery

This chapter will serve as a brief introduction to the tools available in a drug discovery program.

1.1 Introduction

Screening of new compounds is an important part of any drug discovery program. Screening generally involves purchasing molecular libraries, quantifying protein binding of those molecules, and some discernment and investigation into the properties that positively or negatively affect said binding. Finding weak binders amongst a large library of compounds is a tedious and difficult task, but is central to finding “hits”, molecules which show some activity against a protein of interest, and is the central task of screening. Screening results typically find 0 – 3% hits almost none of which will contribute to what eventually become lead compounds. Lead compounds are generally medium to strong binders with a specificity and $IC_{50}$ that begin to approach marketable drug levels. The research that occurs between screening, hit, and lead compound is largely the realm of the medicinal chemist and biologist. A considerable amount of time and funding occurs during this period, a period that can be greatly accelerated through computational methods, namely virtual screening. Virtual screening involves techniques that are analogous to traditional screening methods. Figure 1 outlines the process in silico. (Rester, 2008)
Computational methods including virtual screening fit well with the beginning of a drug discovery project, and especially during target selection and validation, hit/lead finding, and lead optimization.

Virtual screening can reduce the burden on medicinal chemists and structural biologists by rapidly and relatively inexpensively screening larger libraries than what would be possible in vitro. Large libraries from various sources including the National Center for Biotechnology Information and structures available through the protein databank, both NMR and X-ray crystals, are vital for a virtual screening program. Virtual screening consists largely of molecular docking, a simulation of protein binding of library compounds to the proteins of interest. There are five essential steps in the virtual screening process: ligand preparation, protein preparation, pose search (molecular docking), pose scoring (molecular docking), and data analysis. Each of these parts has a myriad of available options and techniques, each with its own benefits and downsides. There is a long list of techniques which can support a screening program such as molecular modeling, homology modeling, molecular dynamics, 2D and 3D QSAR.
screening, pharmacophore screening, ab initio quantum mechanics, both ligand and structure based drug design, and fragment or combinatorial based screening to name a few. All together these techniques and methodologies attempt to achieve many of the same goals as in vitro high throughput screening.

**Virtual Screening**

- Protein Preparation
- Ligand Preparation
- Pose Search (translation, rotation, etc.)
- Scoring
- Data Analysis
- Protein Prep Wizard
- Ligprep
- GLIDE Docking
- GLIDE Docking

**Figure 2** Molecular Docking follows the above procedure. Listed to the right in green are the programs that are used by my group.

The first virtual screening program was DOCK, which was released by Tack Kuntz’s group at University of California at San Francisco in 1982. Since then, both academic and corporate groups have released time more than 16 different molecular docking programs including DOCK, AutoDock, Glide, GOLD, FlexX to name a few, Table 1. A particular issue with having so many molecular docking or virtual screening
programs is that each has a specific instance where it is the most predictive program. All perform similar functions and give an output that is in the form of one or more ligand conformations within a defined binding site area and a docking score. This output cannot at this time be used blindly because scoring functions, despite a long and involved evolution, still have large amounts of error. Often, very similar molecules will have wildly different scores despite being able to reproduce a known conformation. The pose, conformation of the ligand in the appropriate orientation, then becomes very important and being able to visually inspect each conformation proves very valuable, especially when considering pharmacophore design and discerning Structure Activity Relationships (SAR).

Table 1 Table of Major Docking Programs

<table>
<thead>
<tr>
<th>Company</th>
<th>Molecular Docking Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scripps</td>
<td>AutoDock</td>
</tr>
<tr>
<td>UCSF</td>
<td>Dock</td>
</tr>
<tr>
<td>BioSolveIt</td>
<td>FlexX</td>
</tr>
<tr>
<td>Schrodinger</td>
<td>GLIDE</td>
</tr>
<tr>
<td>CambridgeSoft</td>
<td>GOLD</td>
</tr>
</tbody>
</table>

1.2 Ligand Preparation

Potential ligands can come from many different sources including libraries from chemical companies, libraries from National Institutes of Health (NIH), libraries from medicinal chemists or combinatorially derived libraries. The National Institutes of Health have one particularly useful set of compounds known as the NCI Diversity Set. The NCI diversity set is a subset of the 140,000 compounds available from NCI as part of the NCI plated set. The purpose of this set of compounds is to provide a diverse library of chemical structures. The NCI diversity set of compounds was created by taking all of the compounds in the NCI plated compounds. Reducing them based upon their ability to
fulfill orders of the compounds that lead to the selection of around 76 thousand compounds. These compounds were then aligned based upon a three point pharmacophore with each point representing a particular interaction. Over a million possible pharmacophores were produced but then determining whether the conformations were of an acceptably low energy reduced this further. Compounds were then selected on whether they added at least 5 more pharmacophores to the diversity set. This lead to an initial set of 1990 compounds which known as NCI Diversity Set I. This set of compounds has undergone 3 revisions and is subject to change as compounds that make up the diversity set become unavailable. This has resulted in a drop in the number of compounds from 1990 compounds to 1597 compounds. In other parts of this document, the revision will be noted after the name Diversity Set such as Diversity Set II*.

<table>
<thead>
<tr>
<th>NCI Diversity Set Revision</th>
<th>Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI Diversity Set I</td>
<td>1990</td>
</tr>
<tr>
<td>NCI Diversity Set II</td>
<td>1564</td>
</tr>
<tr>
<td>NCI Diversity Set III</td>
<td>1597</td>
</tr>
</tbody>
</table>

**Table 2 Revisions of NCI Diversity Set and the number of ligands contained in each.**

Combinatorial techniques can be used to create a diverse set of compounds from a set of fragments or from a group of ligands. In combinatorial screening, both the scaffold, also known as the core, can be altered as well as functional groups or attachments. BREED, a script within the Schrödinger Software Suite, is another type of combinatorial technique, which involves overlaying docked compounds and matching areas of overlap and exchanging atoms and even entire functional groups. BREED tolerates some difference in angle and distance when calculating overlap. Both produce new libraries of compounds, although combinatorial methods offer the researcher more control. (Pierce, Rao, & Bemis, 2004)
The importance of screening a diverse set of ligands lies in that the chemical space of small compounds is vast. As of July 2009, 49 million compounds have been reported in the scientific literature, and there is an approximate theoretical limit of approximately $10^{60}$ possible small organic molecules. (Bohacek, McMartin, & Guida, 1996; Dobson, 2004) The space encompassed by chemical space is so large that it would be physically impossible to produce every molecule possible. There are $10^{82}$ atoms in the universe (Villanueva, 2009) and the vast majority of those atoms are either helium or hydrogen as contained within the stars. The ratio of carbon to hydrogen atoms in the universe is $2.37 \times 10^{-4}$ M. (Croswell, 1995) It would take many billions of years of fusion within the stars to produce a ratio of carbon necessary to produce even one percent of the possible compounds. Even with no timetable to finish the conversion, the energy required to fuse hydrogen into carbon would be phenomenal. It would necessary to
produce at least a 10 µM solution or $6.04 \times 10^{18}$ of each molecule per milliliter for screening. If the right proportion of carbon, nitrogen, and oxygen were achieved, it might take 10 to 20 universes to make enough material to screen every small molecule once! It may be possible to screen a significant portion of these compounds *via in silico* screening in the near future. If Moore’s law holds true, 2000 compounds on single processor in 3 to 4 hours today would become $3.35 \times 10^{82}$ on a single processor in 3 to 4 years in just 25 years using today’s screening methods.(Brock & Moore, 2006) With large multiprocessor computers or computational clusters, screening all possible small molecules will become possible even sooner.

1.3 Protein Preparation

The contribution of structural biology to the field of computational chemistry cannot be overstated. With X-ray crystal protein structures or NMR protein structures, it becomes very difficult to have a virtual screening program. In the absence of a protein, with a large library of known inhibitors ligand based screening can be conducted where known activities are matched to ligands via 3D Quantitative SAR (QSAR) in an attempt to develop a pharmacophore with which to screen against. There are several kinds of QSAR that can be done computationally. The number dimensions may refer to spatial dimensions or refer to a set of attributes of considerations. For instance, 1D QSAR is not a QSAR in one-dimensional space but QSAR developed using a parameter such as Log P. 3D QSAR does in fact refer to the three dimensional coordinates of a overlaid drug molecules from which structural motifs such as hydrogen bond donor or acceptor and hydrophobic residues become apparent. In order for the overlapping structures to be
given meaning, they must be understood in the context of empirically derived activity. (Verma, Khedkar, & Coutinho, 2010)

In addition to this technique, if a similar protein exists, homology modeling can be used to build protein structure using another closely related structure as a template. Because protein structures are dynamic mobile molecules, it is helpful to have many different conformers of the same protein. Screening against many conformers is a way to take into account protein flexibility and a way to gain a consensus of the best ligand conformations that best represent the *in silico* or in vivo experiment. A consensus can be made through averaging of scores or creating clusters of very close scores.

