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4-ISOXAZOLYL-1,4-DIHYDROPYRIRINES BIND THE MULTIDRUG-RESISTANCE TRANSPORTER

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ABSTRACT

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4-Isoxazolyl-1,4-dihydropyrirines bind the multidrug-resistance transporter

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The development of multidrug resistance in tumor cells has been recognized as a major obstacle to successful cancer treatment. Tumor cells *in vitro* and *in vivo* can develop multidrug resistance (MDR) to the lethal effects of a variety of cytotoxic drugs used to treat cancerous tumor cells. The over expression of multiple drug resistance gene 1 has been correlated with the expression of multi drug resistance protein 1(MDR1, also known as P-glycoprotein or P-gp). MDR1's role in altering uptake, distribution and bioavailability is considered a significant factor when examining drugs for clinical administration, and represents a viable drug target for the reversal of MDR.

MDR1 is driven by ATP hydrolysis and as such it shares both sequence and structural homology with proteins that are energy-dependent efflux transporters driven by ATP hydrolysis, making MDR1 a member of the ATP binding cassette (ABC) super family. Because, MDR1 transports substrates that are often toxic xenobiotics, MDR1 is thought to fulfill a cellular detoxification function. As such it is expressed in several tissues in the body such as the liver, pancreas, kidney, colon, intestinal mucosa, and in the blood brain barrier. Due to its presences in a wide variety of cells it has been suggested that MDR1 is involved in protection of the organism as a whole. Consequently, the overproduction of MDR1 is seen in cancer cells. MDR1 has been shown to transport a wide variety of lipophilic agents, of importance, MDR1 effluxes chemotherapeutics agents out of the cell resulting in a low and ineffective intracellular drug concentration. Thus, the over production of MDR1 in cancer cells can then be thought of as a protective factor for cancer cells, and as an effect causes MDR cancer cells. Therefore, understanding MDR1's function is important for controlling the bioavailability of drugs and for improving anticancer chemotherapy.

Reversal of multidrug resistance is of interest, and MDR reversing agents have been under intensive investigation. The 4-Isoxazolyl-1, 4-Dihydropyridines (IDHP's) have been shown to exhibit inhibition of MDR1. A novel series of IDHP compounds have been prepared and found to inhibit MDR1. The synthesis, MDR1 assay results, and relevant controls will be discussed. If successful, halting the function of MDR1 will stop the outward efflux of chemotherapeutic agents. In combination with chemotherapeutic treatments IDHP agents could allow for greater effectiveness of pharmaceutical intervention. This research would give an option in the treatment of cancer that would normally never exist for MDR cancer patients, and would allow for far more effective treatment via pharmaceutical means.

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MDR1 Background: Why do we care about MDR1 and what does it have to do with cancer

1.1 The ABC Superfamily

The mammalian multidrug efflux pump Multidrug-Resistance Protein 1 (MDR1) is a membrane transporter that is the most widely studied member of the ABC super family of proteins. This 170 kDA protein is most commonly observed in cells that are over expressing the multiple drug resistance gene 1 (MDR-1) or ABCB1 gene. MDR1 was first identified in Chinese hamster ovary cells that were selected for colchicines resistance.[1] These cell developed into multiple drug resistant(MDR) cell lines that had a reduced rate of drug uptake. [1] The ABC super family is the largest protein family in many organisms, and the family of transporters carries out a wide variety of processes in both prokaryotes and eukaryotes. [2] Over 80 ABC proteins have been seen in *E.coli* [3] and 48 analogous proteins have been observed in the human genome.[4] ABC proteins are typically composed of four course domains: two α-helical transmembrane domains (TMD), and two cytoplasmic nucleotide binding domains (NBD) that hydrolyze ATP. [5] In eukaryotes, the four domains are commonly expressed as a single polypeptide, while in prokaryotes they are synthesized as two or four separate subunits depending on the area of expression.[5]

Bacterial ABC proteins have been shown to have importer and exporter characteristics, while mammalian ABC proteins are exclusively exporters by nature. [6] All of the ABC proteins have been shown to actively transport substrates against their concentration gradient. [6] Most ABC transporters have been shown to have broad substrate specificity, with MDR1 and the breast cancer resistance protein (BCRP) being the most important clinically. Due to the clinical significance of the ABC proteins they

have been studied extensively in the hopes of reversing the MDR phenomenon. Mutations in 17 different human ABC transporters have been implicated in several disease states to date, [7] making the identification and examination of compounds that will selectivity inhibit these transporters of clinical interest.