Proteins are made available through the Protein Data Bank (rcsb.org) (Berman et al., 2000; Bernstein et al., 1977) in the form .pdb files. Crystallographers and NMR structural biologists deposit these files along with the related academic papers as a requirement to publish. These .pdb files cannot be used directly and the data must go through significant processing before being used to screen. The resolution of the X-ray diffraction method is too low to detect chemical features like hydrogen atom positions and because proteins are flexible, there is some variability to the position of side chains making it difficult to process X-ray diffraction data. Bond information is not explicitly contained within the .pdb file either and is inferred from inter-atomic distances. Missing information must be derived using protein prediction programs such as Prime from Schrödinger. (Andrec, Harano, Jacobson, Friesner, & Levy, 2002)
1.4 Docking Methods (Search Algorithms)

The goal of conformational search within a docking protocol is to find the pose that most accurately represents the interactions found \textit{in vivo}. One way to check the accuracy of this search is to compare root mean square deviation (R.M.S.D.) with an available crystal structure and molecular dynamics can be used to test the stability of a protein confirmation as well. Large discrepancies between the X-ray crystal coordinates and the processed protein coordinates should be avoided because the X-ray coordinates represent empirically derived information that is the closest to in vivo structures. When this is done using both the ligand and protein from a single crystal structure, this is called self-docking. Most virtual screening programs are developed to screen novel molecules for which the activity is unknown.

There are many approaches to search algorithms in molecular docking. Listed here are approaches to conformational sampling. Along with conformational sampling rotational and orientational sampling are done. Rotational and orientational sampling occur prior to conformational sampling and is done at an optimized level representative of the possible rotational and orientational positions possible. (Goodsell & Olson, 1990)

It would be impossible to sample at every angle and every point in space.

A full search, a so called systematic grid search will sample more conformational space but takes so long that it is not reasonable for screening anything but a handful of compounds at a time. Furthermore within the full population of conformations of any given ligand, most conformations will be unwanted, having unlikely and energetically unfavorable interactions. To remedy this potential waste of computational power, several methods have been devised including genetic algorithms, favorable interaction search,
and simulated annealing, a modification of a technique used in molecular dynamics where the ligand in this case would be heated and cooled in order to sample conformational space. (Lipkowitz & Boyd, 1990)

Genetic algorithms search conformational space by mimicking natural selection by creating candidate solutions that are organized in a genetic representation and allowed to exchange information in a genetic way. Genetic means that a random mixing of elements from previous solutions produces each subsequent solution. These newly produced populations of solutions are then evaluated by a fitness function, a function which assigns a score in an attempt to rank poses by lowest energy, validity, or other metric. (Lipkowitz & Boyd, 1990)

Incremental construction attempts to match functional groups to favorable interactions and reconstruct the ligand by joining fragments. A central node is chosen from the central fragments are then built from until the original molecule is constructed. The best solution is selected by evaluating each solution via a fitness function. This is a methodology employed by FlexX. (Sato, Shewchuk, & Tang, 2006; Trott & Olson, 2010)

A molecular dynamics approach can be used employing simulated annealing, which a molecular dynamics simulation in which alternating heating and cooling periods move ligands between potential energy wells. This is one method that is part of the very popular molecular docking program AutoDock Vina. (Trott & Olson, 2010)

Metropolis - Monte Carlo Methods are still the preferred methods when studying a few molecules and geometry is absolutely important. This method uses Markov chains to build a set of conformations that are representative of a population of conformations.
It is also one of the slowest methods. Monte Carlo is very well suited for single molecule study. (Lipkowitz & Boyd, 1990)

In addition to the above search mechanism, there exist hybrid methods, which attempt to optimize both speed and search completeness. Schrödinger’s Glide uses a hybrid of an exhaustive Monte Carlo search and favorable interaction search. Schrödinger refers to this as “greedy search” and involves an inverted pyramid like narrowing of conformations until only a few are reported. (Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)
Figure 4 Protein preparation proceeds from the top structure that was downloaded from the Protein Data Bank to the bottom structure, the prepared structure.
1.5 Molecular Docking (Scoring Functions)

Scoring functions attempt to quantify binding and are related but not necessarily equivalent to the fitness functions that evaluate docking search solutions. Results are typically reported in Kcal per mole as a measurement of binding affinity (i.e., binding free energy). There are many scoring functions and more being created all the time. The problem with using any particular scoring function is that it may fail in a particular case of interest. For this reason it is often advisable to employ consensus scoring with many different scoring functions as possible.

Scoring functions are generally of three types: force field, empirical, and so-called knowledge based methods. Force Field based methods are derived from AMBER(Yang et al., 2006), CHARMM(Brooks et al., 2009), or OPLS(Kaminski, Friesner, Tirado-Rives, & Jorgensen, 2000) force field non-bonded interaction terms including van der Waals and electrostatic terms. Electrostatic terms are often complimented by Generalized Born equation term (GBSA) for calculating the effects of solvation. This methodology is a part of the Dock program.(Lipkowitz & Boyd, 1990)

Empirical functions are scoring functions that calculate an estimate of the free energy of binding by summing a list of interactions that are weighed by experimentally derived coefficients. An interaction may be identified as an advantage or a penalty. For example electrostatic interactions are then broken into hydrogen bonding, ionic interactions, and buried charge penalties. GOLD further breaks hydrogen bonding into twelve separate categories that all have different constants associated with them.(Jones, Willett, Glen, Leach, & Taylor, 1997)
So-called “Knowledge-based” scoring functions are simply scoring functions in which score is based upon how closely a novel ligand matches known binders contained within the Protein Data Bank. (Berman et al., 2000; Bernstein et al., 1977)

Hybrid scoring functions such as the one employed in GLIDE use two or more functions to arrive at a score. GLIDE’s Glidescore is both a force field and an empirically derived scoring function. It takes electrostatic and van der Waals terms from a OPLS force field based score and modify it with advantages and penalties from an empirical based function.(Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)
Another way scoring functions can differ is whether they are all-atom based functions or employ a “grid,” a grid is a lattice of points within the binding site at which all interactions are accounted for at that point. Scoring functions that employ a grid are twenty-fold faster than all atom based scoring functions. They have the added benefit of being separable, as each can represent all of the interactions of the protein without having the ligand within the binding site. (Lipkowitz & Boyd, 1990)

1.6 Data Analysis

When analyzing the data from virtual screening, the most important thing to remember is that nothing can be considered in isolation. The scoring functions as stated above are often accurate in a particular set of experiments and not predictive or able to correlate under a different set of conditions. Visual inspection of the binding pose must be taken along with docking score in order to make the determination of how well a ligand binds to a target.

The best ligands have a ligand efficiency of around 1.5 Kcal/heavy atom/mole. This quality of score is atypical to early screening near the beginning of a medicinal chemistry program. Finding weak binders, or hits, takes a bit more interpretation and the identification of important functional groups and interactions.

The docking scores attempt to predict the energy of binding and that energy should correlate with biophysical measurements in the form of IC\textsubscript{50} values or K\textsubscript{i}.

$$K_i = \frac{IC_{50}}{S}$$

\[ \frac{1}{K_m} + \frac{[S]}{K_m} \]

Figure 6 This equation describes the simple relationship between Ki and IC50.
A two kilocalorie per mole difference in Glide docking score represents about a one order of magnitude difference in IC$_{50}$ concentration. Two situations will arise where this is not the case: 1. All the ligands are extremely weak binders and then making it quite difficult to find a signal in the proverbial noise. 2. The scoring function is poorly suited for the protein and ligand combinations being screened, and the researcher should consider using a different scoring function or obtaining consensus with another scoring function.

1.7 Protein Flexibility

Inclusion of protein flexibility is a relative new development in molecular docking which typically uses static proteins. Using static proteins greatly reduces the computational cost of molecular docking but does not represent the reality of induced fit effects, the lock and key mechanism of ligand – protein interaction. Not only can proteins have mobile side chains but can also have mobile domains. One class of protein is in fact so mobile it is known as intrinsically disordered. Intrinsically disordered proteins are some of the most difficult proteins to study because they are difficult to capture via x-ray crystallography. Intrinsically disordered proteins represent some of the most important molecules however and include P53 and MDM2 proteins whose study holds the key to creating better cancer drugs.(Dunker & Uversky, 2010)

One simple but crude way to consider protein flexibility is to dock to different conformations of a protein. This is possible because of the wealth of structures now included in the protein data bank. The biggest problem with this way of considering protein flexibility is that it is probably not totally representative of the population of
conformations that occur naturally. However, in order for crystallization to occur, the conformations must be of relatively low energy and structurally stable.

Yet another way but still quite incomplete is to allow side chains to move partially or fully. To fully appreciate the movement of a protein, either molecular dynamics or low frequency modes of vibration should be considered. With either technique, several frames, or protein conformations, are produced which then can be docked to produce a docking ensemble.

1.8 Conclusion

As stated previously, the docking techniques presented here do not represent the totality of what is available and give a framework for understanding the rest of this dissertation. The field of virtual screening is constantly changing and new techniques are being developed all the time. One thing that guides the purpose of all these techniques are the simulation of natural phenomena using computational techniques, and one must never lose sight that although these techniques can be useful, they should never be taken without considering the empirical evidence that underlies the simulation.

For most of the experiments included in this dissertation, the Schrödinger Suite of Software was used. Maestro is the molecular modeler and viewing software. Glide is the virtual screening software. Lig Prep and Protein Preparation are wizards to prepare ligands and proteins for use in Glide respectively.(Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)
1.9 References


Schrödinger. (2011). CombiGLIDE: Schrödinger, LLC.


20
Chapter 2: P53, MDM2, and MDMX

2.1 Cancer

Cancer is a genetic disease where cells gain self-sufficiency and ultimately leads to tissue destruction and death. Because of the large array of control systems, the evolution of a cancer from genetic abnormality to full-blown neoplasia is a complex one that involves specific mutations in both oncogenes and tumor suppressor genes in order to undermine normal mechanisms for maintaining controlled cellular development. As these genetic abnormalities accumulate, allow for angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, and limitless replication.

Knudsen’s two hit hypothesis suggests that both copies of a gene must be mutated in order cause dysfunction. A mutation in oncogenes leads to a gain of function mutation, a mutation where a cell learns to perform a metabolic function not associated with its cell line. A mutation in tumor suppressor genes leads to a loss of function in a critical genetic caretaker that results in incomplete inhibition of pro-tumor process or protein. Oncogenes typically mutate in a way to suppress tumor suppressors and tumor suppressors mutations cause a decrease in their activity as promoters of apoptosis. (Nordling, 1953)
2.2 P53 and Apoptosis

Two tumor suppressors that are being intensely studied at this time are the BCL2 family of proteins and P53 (Hollstein, Sidransky, Vogelstein, & Harris, 1991) and its regulators MDM2 (Momand, Zambetti, Olson, George, & Levine, 1992). Actually some members of the BCL2 family are tumor suppressors (BAX, BAD, BIM) and others are proto-oncogenes such as BCL-2, BCL-XL, and MCL-1 (Chao et al., 1995). P53 is the chief of the tumor suppressors as it alone initiates apoptosis, however, it is a regulator of its function MDM2 and its analog MDMX are proto-oncogenes, as they cause a decrease in P53 levels when mutated. (Hollstein et al., 1991)

P53 is the gatekeeper of the cell and chiefly responsible for destruction of a cell whose ability to survive has been compromised. Apoptosis, the process by which cells initiate death becomes necessary under five conditions: lack of oxygen, lack of nutrition, heat or cold shock, mitotic spindle damage, and genetic instability. P53 acts as a transcription factor for many different apoptosis related proteins. Each process has its own unique mechanism of action that cross at the P53 protein with four outcomes possible: senescence, DNA repair, cell cycle arrest, or cell death. (Hollstein et al., 1991)

P53 consists of three functional domains. The first of which is the transactivation domain, residues 1 – 42, which is responsible for up-regulation of pro-apoptotic genes. This is the portion that binds to the regulatory molecule MDM2 at the P53 domain. The P53 binding domain, residues 102 – 292, is where 90% of mutations occur. This region is required for sequence specific binding. Lastly there is the Oligomerization domain,
where P53 forms a tetramer. (Hollstein et al., 1991; Momand et al., 1992; Vassilev et al., 2004)

Figure 7 P53 acts to initiate apoptosis by binding to promoter regions of DNA. Both MDM2 and MDMX act to counter its pro-apoptotic effects and keep it in low concentration within the nucleus.

2.3 MDM2

It should also be noted that P53 is responsible for differentiation of certain cell lines and especially important in cancers of blood cells. In cancers such as multiple myeloma, higher levels of P53 are linked to worst outcomes and survival. Apoptosis mediated by P53 can be inhibited by MDM2 and MDMX. These act to remove P53 from the nucleus and mediate its destruction. Because of the importance of P53, cancers in up to 50% cases contain some dysregulation of P53. MDM2 stands for Mouse Double
Minute 2 and was discovered along with several DNA binding proteins. Double Minute refers to length of time it took to transverse a chromatography gel.(Lenos & Jochemsen, 2011)

P53 binds to MDM2 and MDMX at the P53 binding domain via the trans-activation domain, a short disordered loop which forms a alpha-helix upon binding. The RING domain of MDM2 binds to a ubiquitin ligase that is responsible for marking P53 for destruction. MDM2 can also act to mark other MDM2 proteins and the MDM2 analog, MDMX, for destruction by the same mechanism. Some conformational change is thought to occur which allows both of these actions to happen, but little is known about the mechanics within the disordered residues of MDM2. This kind of conformational change is not uncommon in MDM2/MDMX or P53.(Dunker & Uversky, 2010)

Docking studies showed that a indole-like group was necessary to bind to the tryptophan binding pocket. The Leucine binding pocket is very shallow, and therefore difficult to model accurately. The volume of the Leucine pocket is less than one square angstrom.

P53 levels directly lead to increased MDM2 levels. MDM2 levels are regulated by MDM2 – MDM2 complex formation and the ubiquitination of MDM2 when levels are high relative to P53 or MDMX levels. MDMX does not contain ubiquitin ligase and interaction with MDM2 does not lead to its destruction. This increase in MDM2 by an increase in P53 is an example of a feedback loop. This feedback loop is disrupted in cancer. 50% of human cancers contain a mutation in P53. Additionally, 50% of cancers contain a mutation of MDM2. Drugs that can reactivate P53 would be the most direct
route to killing cancer cells and restoring homeostasis, the natural functioning of the body. (Vassilev et al., 2004)

One of the early scientific successes against MDM2 were the Nutlin compounds, especially Nutlin-3A. Nutlin-3A was the first molecule to achieve sub-micromolar inhibitory concentrations against MDM2. This author’s first attempts at inhibitor design were centered on screening Nutlin-3A analogs and molecules inspired by the Nutlin series of compounds. (Vassilev et al., 2004)

**Table 3 Table of Nutlin Compounds**

<table>
<thead>
<tr>
<th>Title</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang-Nutlin-1</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Wang-Nutlin-2</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Wang-Nutlin-3B</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Wang-Nutlin-3A</td>
<td>![Structure Image]</td>
</tr>
</tbody>
</table>
2.4 MDMX

The project was expanded to include MDMX, a close analog and regulator of MDM2. MDM2 and MDMX differ at the RING domain, as MDMX does not have a binding domain for ubiquitin ligase, but still retains the ability to export P53 from the nucleus to the cytosol. MDMX has a binding site which is particular problematic, as it has a shallower binding pocket when compared to MDM2. (Linke et al., 2008) Also problematic are that MDMX levels rise along with MDM2 levels and causes loss of activity of MDM2 inhibitors as it makes P53 unable for promotion of pro-apoptotic factors by exporting it from the nucleus.
Figure 9 Model of binding site of MDMX showing auxiliary binding pocket to left and standard grid placement that does not include the auxiliary pocket.

In addition to small molecule inhibitors cyclic compounds and alpha-helix mimetics from the McLaughlin and Del Valle groups were screened against MDM2 and MDMX, both containing residues that are hydrophobic.
Later MI-XX series of molecules were discovered. These compounds were used as a basis of a second generation of molecules screened against MDM2. Moffitt cancer produced a series of crystal structures of high affinity optimized peptides based on the P53 amphipathic trans-activation alpha helix. These high-resolution structures were then used to find small molecule inhibitors.(Canner et al., 2009)

Figure 10. MDMX overlayed with MDM2. MDMX with carbon in blue and MDM2 with carbon in gray.