1.2 MDR1's Physiological and Pharmacologically Important

The multidrug ATP- binding cassette (ABC) transporters mediates the ATP dependent efflux of cytotoxic agents out of the cell and away from their intercellular targets. [8] There are several members of the ATP driven transporters that are expressed at varying tissue barriers found thought out the body thus, the ABC binding cassette super family has a profound influence on the uptake and elimination of drugs. [9] Low levels of MDR1 expression have been observed in most tissues, however, MDR1 is the most abundant in the apical membranes or polarized epithelial cells with excretory function, such as, the intestine, kidney, liver and pancreas.[10][11] MDR1 is also located in the endothelial cell membrane of blood-tissue barriers, including the blood-brain barrier (BBB), placenta, blood-testis, blood ovary and blood-nerve barriers. [12] The tissues where MDR1 is expressed are important barriers in the absorption, distribution, metabolism and elimination (ADME) of many clinically important drugs, including those used in chemotherapy treatment. The exact physiological role of MDR1 in each of these tissues is not known with certainty and is complicated by the multiplicity and promiscuity of substrates that MDR1 can bind. It is though that MDR1 may play a role in protecting the body from toxic compounds and is considered a cellular "vacuum cleaner" for both endogenous toxins and xenobiotics. This protective factor is seen experimentally as well. Experiments with double –knockout mice, mice that don't express MDR1, but still

display a normal phenotypes where exposed to ivermectin. Ivermectin is an anti-parasitic agent; it binds and activates glutamate-gated chloride channels in the CNS. Ivermectin will lead to ataxia and death if given in high doses. Given that MDR1 was not present in the double –knockout mice the ivermectin was allowed access into the CNS and the mice died.[13] While the wild type mice, mice that have MDR1, survived. [13] The knockout mice couldn't expel the neurotoxic ivermectin which accumulated in the brain, whereas wild type mice where protected by the presence of MDR1 in the BBB. This can also be observed in non-knockout animals, Collie's (*Canis familiaris*) especially rough and smooth Collies, have a naturally occurring fame shift mutation that result in a lack of MDR1 expression. As one would expect they also display high sensitivity to ivermectin relative to other breeds of dogs. [14]

Originally the ABC super family has been linked to development of multidrug resistance in tumor therapy. [20] [21] As mentioned the mammalian MDR1 is an ATP-driven pump that transports structurally unrelated compounds, which include many anticancer and antimicrobial agents. [22][23] Thus, MDR1 has a vital pharmacologically role in conferring drug resistance to cells by catalyzing the efflux of these cytotoxic compounds. [7,15-18] It has become clear that MDR1 can transport many chemically and structurally unrelated drugs and agents [24], resulting in the MDR phenomenon that accounts for chemotherapeutic failure seen in the treatment of cancer. Due to this observation MDR1 has also been recognized as an important factor in novel drug development, so much that the Food and Drug Administration (FDA) suggested that new drug candidates should be routinely be screened for MDR1 interactions. [19]

1.3 MDR1 and Multidrug Resistance

Tumor cells can become resistant to a wide range of anti-cancer drugs in a condition known as MDR. The development of MDR in tumor cells has been recognized as one of the major obstacles to successful cancer treatments. [25][26] It has been observed that MDR1s occurrence is a precursor for the development MDR. [27-29] There are thirteen known ABC transporters including MDR1, that have been reported to be involved in the development of MDR. [30] MDR can arise from a variety of cellular mechanisms including altered drug metabolism, p53 mutation an altered DNA repair processes, but drug efflux resulting from MDR1 over expression is thought to be one of the core causes. [31][32] MDR1 expression levels are correlated with resistance to several anti-cancer drugs in the U.S. National Cancer Institute collection of tumor cell lines, and it is believed to contribute to chemotherapy drug resistance in at least 50% of human cancers.[33][34] Some cancers, including those of the colon, liver, pancreas and kidney, cell lines that express high levels of MDR1, tend to be inherently drug-resistant. While others such as leukemia, myelomas, ovarian and breast cancers most often develop MDR subsequent to chemotherapy treatment. [35] MDR1 has been reported in many of these tumors and in some cases, its expression increases after one or more rounds of chemotherapy. [35] Given the clinically relevant evidence of MDR1 and it role in MDR development it illustrates the continuing need for a selective MDR1 inhibitor.