The Moffitt series of crystal structures were insightful because one X-ray crystal structure contained the peptide PDIQ that appeared to bind to an auxiliary pocket of
MDMX that had not been seen before. (Phan et al., 2010) In addition, the crystal structure of MI-63 bound to MDMX contained two bound molecules one bound to the auxiliary pocket. (Canner et al., 2009) Much effort was put into creating a suitable linker to take advantage of this auxiliary binding site.

![Figure 11 Model of crystal structure containing two bound copies of a MI-63 analog.](image)

Another interesting feature of MDMX/MDM2 are two residues that appear to have much flexibility that separate the usual Phenylalanine, Tryptophan, and Leucine pockets from above mentioned auxiliary pocket. MET – 53 and TYR – 100 on MDMX
appear to be able to move enough for a small molecule inhibitor or peptide inhibitor to displace them and increase binding area. One of the first computational experiments regarding MDM2 was better understand this interaction.

Crystal structures of several peptides were produced by the Moffitt Cancer Center as part of MDM2/MDMX program. Among these structures is one peptide PDIQ (PDB ID: 3JZQ showing novel binding to previously unknown site that bound to Leucine side chain of PDIQ.

2.5 Methods

Virtual Screening. As part of a large screening program to identify inhibitors of MDMX, there was a collaboration with Schrödinger Inc., where the NCI plated set, a set of plated compounds that are available from NIH was screened against MDMX. Schrödinger chose an “open” conformation of MDMX chosen by observing the distance between TYR and MET and the proposed auxiliary binding site. From the Schrödinger screening of the NCI plated set, 5 potential inhibitors of MDMX.

Two of these inhibitors, E12 and A6, are potential binders of the auxiliary binding site. There was intense interest in producing tighter binding analogs of these two. E12 was the only one that showed binding to MDMX at 50 um concentration despite repeated trials. As part of this screening regimen, induced fit effects of binding to the MDMX binding site were examined in an effort to demonstrate how a small molecule might bind to the auxiliary binding site.

Five molecules were docked via the induced fit protocol, a part of the Schrödinger Suite of Programs allowing full flexibility in Met 100 and Tyr 53. Each was also docked
to open and closed confirmations of MDMX via Glide docking using both standard precision (SP) and extra precision docking (XP).

Table 4 Schrodinger selected compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Title</th>
<th>XP Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>B5</td>
<td>-5.725</td>
</tr>
<tr>
<td>E12</td>
<td></td>
<td>-5.419</td>
</tr>
<tr>
<td>C11</td>
<td></td>
<td>-4.262</td>
</tr>
</tbody>
</table>

The five compounds chosen by Schrodinger that were found by their virtual screening that also potentially interact with the auxiliary binding pocket.

Software. All software used was part of the Schrödinger Software Suite. (Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)

Hardware. Dell Precision 490 workstation with dual quad-core processors running at 2.23 GHz and having 9800 GT graphics and 1 TB hard disk.
2.6 Conclusion

Two generations of analogs were produced based upon virtual screening. An additional two generations of compounds were made based upon the MI-63 compound. From screening, we were able to produce an inhibitor with superior MDMX IC50 concentration from a MI-63 analog compound of 3nm IC50 concentration. (Canner et al., 2009)

It is very possible that a strong enough binder or large enough ligand would have enough energy to offset the energy cost of displacing the two side chains. The Tyrosine 8.3 A and the Methionine sulphur moved 3.8 A. The difference in energy between the closed and open conformations is about 3 Kcal. The additional hydrophobic interaction is worth a difference of 3 Kcal for the native ligand. Further study will be needed to fully uncover whether this additional binding site is a worthwhile target.

2.7 References


3.1 Lenalidomide causes dose dependent increase in MDM2

Lenalidomide is an analog of the notorious Thalidomide that was never approved in the United States but was widely used in the United Kingdom and imported into the United States through illicitly importing the drug from United Kingdom. Thalidomide was largely hyped at the time as a super drug. It eased the pains of leprosy, as well as quelling nausea and vomiting of morning sickness. (Franks, Macpherson, & Figg, 2004; Macpherson et al., 2003)

![Chemical structures of Lenalidomide and Thalidomide](image)

**Figure 12 Lenalidomide (left) and Thalidomide (right)**

Lenalidomide is a drug that suppresses multiple myeloma cell differentiation. It directly causes cell to undergo apoptosis within cells by binding to the DDB1, Cullin-4A, and ROC1 complex. This complex is activated when ubiquitinates several unknown
proteins which through a largely unknown mechanism causes upregulation of fibroblast growth factor FGF8. Within patients having multiple myeloma, MDM2 levels were seen to rise dose dependently. This is a curious development as the mechanism of action of lenalidomide is thought to act via cereblon, a ubiquitin ligase (Ito & Handa, 2012).

Patients at the Moffitt Cancer Center who undergo treatment for multiple myeloma take Lenalidomide to prevent the maturation of Plasma Cells that are cancerous in this condition. Plasma cells mature from B cells and produce immunoglobins which provide humoral immunity to pathogens. Patients were found to have increased levels of MDM2 in cellular assays. This finding is particularly significant because it is known that deficiency in P63, a homolog of P53 that also binds to MDM2 causes mice to be born without limbs and human beings to be born with electrodactyly, lobster claw hand syndrome. The investigation centered around the possibility that lenalidomide and thalidomide exerted its effect across ubiquitin ligases, and that in fact P63 may have been the cause of limb deformities with or without stabilization of cereblon. (van Straten & Butow, 2013)

3.2 MDM2 / MDMX / P53 regulation

MDMX, a closely related protein to MDM2, has similar activity as MDM2, except it does not contain ubiquitin ligase activity. The RING Domain of MDM2 and MDMX lie at their C terminus. The region acts a regulatory domain for MDM2, MDMX, and P53. MDM2 self regulates through an auto-ubiquitination pathway whereby it binds to another molecule of itself and recruits a ubiquitin conjugase enzyme, UbcH5b, which tags MDM2 with ubiquitin. This marks it for destruction by the
proteosome. P53 and MDMX is regulated in a similar manner where MDM2 ubiquitination by MDM2. MDMX can bind MDM2, P53, and other MDMX molecules but cannot mark any of them for destruction. It can however still remove bound molecules from the nucleus, preventing them from promoting transcription.(Linke et al., 2008)

MDM2, MDMX, and P53 are subject to post-translational modification as a methodology that grants or denies activity; however, it is the MDM2 protein which responsible for sequestration and ubiquitination. What enzyme becomes ubiquitinated is subject to the concentration of each enzyme. When MDM2 is in high concentration relative to MDMX or P53 it will act upon other MDM2 molecules. When P53 is in high concentration relative to MDM2 or MDMX, the P53 will be ubiquitinated and subsequently destroyed by the proteosome. When MDMX is in relative abundance MDM2 and P53 will be stabilized, as MDMX does not have the ubiquitin ligase functionality.(Linke et al., 2008)

The investigation started with a survey of crystal structures available from the pdb. While there were more than 30 structures for the N-terminal P53 binding domain. Only two related structures of the C-terminal RING domain were available (PDB ID: 2VJE and 2VJF) from the C.L. Day lab at the University of Otago, New Zealand. Knowing that the key regulatory portions of MDM2 were that of the RING Domain, and tow zinc finger domains, our investigation began with these structures.(Linke et al., 2008)
Figure 13 MDM2 domains are shown in color. The two unlabeled central regions are zinc finger domains. The white portions between the colored segments represent disordered portions of the protein.

Figure 14 Regulation of MDM2 and MDMX are concentration dependent.
Figure 15 An Assay showing the MDM2 mediated decrease in P53 levels and the effect of lenalidomide.

3.3 Methods

Because lenalidomide caused an increase in MDM2, our hypothesis was that lenalidomide may exert its action via the RING domain ubiquitin conjugase binding region. A similar region exists on Cereblon a well-reported target of Lenalidomide. Firstly, Lenalidomide binds to Cereblon an enzyme containing Ubiquitin Conjugase Binding domain similar to MDM2.

**Structure Preparation.** There are two X-ray crystallography structures that contain the MDM2/MDMX heterodimerization of the RING Domain. PDB ID: 2VJE and PDB ID: 2VJF differ by only be four residues. These residues lie at the very end of the N-terminus and C-terminus of both proteins and so affect the binding site very little. The homodimers were constructed by aligning MDM2 monomer with MDMX monomer and vice versa, producing a MDM2 pair and copy as well as MDMX pair and copy.

Lenalidomide structures were prepared for docking using Schrödinger’s LigPrep 2.5 utility. LigPrep creates a set of 3D computer models that are physiologically relevant. Thus, protonation states that are appropriate for pH’s between 5 and 9 are assigned to ionizable groups. Relevant tautomers are produced and stereoisomers are generated if stereochemistry is not known.
**Molecular Dynamics.** A short ten-nanosecond run was done via Desmond molecular dynamics to examine the strength of the heterodimer and the homodimers.

**Virtual Screening.** SITEMAP predicted two possible binding sites on MDM2 and one on MDMX. Both contained a possible binding site near dimer interface where a alpha-helix resides. MDM2 additionally contained a possible binding site near the zinc finger domain which is also the site that binds to ubiquitin ligase.

Taking the consideration of the SITEMAP output but also wanting to be thorough, grid files were calculated using Glide molecular docking for every part of dimer and each of the monomers. Glide has a built in limitations regarding the number of grid points that despite a long effort was met with no success. Higher grid point density led to better docking pose reproduction with x-ray crystal. So, instead six grids were made for the dimer and two grids for each of the monomers of MDM2 and MDMX. Each grid contained large areas of overlap in order to prevent some areas from not being docked to. This methodology was intended to function as a chemical probe might.

Computational docking of Lenalidomide was performed against the MDM2 and MDMX’s RING Domain as receptors. S and R enantiomers of Lenalidomide were docked separately. The X-Ray Crystal Structure (PDBID: 2VJF) for the MDM2 and MDMX’s RING DOMAIN was prepared using Schrödinger’s Protein Preparation Wizard. Docking was performed on monomeric MDM2 and MDM2 as well as the heterodimer of the two RING domains. Since no binding site had previously been described, Schrödinger’s SiteMap program was used to find potential binding sites. (Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)
3.4 Results

Output from SiteMap indicated a potential binding site may exist in a shallow hydrophobic pocket between the E2 conjugase binding and dimerization regions. Docking was performed using Schrödinger’s Standard Precision (SP) GLIDE software\(^2\). The best docking poses were chosen by highest binding affinity / lowest energy.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Docking Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenalidomide (S)</td>
<td>-5.2 KCal/mole</td>
</tr>
<tr>
<td>Lenalidomide (R)</td>
<td>-5.0 KCal/mole</td>
</tr>
</tbody>
</table>

Linke et al published results along with crystal structure that suggest that hydrophobic interactions were critical to E2 Conjugase recruitment. When I440, L468 and P476 is mutated, recruitment is abolished and MDM2 mediated degradation is not observed. The Docking pose of Lenalidomide is seen (Figure 2) binding to the hydrophobic pocket just beyond the E2 conjugase binding region suggested by Linke et al. (Linke et al., 2008) In all cases, the S enantiomer has a lower docking score than the R enantiomer. However, the difference in docking score is not greater than the 2.0 KCal/mole absolute error. The S enantiomer forms a hydrogen bond within the proposed binding pocket while the R enantiomer forms a hydrogen bond to an area not likely exposed in full length MDM2. It is evident that the R enantiomer binds with a lower affinity than docking would suggest.

Currently there is an effort to quantify binding biophysically, but that work is ongoing. Many questions remain about the whether lenalidomide binds to MDM protein monomers or to the dimer. The docking would suggest that the dimer is necessary. Expressing the MDMX/MDM2 dimer has not been done yet, but should be done in the near future.
Figure 17 Figure showing the binding of Lenalidomide to MDM2/MDMX heterodimer.
Figure 18 In this figure lenalidomide is docked to the binding site found by SiteMap. The S enantiomer (right) interacts with the ubiquitin ligase site and the R enantiomer (left) does not bind to the pocket.

3.5 References


Chapter 4: BCL-2 Family

4.1 BCL-2 Family

The BCL-2 family of proteins contains many oncogenes and tumor suppressors that act together to regulate apoptosis, especially apoptosis initiated by c-myc. Although c-myc is a proto-oncogene it actually is an inducer of apoptosis; it however, also causes telomerase activation. Through inhibition of BCL-2 family proteins apoptosis is inhibited while the telomerase activity is preserved. This is a pathway by which cells become immortalized. The BH3 domain members of the BCL-2 family are pro-apoptotic tumor suppressors that are regulated by binding to a long hydrophobic cleft in pro-survival MCL-1, BCL-XL, and BCL2 proper. In similar fashion to P53, pro-apoptotic factors of the BCL-2 family can be sequestered by BCL-XL and MCL-1, anti-apoptotic BCL-2 family members and potential proto-oncogenes. The pro-apoptotic factors Bim, Bid, Bad, Bik, Puma, and Noxa regulate cell permeability and control the release of cytochrome C and other death factors. BCL-XL and MCL-1 regulate these other members of the BCL-2 family by binding to amphipathic helix at the C-terminus. Disrupting this regulation is of great importance to inducing apoptosis in transformed tumor cells.(Reed, Zha, Aime-Sempe, Takayama, & Wang, 1996)
Figure 19 This figure illustrates the 4 hydrophobic binding pockets of MCL-1. These 4 hydrophobic residues of a region of BIM which binds to MCL-1 form the basis of inhibition design.

The drug discovery focus is this regard are the design of small molecule inhibitors which mimic the BH3 domain and disrupt this domain from binding to MCL-1, BCL-XL, and BCL2. Generally the search has centered around producing a pan-inhibitor of all three, however the most well known BH3 domain inhibitor is the Abbott compound ABT-737 which binds with high specificity to BCL-XL. (Kline et al., 2007)
Figure 20 MCL-1's binding is considerably more shallow than BCL-XL.

Binding to BH3 domain is governed by binding to a series of three to four adjacent hydrophobic pockets, which correspond to two isoleucines, a leucine, and phenylalanine on the BH3 domain.
Figure 21 BCL-XL and MCL-1 are responsible for BAX and BAK regulation.

4.2 Methods

To support the *in vitro* screening of efforts of Del Valle and Manetsch lab, several compounds were docked to MCL-1. The Manetsch lab in particular was interested in screening many compounds via a click chemistry methodology, which involved the reaction between a sulfoazide and a amino-acetal group. This reaction occurs only if both fragments bind the site of the reaction in a geometrically favorable position, which leads to reaction completion. This is known as Kinetic TGS. (Namelikonda & Manetsch, 2012)

Click chemistry is a technique first described by Nobel Laureate Dr. K. Barry Sharpless of Scripps Research Institute in 2001. It involves using simple reactions with common conditions in either water or solvent. TGS is an expanded idea of Click Chemistry in that using these simple reactions to perform combinatorial chemistry. In
this case the reaction occurs between an azide and a thio-acid, Figure 20. (Namelijkonda & Manetsch, 2012)

![Chemical Reaction Diagram]

**Figure 22 General scheme for TGS synthesis**

Combinatorial Screening. All combination of fragments produced by Manetsch lab were screened using Schrödinger’s Glide SP. We determined early on by comparing hits to IC50 values that Glide SP was the scoring function which best correlated with the inhibitory concentration for this particular reaction.
Methods. Schrödinger Glide1, 2 (version 5.0) dockings (Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004) were performed at standard precision for TGS compound structures containing one each of thiol acid (TA) and sulfonamide azide (SZ) fragments. PDB 2NL9 (Czabotar et al., 2007), a 1.55 Å resolution structure of Mcl-1 complexed with the Bim BH3 peptide, was downloaded from the Protein Data Bank (PDB) (Berman et al., 2000; Bernstein et al., 1977) and prepared into grid files for docking. Correlation of docking scores and IC50 values yielded $R^2 = 0.44$, an adequate value for use of this model. The top 2 kcal/mol docking modes consisted of 45 of 58 (77%) of the compounds. Fragment and cluster analysis of the top 2 kcal/mol ranked docking poses identified SZ31 to be most frequent among the SZ fragments while structures containing...
SZ15 were next in number to provide a similar binding mode as the SZ31 cluster of
docking modes. These findings corresponded well with IC50 data where all compounds
with the SZ31 fragments exhibited a range of 3.5 – 16.4 µM.

Overlaying the SZ31 fragment-containing structures in the top 2 kcal/mol docking
modes revealed one cluster with 1) an acyl-sulfonamide hydrogen bonding with Arg263,
a conserved residue of Mcl-1 known to hydrogen bond with Asp67 of the Bim BH3
peptide,3 2) the thio-phenyl and benzo-dioxalane groups of the SZ31 fragment occupying
the P1 and P3 pockets, respectively, and 3) the TA fragments occupying the binding
region between Arg263 and the P4 pocket.