Consequently, the overproduction of MDR1 is observed in cancer cells. The over production of MDR1 in cancer cells can then be thought of as a protective factor for cancer cells, and a cause of MDR seen in cancer. Therefore, understanding MDR1's function is important for controlling the bioavailability of the drugs and for improving anticancer chemotherapy. MDR1 has been shown to efflux a remarkably broad range of

substrats, and transport numerous structurally and functionally diverse compounds across the cell membrane. [36] MDR1 and its ligands are therefore extensively studied both in respect to reversing multidrug resistance in tumors and for modifying ADME properties for other novel drug candidates. [37] A novel compound that would hinder MDR1 ability to efflux chemo selective agents out of cancer cells would allow for the repurposing of out-of-date agents where resistance has been developed. It also allows chemo selective agents to be used at lower and safer doses, if co-administration was utilized. The exploration and validation of this hypothesis will follow in due course.

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Chapter 2:
Computer Modeling of MDR-1 and its uses as a predictive model

2. Computer Modeling

A key method for the prediction of receptor-ligand complexes in the drug discovery process is molecular docking. [1] The first atomic orbital calculation was done in the UK in the early 1950[71] since then the process has been refined and is now widely used as a virtual screening tool in the early stages of drug development. It allows for the reduction of complicated interactions to be refined down to SARs that directs early synthetic efforts towards more potent and selective compounds. The docking process itself involves two phases. The first phase, sampling, covers the generation of ligand configurations and orientations of a ligand relative to the target binding site. These are referred to as poses. When receptor flexibility is taken into account, sampling also involves the variation of the receptor configuration. The second phase, scoring, is a calculated estimation of the ligand's binding affinity or activity. When docking is applied to a library of virtual compounds, the compounds are ranked according to the best scored poses. This process is called ranking. Scores are calculated by evaluating the free energy that is estimated from the binding of the receptor-ligand complex. There-by, the complexity of the receptor ligand interaction is immensely reduced and quantified to allow for a simplified list of potential target compounds. Most of the contemporary scoring algorithms focus on enthalpy terms, whereas molecular associations are driven by enthalpy and entropic effects. Often docking programs used simplified structural representations and reduce if not neglect protein flexibility as well as the participation of solvent molecules in binding. Additionally, most docking programs assume a certain static protonation state and consider a fixed distribution of charges among the atoms. The lengths and, except for the torsions of rotatable bonds, angles between covalently bonded

atoms are kept fixed. [2] Even considering the limitations, the predictive power and benefit of molecular docking has been demonstrated in many studies.

2.1 Computational Chemistry Basics

Molecular docking attempt to assess the standard binding free energy of complex formation. For the estimation of receptor-ligand interactions the free energy is the most important thermodynamic characteristic. [24-26] In general, it describes the driving forces of most biological processes such as the folding of proteins, osmotic forces and the formation of receptor-ligand complexes. Knowing the basic physics involved in these processes would enables us to calculate the corresponding binding free energy. However, the complexity of biological systems renders the exact calculation untenable for truly realistic biological systems. Despite this, there are many systems where it is possible to construct a virtual model system that reflects the relevant and inherent properties of the real target system. [23] Modern computer based techniques use parameter based model systems and several computational and mathematical "tricks" for the generation of structural ensembles corresponding to a series of structures that are represent the dynamical processes seen in biological systems. Such simulations are expected to correspond to the dynamics of the target system. When such a biological process can be modeled and covered by the simulated timescales, simulations can be used to approximate the binding free energy involved in these processes. Nevertheless, the size of typical biologically systems and the timescales on which drug binding takes place cause an enormous amount of computational power.

2.2 Scoring Functions

The scoring function is one of the central concepts in molecular docking. This function enables a docking algorithm to rapidly describe and quantify the interactions between ligand and receptor. During the sampling phase the docking algorithm produces different ligand configurations and orientations within the target site and assigns a score by evaluating the scoring function. An ideal scoring function would provide the lowest scores for the energetically most favorable receptor ligand configurations. Assuming that these configurations represent the interactions that mainly promote the ligand binding, they give some insight into the underlying molecular mechanisms. An overview over a broad spectrum of scoring functions is given, [28] there are mainly three different types of scoring functions.