**Specificity.** Mcl-1 over Bcl-xL. Similar docking and analysis of the TGS compounds
were performed on PDB 3FDL(Merino et al., 2009), a 1.78 Å resolution structure of Bcl-
xL. Fragment analysis of the top 2 kcal/mol identified a high-number clustering of TA11
fragment compounds not found in the Mcl-1 docking poses. The SZ15 docking modes of
the top 2 kcal/mol structures also provided a cluster of poses; however, since similar data
also appeared in the Mcl-1 dockings, the TA11 fragment cluster suggests a flatter
substituent for specificity towards the shallower P4 pocket of Mcl-1. Analysis of SZ31
molecule docking modes between Mcl-1 and Bcl-xL revealed that specificity can be
attained for Mcl-1 by having a flat TA fragment coupled to SZ31 by increasing ligand
strain energy due to the deeper P2, P4 pockets of Bcl-xL.

The Mcl-1 top 2 kcal/mol cluster led to further investigation of SZ31TA2 using
CombiGlide to first explore potential linkers then suitable R-groups for extension of the
SZ31 fragment through the h1 pocket to secure a hydrogen bonds with Ser245 and/or
Arg248. Further, cross-docking studies data recognized TA11 as a potential structure to
optimize for specificity of Mcl-1 over Bcl-xL. Recommended to the Manetsch Lab 1) the synthesis of a para-sulfonamide linker to the thio-phenyl group of SZ31 together with an para-ethyl-benzoic acid and 2) the analog library development of flat aromatic system for the TA fragments.

Table 6 Top scoring TGS compounds.

<table>
<thead>
<tr>
<th>TGS Compound</th>
<th>Specificity Index (glide score / glide score)</th>
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</thead>
<tbody>
<tr>
<td>SZ31TA02</td>
<td>60.6</td>
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<tr>
<td>SZ31TA08</td>
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<td>SZ31TA07</td>
<td>5.66</td>
</tr>
<tr>
<td>SZ31TA06</td>
<td>3.29</td>
</tr>
</tbody>
</table>

Figure 24 Correlation between SP Docking and IC50 for TGS compounds
We speculated that Glide XP that includes much steeper penalties for solvent exposure was over penalizing hydrophobic atom’s exposure to water. Most protein-protein interaction binding sites have large shallow pockets where high penalties for solvent exposure are probably not appropriate.

Taking the two best compounds at the time, SZ15TA03 and SZ31TA02, we employed combiglide enumeration with the Schrödinger fragment library in order to extend each compound with a hydrophilic substituent that would increase activity with Arginine 248 of MCL-1 and decrease solvent exposure in the Phenylalanine pocket.

**Figure 25** ARG 248 looked to be a site for potential extension of SZ31
Software. All software used was part of the Schrödinger Software Suite.

Hardware. Dell Precision 490 Workstation with dual quad-core processors running at 2.23 GHz and having 9800 gt graphics and 1 TB hard disk.

Extension toward Arginine 248. Several new libraries were created to based upon the best compounds, SZ31TA02 and SZ15TA03 with the intention of increasing the binding to MCL-1 and possibly increasing specificity. These new libraries were screened against 2NL9 using GLIDE.(Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004)

![Figure 26 This site is highly solvent exposed](image-url)
4.3 Conclusion and Future Direction

Although our modifications did lead to a decrease in IC$_{50}$ to 11 µM it was six times more specific for MCL-1 than previous compounds. Despite having a higher overall docking score, the ligand efficiency decreased. This demonstrates a fundamental problem of scoring functions in that higher molecular weight compounds tend to have higher docking score because more ligand atoms contribute to the calculation. Our speculation is that by focusing on ligand efficiency in the future this problem can be avoided.

The current focus of this project is on predicting the occurrence of click reactions on the BCL-XL surface. The current methodology is to dock molecules into each of the four pockets and then using scripts to calculate the distance from reactive sulfur to
nitrogen. By producing a list of structures ranked by their distance, we hypothesize the lower distances will correlate with probability for reactions occurring. One issue that has come to light is the relative non-specificity of the largely hydrophobic fragments. Our findings will be published in a paper in the near future.

4.4 References


Chapter 5: Virtual Screening against Deoxycytidine Kinase

5.1 Introduction

One of the most important enzymes for cancer drug discovery is the enzyme Deoxycytidine Kinase (DCK). DCK's function is to catalyze the phosphorylation of nucleosides. Once nucleosides have been phosphorylated they become monophosphorylated nucleotides. The nucleotides will then be phosphorylated a further two times to form triphosphorylated nucleotides. Finally triphosphate nucleotides are used as energy currency in the cell and also the building blocks of DNA and RNA. DCK phosphorylates deoxyribonucleosides specifically for incorporation into DNA. Because DCK functions to build genetic building blocks, it is of interest in both producing medicines for cancer and antimicrobial agents. DCK is therefore said to be a promiscuous enzyme because it phosphorylates deoxycytidine along with several other deoxynucleosides. The ability of DCK to phosphorylate a wide range of both purine and pyrimidine nucleosides makes it a good target.(Usova, Maltseva, Foldesi, Chattopadhayaya, & Eriksson, 2004)

Table 7 shows many of the prodrugs that already exist that use DCK as activator of their functionality. These drugs are known as anti-metabolites including acyclovir and ganciclovir. These anti-metabolites become incorporated into the DNA of the host organism but also deplete ATP molecules because DCK phosphorylates the fraudulent bases.(Rejiba, Bigand, Parmentier, & Hajri, 2009; Usova et al., 2004)
Table 7 List of Deoxycytidine Kinase Activated Compounds

<table>
<thead>
<tr>
<th>Citidine Antimetabolites</th>
<th>Characteristics</th>
<th>Category</th>
<th>Treats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine (Rejiba et al., 2009)</td>
<td>Hydrogen atoms on 2’ C of deoxycytidine are replaced by Fluorine.</td>
<td>Chemotherapy</td>
<td>breast cancer, ovarian cancer, non-small cell lung cancer, and pancreatic cancer</td>
</tr>
<tr>
<td>Cytarabine (Wang et al., 1997)</td>
<td>a.k.a cytosine arabinoside; kills cancer by interfering with DNA synthesis</td>
<td>Chemotherapy</td>
<td>acute myeloid leukemia and non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>Clofarabine (Zhang, Secrist, &amp; Ealick, 2006)</td>
<td>kills leukemia cells in blood</td>
<td>Chemotherapy</td>
<td>relapsed or refractory acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>Acyclovir (Santos et al., 2009)</td>
<td>acycloguanosine</td>
<td>Antiviral</td>
<td>Treats Herpes Simplex and Herpes Zoster</td>
</tr>
<tr>
<td>Ganciclovir (Hible, Daalova, Gilles, &amp; Cherfils, 2006)</td>
<td>9-(1,3-dihydroxy-2-propoxymethyl)guanine</td>
<td>Antiviral</td>
<td>Treats cytomegalovirus</td>
</tr>
<tr>
<td>Zalcitabine (Horwitz, Chua, Noel, &amp; Donatti, 1967)</td>
<td>dideoxycytidine</td>
<td>Antiviral</td>
<td>HIV</td>
</tr>
</tbody>
</table>

DCK is found to have increased expression in a few important cell lines. It is found increased frequency in cells of the thymus and those of lymphocites, white blood cells. This makes drugs activated by DCK important for drug discovery against Acute Myeloid Leukemia (AML) and Acute Lymphocytic Leukemia (ALL). In Herpes Simplex Virus (HSV), Deoxycytidine Kinase is induced in order to speed the replication of new viral DNA. (van der Wilt et al., 2003)

There are quite a lot of anti-metabolites as marketed drugs whose function is governed by DCK. The list includes drugs that act on herpes viruses and lymphocytic cancers. Resistance to DCK occurs through two distinct mechanism. One mechanism involves the alternative splicing of DCK. The other is through mutation in the genes that
control the HERT transporter genes. The HERT transporters are responsible for transporting Nucleosides into the nucleus.

5.2 Methods

Software. All software used was part of the Schrödinger Software Suite.

Hardware. Dell Precision 490 workstation with dual quad-core processors running at 2.23 GHz and having 9800 GT graphics and 1 TB hard disk.