Force-field based scoring functions are designed based on underlying physical interactions such as van der Waals (VDW) interactions, electrostatic interactions they also take into account the bond stretching, bending and torsional interactions of the calculated compounds. The force field parameters are usually derived by both fitting to empirical data and *Ab initio* calculations. A typical force-field based scoring function is implemented in the DOCK algorithm whose energy function is the sum of VDW and coulombic energy contributions:

$$E = \sum_{i,j \neq i} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + \frac{q_i q_j}{\epsilon r_{ij}}\right)$$

Here Aij and Bij are VDW parameters, qi and qj the charges and rij is the distance between the particles i and j this is often referred to as the dielectric constant.[29] The scoring function does not include the energetic costs of desolvation which is a consideration for many body interaction terms and depends on the chemical environment. As such, to account for desolvation, further terms are usually added based on the solvent-

accessible surface area of the ligand and possibly the receptor.[30] When energy terms of VDW and coulombic interactions are used in a scoring function, they need to be empirically weighted, this will account for the difference between energies and free energies,[31,32] and also account for the different methods used to calculate the varying terms.

Empirical scoring functions estimate the binding free energy Δ G of a receptor-ligand complex by a sum of weighted energy terms:

$$\Delta G = \sum_i \omega_i \Delta G_i$$

The energy terms Gi can represent VDW and electrostatic interactions, hydrogen bonding strength, entropy changes, hydrophobic interactions or desolvation energies and energy terms. The weighted term ωi is derived from known experimental data of a previous training set. In 1994 Büohm developed an empirical scoring function consisting of hydrogen bonds, polar interactions, the lipophilic contact area between ligand and receptor and the number of rotatable bonds in the ligand.[30] The weighted terms were calculated with a dataset of 45 protein-ligand complexes.[30] This scoring function was further improved when Eldridge[31] developed the ChemScore scoring function that includes terms for hydrogen bonds, metal atoms, lipophilic contacts as well as the number of rotatable bonds in the ligand. But, due to the number of terms in the empirical scoring function it becomes more and more difficult to avoid double counting specific interactions. Thus, the applicability of empirical scoring functions may depend on the data used in the training set. As such empirical scoring functions that are fitted to larger training sets should be more generally applicable. Knowledge based scoring functions use terms that weight the receptor-ligand complexes by the occurrence frequencies of

particle-particle pairs in a database of known complexes[32]. The idea behind the knowledge based scoring function is that large numbers of different particles will somehow distribute themselves into a gas phase at temperature T if the interactions are purely pair wise, the distributions can be described by the equilibrium pair wise density ρij (r) between any two particle types i,j at distance r. In this case, the interaction free energy, wij(r), can be calculated from the observed densities by the inverse Boltzmann relation:

$$\omega(r) = -k_B T \ln \left(\frac{\rho_{ij}(r)}{\rho_{ij,0}}\right)$$

Where ρij (r) is the pair density of a particle pair at distance r and ρij, the pair density of a reference state where the inter-atomic interactions are zero[27,32]. Since these potentials are extracted from the structures rather than from known binding affinities by fitting, and the training structural database can be large and diverse, the knowledge-based scoring functions is considered to be more reliable to a given training set[27]. Because of the pair wise interaction scheme the knowledge based scoring functions can be as fast as the empirical scoring functions. However, atoms in protein-ligand complexes are not particles in the gas phase and the frequencies are not independent from each other. Therefore, the calculation of accurate reference states ρij, is a challenging task in the development of knowledge based scoring functions.

Hybrid scoring functions are implementations of mixtures of the different flavors of scoring functions. They combine force field terms and empirical energy terms. This is done, in the program like eHiTS[33,34]. Notably, all currently applied scoring functions require a significant degree of empirical fitting. Therefore, scoring functions are not

necessarily generally applicable to all kinds of drug targets and should be standardized and possibly optimized.

2.3 Molecular architecture of MDR-1

There are a number of techniques that can be used to derive the structure of a protein. Including, cryo-electron microscopy(EM), X-ray crystallography, nuclear magnetic resonance (NMR), and bioinformatics-based approaches can all aid in the elucidation of the structure and function of an unknown protein. Historically, our understanding of drug transporter structure originated from single particle and 2D cryo-EM. As such, the first three dimensional structure of MDR1 was obtained by "two-dimensional" cyo-EM.[5] The two-dimensional proteins [3,4] were observed in the presence and absence of AMP-PNP, a non-hydrolyzable analogue of ATP that has been shown to bind to the NBD at the same site as ATP. Two dimensional cyo-EM trapped MDR1 at different steps of the transport cycle. These data were interpreted to suggest that the transmembrane α-helixs undergo conformational changes as a result of the ATP binding, followed by subsequent hydrolysis and substrate release. [5] This led to many attempts to correctly describe the conformational change, ultimately leading to the alternating access hypothesis that is supported today. It was also noted that this conformational change was responsible for the known efflux phenomenon of the transporter that leads to MDR.

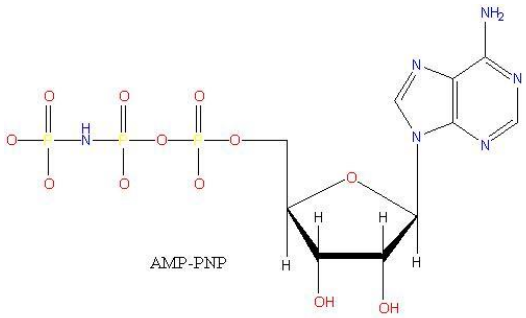

Figure 2-1: AMP-PNP

2.4 Topology of MDR1

The mammalian MDR-1 topology, was first described by Danø[35] and Juliano and Ling.[36] MDR1 is a single 1280 amino acid polypeptide organized into two semi-

homologous halves. With mammalian MDR1 characterized by 12 transmembrane domains and two nucleotide binding sites. [47] Each half of the protein contains six transmembrane domains and one nucleotide binding site for each half. [38] The two semi-homologous halves of the protein are separated by an intracellular flexible 60 amino acid linker polypeptide loop. [38]

In general the two NBD's have been shown to work in a cooperative matter to allow for the hydrolyzing of ATP. [39] The binding and hydrolysis of ATP is the energy source that is used to functionally couple the outward efflux of drugs substrates against the concentration gradient. [40, 41] Several models have been proposed to explain the energetic coupling between the NBDs and the efflux of drugs out of the cell. [39] The most widely accepted model is known as the alternating access or the rocking banana mode of action.

The common transport of substrates against a concentration gradient via ATP hydrolysis is a common function of ABC transporting proteins. It is not surprising that there are shared structural features. As described previously the focus of our research is the NBD due to the previous observations that DHPs have been shown to bind in this location. There is also a relatively high degree of conservation between the NBD across species, which is expected given the common mechanism of ATP driven transport. This also makes targeting the NBD a promising lead in that most MDR1 proteins will be affected by competitive binding at the ATP site. NBD1 and NBD2 have 25% and 28% sequence identity respectively, with a 50% similarity across the ABC superfamily. [42] [43] The NBD of many proteins that bind and hydrolyze ATP contain a sequence termed the Walker A and Walker B motifs, [44] with a unique motif, the signature C motif being

specific to MDR1.[45] MDR1 also contains highly conserved amino acids in the A, Q, H, and Pro-loop of the NBDs. These loops along with the Walker A and B regions and the signature C motifs, are involved in the binding and the coordination and hydrolysis of ATP. [46] The Q and P-loops have also been shown to be involved in the interdomain communication and coupling ATP hydrolysis to the TMD. [47] It must also be mentioned that the other potential binding sites in MDR1 don't show the same level of conserved, with low level of sequence similarity in the TMDs. This is not surprising due to the substrate promiscuity of MDR1.

2.5 X-ray Crystal structure of MDR1

Mammalian MDR1 has recently been crystallized [48] (PDB: 3G5U). Earlier attempts to model the 3D structure of MDR1 suffered from low sequence identity to the template protein, a prime example being the bacterial ABC transporter MsbA.[49,50] The high resolution X-ray crystallographic structure are available for the bacterial lipid A flippase MsbA in several nucleotide bound states,[51] and the transporter Sav 1866 from *S. aureus* with bound AMP-PNP.[52,53] Both MsbA and Sav1866 show the highest sequence similarity with MDR1 of all ABC transporters.