Through our collaboration with the Southern Research Institute and the labs of Dr. Steven Ealick and Dr. John Secrist, III we received a set of potential DCK ligands for screening. These were all potential prodrugs that could be activated by DCK.

PDB ID: 2A7Q is a DCK structure solved by the Ealick group using X-ray diffraction methods and was used as the sole target of our virtual screening.

Because anti-metabolites are not inhibitors but active substrates of DCK, the overall magnitude of docking score can not be the sole deciding factor in choosing a set of ligands for screening. Careful consideration of the number of hydrogen atoms and ligand geometry must be considered as well. With so many market drugs that work through activation of DCK, a statistical analysis can be done comparing those scores with our set of potential anti-metabolites.

From the initial screening several molecules showed strong activity. Oddly some molecules with very similar structure show no potential activity. In this case our screening focus was changed to that of an investigation of interactions with DCK and the formulation of a hypothesis explaining the differences in interaction.
5.3 Results

Table 8 Activities and Docking scores for the best compounds.

In Figure 1 we have the best compounds from the set compounds from the Ealick group. Despite several of the molecules sharing very similar structures (Figure 2) the activities vary widely.

Activities seem to decrease with polarity of the sugar group. Where as the nucleoside base group seems to make less difference in activity. When looking at the overall docking score, activities are relatively the same. Looking at docking pose however reveals a greater number of hydrogen bonds with the more polar sugar group in
less active compounds. The less active compounds contain an additional fluoromethyl as in figure 2 or hydroxymethyl at the first position of the sugar portion of the nucleoside.

![Image](image.png)

**Figure 28** These two molecules differ only in the placement of one Fluorine Atom. Yet one is active (31 nm) and one is inactive (800 µm). The docking scores are within 1.2 Kcal/mole of one another.

**5.4 Conclusion**

Through screening ligands against the Ealick Crystal Structure (PDB ID: 2A7Q) we found that overall score was not as important as our hypothesis. From the list actives and related molecules we found that in all cases the inactive analog contained additional hydrogen bonding which led to increase in binding and a possible prolonged interaction within the binding site. This additional hydrogen bonding we proposed would lead to decrease in the dissociation constant leading to an overall decrease in reaction rate. An initial set of compounds was sent to us by the Secrist group for evaluation against DCK. The purpose of this evaluation was to design an inhibitor across several cancer cell lines.
This initial set of compounds was then tested and another set of compounds were then sent to us. From these two sets compounds several possible actives were found. Several very similar compounds to the active compounds were also tested but found to be inactive in the modeling studies. It then became the task of this lab to discern the reason for the inactivity. We found that the inactivity was the result of tighter binding than it's counterpart usually differing by a hydrogen bond. We propose that the additional hydrogen binding leads an decreased reaction rate or in Michaelis Menten kinetics, a decrease in $K_m$.

### 5.5 References


### A. MDM2 Inhibitors and Glide Scores

**Table A-1 List of Docking Scores of Known Inhibitors of MDM2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Glide Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoll-Chalcone-b1</td>
<td>-6.502</td>
</tr>
<tr>
<td>Dudkina-Benzodiazepine-29</td>
<td>-6.294</td>
</tr>
<tr>
<td>Stoll-Chalcone-c</td>
<td>-6.262</td>
</tr>
<tr>
<td>Stoll-Chalcone-a</td>
<td>-6.144</td>
</tr>
<tr>
<td>Hardcastle-Isoindolinone-2y</td>
<td>-6.003</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-10</td>
<td>-5.909</td>
</tr>
<tr>
<td>Title</td>
<td>Glide GScore</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-42</td>
<td>-5.907</td>
</tr>
<tr>
<td>Dudkina-Benzodiazepine-31</td>
<td>-5.899</td>
</tr>
<tr>
<td>Hardcastle-Isoindolinone-2v</td>
<td>-5.782</td>
</tr>
<tr>
<td>Walton-PFT-A</td>
<td>-5.759</td>
</tr>
<tr>
<td>Wang-Nutlin-3B</td>
<td>-5.731</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-40</td>
<td>-5.714</td>
</tr>
<tr>
<td>Walton-PFT-B</td>
<td>-5.702</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-29</td>
<td>-5.671</td>
</tr>
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title: Parks-Benzodiazepine-20
glide gscore: -5.515

title: Parks-Benzodiazepine-14
glide gscore: -5.485

title: Parks-Benzodiazepine-35
glide gscore: -5.481

title: Parks-Benzodiazepine-17
glide gscore: -5.457

title: Parks-Benzodiazepine-15
glide gscore: -5.384

title: Parks-benzodiazepine-5
glide gscore: -5.376

title: Parks-Benzodiazepine-24
glide gscore: -5.355

title: Parks-Benzodiazepine-30
glide gscore: -5.299
<table>
<thead>
<tr>
<th>Title</th>
<th>Glide GScore</th>
</tr>
</thead>
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<td>Parks-Benzodiazepine-6</td>
<td>-4.229</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-41</td>
<td>-4.222</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-26</td>
<td>-4.184</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-1</td>
<td>-4.174</td>
</tr>
<tr>
<td>Parks-Benzodiazepine-33</td>
<td>-3.863</td>
</tr>
<tr>
<td>Dudkina-Benzodiazepine-32</td>
<td>-5.047</td>
</tr>
<tr>
<td>Hardcastle-Isoindolinone-2w</td>
<td>-3.76</td>
</tr>
<tr>
<td>Hardcastle-Isoindolinone-2z</td>
<td>-4.932</td>
</tr>
</tbody>
</table>
title: Hardcastle-Isoindolinone-2z
glide gscore: -4.503
B. Ramachandran Plots From Building RING Domain Homodimers

Figure A-1 Ramachandran plot for the homodimer built only by aligning MDMX monomers.
Figure A-2 Ramachandron plot based on MDMX homodimer after 10 ns MD run.
Figure A-3 The Ramachandron plot for a MDM2 RING Domain homodimer built only by aligning molecules.
Figure A-4 The Ramachandran plot for MDM2 after a 10 ns molecular dynamics run.
C. Plots of NCID2 Screening Against MDM2 RING Domain

2VJF C1 Docking Scores

2VJF C1 NCID 1 Efficiency

Figure A-5 Area C1 Docking into Ring Domain of PDB ID: 2VJF.
Figure A-6 Area C2 Docking into Ring Domain of PDB ID: 2VJF
Figure A-7 Area D1 Docking into Ring Domain of PDB ID: 2VJF
Figure A-8 Area D2 Docking into Ring Domain of PDB ID: 2VJF
Figure A-9 Area CD1 Docking into Ring Domain of PDB ID: 2VJF
Figure A-10 Area CD1 Docking into Ring Domain of PDB ID: 2VJF
Figure A-11 Area CD3 Docking into Ring Domain of PDB ID: 2VJF
Figure A-12 Area CD4 Docking into Ring Domain of PDB ID: 2VJF
Figure A-13 Area CD5 Docking into Ring Domain of PDB ID: 2VJF
Figure A-14 Area CD6 Docking into Ring Domain of PDB ID: 2VJF
D. Synthesis and evaluation of substituted hexahydronaphthalenes as novel inhibitors of the Mcl-1/BimBH3 interaction
Synthesis and evaluation of substituted hexahydronaphthalenes as novel inhibitors of the Mcl-1/BimBH3 interaction

Young B. Kim, Maria E. Balasis, Kenichiro Doi, Norbert Berndt, Courtney DuBoulay, Chih-Chi Andrew Hu, Wayne Guida, Hong-Gang Wang, Said M. Sebti, Juan R. Del Valle

Bcl-2 family proteins regulate apoptosis through their influence on mitochondrial outer membrane (MOM) permeability and the release of cell death factors such as cytochrome c in response to cellular stress. Bax and Bak are pro-apoptotic multidomain (BH1–BH3) family members necessary for apoptosis and are directly involved in binding to the MOM. Other pro-apoptotic Bcl-2 proteins such as Bim, Bid, Bad, Bik, Puma, and Noxa contain only a single BH3 domain and indirectly modulate MOM permeability upstream of Bax and Bak. The anti-apoptotic family members Bcl-2, Bcl-xl, Bcl-w, Mcl-1, and Bfl-1 exert their influence by heterodimerizing with these pro-apoptotic substrates.

Mcl-1 is over-expressed in greater than 50% of hepatocellular carcinomas, pancreatic adenocarcinomas, cervical cancers, non-Hodgkin’s lymphomas, and non-small cell lung cancers. Mcl-1 is known to compensate for the loss of Bcl-2 or Bcl-xL activity induced by selective antagonists and is especially well suited to provide protection from apoptosis due to its relatively short half-life (between 0.5 and 3 h). Mcl-1 is thus a critical survival factor in a variety of human tumors and has emerged as a promising target for small molecule inhibitors.