2.6 Sav 1866 as a template

In 2006 the crystal structure of a bacterial ABC transporter, Sav1866 from *S. aureus*, was published. [7] The first Sav1866 structure was resolved to 3.0 Å, the structure, was a MsbA model that was a homodimeric ABC transporter that is semi-homologous to MDR1. Sav1866 was crystallized in the presence of two adenosine diphosphate (ADP) molecules that bound at the interface of the NBDs. The NBDs of the Sav1866 homodimer are similar in structure to those of other ABC transporters.[7] These domains

show conserved ATP binding and hydrolysis motifs at the shared interface in a head-to-tail arrangement. [8-10] In 2007 Locher published the crystal structure of Sav1866 complexed with AMP-PNP. [11] A comparison and superposition of both structures, the AMP-PNP bound Sav1866 and the ADP bound Sav1866, indicated that these structures are essentially identical and represents the ATP-bound state of the transporter. The different structures of MsbA were indicated to be reconcilable with the Sav1866 when mirrored. [12]

The overall architecture differs from the side by side arrangement of the TMDs and was observed for the ABC transporters BtuCD[13] HI1470/1[14] ModB2C$_2$[15] and MalFGK2.[16] In the ABC exporters the TMDs interact with the helical domains of the NBDs through coupling helices that are located in the loops between TM helices.[17] One intracellular loop of each TMD makes contact with the NBD.[18] The two subunits exhibit a considerable twist [7] the transmembrane helices of MDR1 diverge into two discrete points away from one another towards the cell exterior of the membrane, producing what is known as the inward facing conformation. The inward facing conformation of the transporter is thought of as its resting phase and after ATP is hydrolyzed it transitions into its active phase. This alternative conformation is seen when helices from both TMDs move away from one another.[7]The helical arrangement observed in Sav1886 is consistent with this, except for helices 6 and 12, where cross-linking studies have identified analogous TM helices in human MDR1[7]. Helices 6 and 12 are positioned closer to each other than in the Sav1866 crystal structure. [19] The arrangement of the 12 canonical transmembrane helices observed for Sav1866 is in agreement with the ABC exporter topology.[7] Also, they are consistent with electron density maps of human

MDR1 and cross linking restraints.[20] The ATP bound state of the NBDs, with the two nucleotide binding domains in close contact, is likely to be coupled to the outward facing conformation of the TMDs. In this conformation the helices line a central cavity, which is open to the cell exterior. The three previously published structures from *Escherichia coli*, *Vibrio cholera* and *Salmonella typhimurium* were revised by Chang. Now all models show the analogous topology to Sav1866.[23]

2.7 Homology Model of MDR1

In the absence of a high resolution crystal structural for a human MDR1, an alternative is the generation of a protein homology model. The availability of accurate crystal structures of closely related target proteins is a critical factor for structural homology between a structurally resolved protein, and the unknown. Conformation sequence identity and an identical number of predicted transmembrane spanning helices are important for the selection of a template modeling of membrane proteins [6]. The quality of a homology model is determined by the accuracy of the alignment between the protein of interest and the template protein, and the quality of the crystal structure used as the modeling template.

2.8 Development of MDR1 Homology Model

With the lack of a reliable *Homo sapiens* crystal structure, a homology model based on the inward facing *Mus musculus* model was constructed.

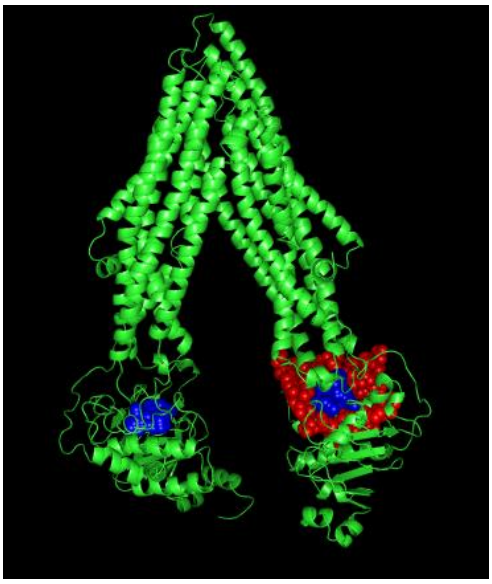

Figure 2-2: Human homology model of MDR1

As the structural difference between the apo protein and the co-crystallized structures was surprisingly low the higher resolved 3G5U structure was utilized as homology modeling template. The Model of the human MDR1 were created using the sequence homologies between the *Mus musculus* (PDB:3G5U) model and the protein sequence of the *Homo sapiens* MDR1 (Uniport: P08183). After the alignment of the sequence with VMD, Modeller 9.4 was then employed to construct 100 different homology models. The lowest energy homology model was then selected as the human model of MDR1.