In our search for new antagonists of anti-apoptotic Bcl-2 proteins, we screened a small in-house library of natural product-like hexahydronaphthalenes that showed activity against the Mcl-1/BimBH3 interaction in vitro. Here, we describe the synthesis of a small library of analogs and their biological evaluation. The most potent inhibitor in the series (19) exhibits an IC50 of 8.3 μM by ELISA and disrupts the interaction between endogenously expressed Mcl-1 and Bim in cultured MDA-MB-468 breast cancer cells.

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Scheme 2. Analog synthesis.

Table 1
Inhibition of the GST-Mcl-1-BimBH3 interaction by ELISA at 25 μM (mean given with standard deviation, n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Compound 1" /></td>
<td>53.8 ± 3.1</td>
<td><img src="image10" alt="Compound 10" /></td>
<td>31.2 ± 7.1</td>
</tr>
<tr>
<td><img src="image8" alt="Compound 8" /></td>
<td>34.8 ± 8.4</td>
<td><img src="image19" alt="Compound 19" /></td>
<td>74.3 ± 6.6</td>
</tr>
<tr>
<td><img src="image12" alt="Compound 12" /></td>
<td>17.1 ± 1.8</td>
<td><img src="image19" alt="Compound 19" /></td>
<td>40.3 ± 8.3</td>
</tr>
<tr>
<td><img src="image21" alt="Compound 21" /></td>
<td>53.9 ± 3.7</td>
<td><img src="image20" alt="Compound 20" /></td>
<td>64.9 ± 4.7</td>
</tr>
<tr>
<td><img src="image22" alt="Compound 22" /></td>
<td>26.5 ± 5.9</td>
<td><img src="image11" alt="Compound 11" /></td>
<td>31.2 ± 1.6</td>
</tr>
</tbody>
</table>
scaffolds for activity against the Mcl-1/BimBH3 interaction. This resulted in the identification of hexahydronaphthalene 1 (Fig. 1) as hit compound for further investigation. Here, we describe the synthesis of analogs of 1 and their biological evaluation as novel Mcl-1 antagonists.

The hexahydronaphthalene core structure was prepared via Diels-Alder reaction between dihydrobezaldehyde derivative 3,7 and (E)-trimethyl(3-methylbuta-1,3-dien-1-yl)oxy)silane under thermal conditions (Scheme 1). Treatment of the intermediate cycloaddition adduct with TBAF in THF promoted silyl ether cleavage and concomitant alkene migration to give core structure 4 as a single diastereomer. Extensive 2D NMR experiments established the structural connectivity of the product while relative stereochemistry was confirmed by X-ray diffraction of reduced derivative 9. Presumably, treatment of the intermediate Diels-Alder adduct with TBAF promotes retro-aldol ring opening and equilibration to the more stable α-OH isomer. While the precise mechanism of the concomitant alkene migration is unknown, this process seems to occur during the retro-aldol reaction and ultimately gives rise to a lower energy dihydrodecalin. The relative stereochemistry of the hexahydronaphthalene scaffold was confirmed by X-ray diffraction of diol derivative 5.

Elaboration of 4 into the desired compounds was achieved through silylation of the secondary alcohol followed by aldehyde reduction and conversion to the methoxymethyl ether 7 (Scheme 2). To assess the impact of the amide substituent of 1 on biological activity, we prepared truncated analogue 8 via silyl ether cleavage and reaction of the resulting alcohol with cyclohexylisocyanate. Hydrolysis of 7 followed by dialkylation afforded analogs 10 and 11. Condensation of 9 with various amines and subsequent carbamoylation gave a series of diversely substituted hexahydronaphthalenes for biological evaluation.

All newly synthesized compounds were tested for their ability to block the interaction between GST-Mcl-1 and the BimBH3 domain in an ELISA assay 19,20 at a single concentration (25 μM). A peptide corresponding to the BimBH3 helix was used as a reference inhibitor and average percent inhibition was calculated from three separate experiments. As shown in Table 1, initial lead compound 1 exhibited >50% inhibition of Mcl-1 at 25 μM. Truncations at either end of the hexahydronaphthalene core as in 5, 9, and 12 resulted in diminished activity, as did the introduction of a more polar morpholine group in place of the isobutanyl amide as in 22. Various hydrophobic substitutions were well tolerated, with the exception of the smaller cyclopentyl and isopropyl carbamates in 17 and 18. The 3-chlorofuryl carbamate derivative 19 exhibited slightly enhanced activity. Compound 10, which is readily accessible from the core scaffold via dialkylation, also showed significant inhibition at 25 μM.

We then selected the three most active compounds from single dose testing for determination of IC50 values. Figure 2 depicts the ELISA dose response curves for compounds 10, 19, and 23 over a range of concentrations between 0.1 and 100 μM. Each compound exhibited dose-dependant inhibition of Mcl-1 in the micromolar range. The most potent compound, 19, showed an IC50 of 8.3 μM in vitro.

We next determined whether the most potent inhibitor in vitro (19) is able to enter human cancer cells, reach its target and disrupt the Mcl-1/Bim interaction. To this end, we treated MDA-MB-468 human breast cancer cells that ectopically express Bcl-xL and Bim with either vehicle (0.1% DMSO) or 19 at 25 or 50 μM, lysed the cells, immunoprecipitated Mcl-1 from the lysates and immunoblotted with Bim as described by us previously.21 As a positive control we used BH3M6, a substituted terphenyl derivative that has previously been shown to disrupt the interaction of various anti-apoptotic Bcl-2 family proteins with Bim in whole cells.22 TPC, an unsubstituted analog of BH3M6 with no appreciable in vitro activity was also included as a negative control. Figure 3 shows that in vehicle-treated MDA-MB-468 cells Bim co-immunoprecipitated with Mcl-1. Similarly, cells treated with negative

![Figure 2. ELISA Mcl-1/BimBH3 dose-response curves for 10, 19, and 23 (95% confidence intervals in parentheses).](image-url)
control TPC also show complex formation between Mcl-1 and Bim. In contrast, 19 inhibited the interaction between Mcl-1 and Bim at both 25 and 50 µM. Compound 19 had similar potency to BH3M6 at both concentrations.

Compound 19 also exhibited approximately 80% growth inhibition toward MDA-MB-468 cells above 100 µM by MTT assay (see Supplementary data). However, increased concentrations did not result in greater cytotoxicity. Poor solubility in culture medium beyond 100 µM precluded an accurate determination of GI50 values. To gain structural insight into the possible binding interaction between 19 and Mcl-1, we performed computational docking experiments using the GLIDE protocol. Since 19 was produced as a racemic mixture, we docked both enantiomers to the X-ray crystal structure of human Mcl-1 (derived from PDB code 2NL9). Figure 4 depicts the top scoring docking poses for each enantiomer, both of which bind to the hydrophobic cleft occupied by the BH3 helical domains of pro-apoptotic partners. Notably, the two large hydrophobic groups of the 1R,4aS,6R,8aS enantiomer (isobutyl and 3-chlorophenyl) bind to the sites on Mcl-1 that normally accommodate the Leu62 (i+4) and Phe69 (i+11) side chains in human Bim (Fig. 4A). While the 3-chlorophenyl substituent in the 1S,4aR,6S,8aR enantiomer also appears to mimic the Phe69 residue, the isobutyl substituent resides well outside of the Leu62 pocket (Fig. 4B). In both docked structures, the methoxymethyl ether substituent of 19 makes extensive stabilizing contacts with Aes260 and Arg263 in Mcl-1. Furthermore, this functional group overlays well with the carboxy side chain of Asp67 (i+9) in human Bim.

In summary, we have described the synthesis and preliminary SAR for a series of hexahydropyranalenes that disrupt the Mcl-1/BimBH3 interaction in vitro. The most potent inhibitor in the series (19) exhibits an IC50 of 8.3 µM by ELISA. Compound 19 also disrupts the interaction between endogenously expressed Mcl-1 and Bim in intact MDA-MB-468 breast cancer cells. Computational docking suggests that 19 interacts with the BH3-binding hydrophobic cleft in human Mcl-1. Efforts toward the synthesis of enantiopure 19 and other analogs for evaluation against a wider panel of anti-apoptotic Bcl-2 family proteins are currently underway.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bml.2012.07.050.

References and notes