Traditionally, experimental assays are used to assess novel drug candidate's interactions [61]. The major drawback to this process is that experimental assays are expensive and time-consuming. A reliable *in silico* model would be an invaluable tool to rapidly and more cost effectively screen potential compounds. This process would identify MDRR candidates and has been recognized as a valuable tool to those in industry

[62-64]. Computational approaches and model based approaches have been attempted but have only shown limited success [65-73].

Computer modeling was used to provide a convenient means of investigating aspects of the SAR. This emerging analysis allows for supporting explanations of pharmacological date from relevant experimental data. Computer modeling also allow for the ration design of the potential pharmaceutical agents. A number of studies have been conducted and have given some insight into what novel structures should be pursued [58] [59]. In this respect a reliable homology model of MDR1 would be a great asset in drug discovery [60].

This human model was then utilized to evaluate established SAR that has been established, along with being the current working model of the remainder of the *in silico* studies performed for inhibitors.

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Chapter 3:
4- Isoxazolyl-1,4-dihydropyridines as inhibitors

3.1 MDR1 inhibitors and Current Clinical Trials

There have been a number of compounds that have been identified as MDR1 inhibitors.[1] [2] The majority of these novel compounds that are known to inhibit MDR1 have not yet advanced to being utilized in the clinic due mainly to severe side effects and lack of selectivity. Never-the-less, reversal of multidrug resistance is of widespread clinical interest and multidrug-resistance reversers (MDRR) are currently under intensive investigation. Given MDR1's involvement in MDR it is a viable drug target for the reversal of MDR.

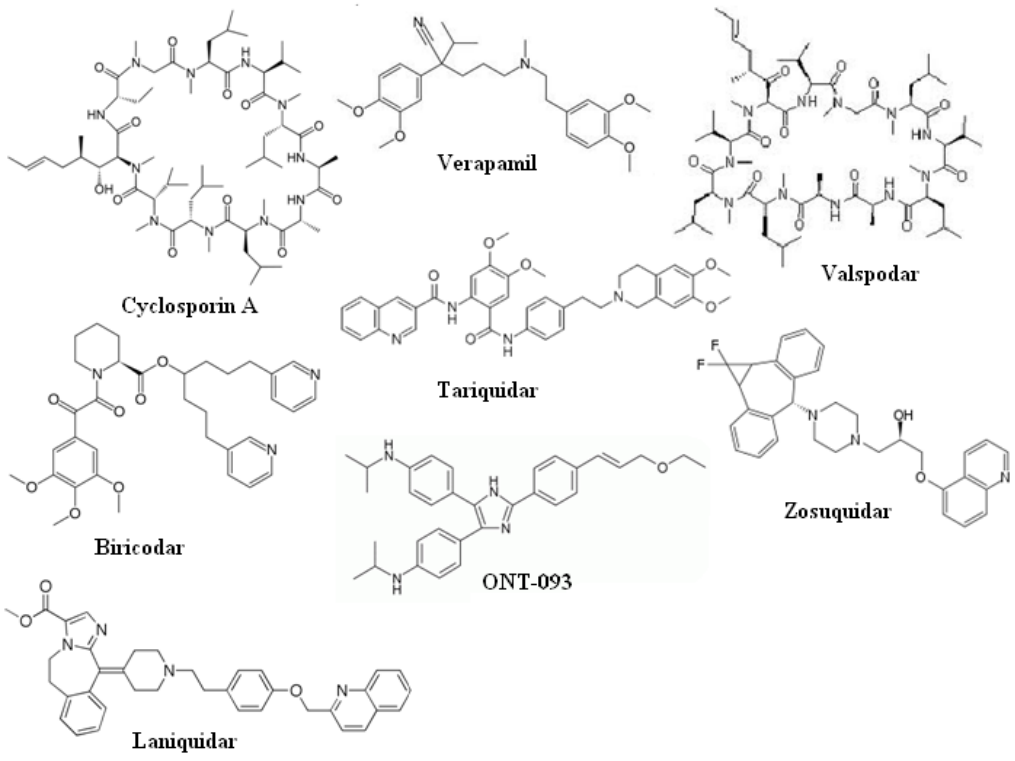

Figure 3-1: Known inhibitors of MDR1

First-generation inhibitors such as cyclosporin A, and verapamil suffered from unacceptable high toxicity, and were dropped as potential inhibitors after phase II clinical trials. [3] The second-generation agents: valspodar, and biricodar have better toxicity

profiles, but showed cross reactivity and unpredictable pharmacokinetic interactions with other transporter proteins [3]. Third-generation inhibitors such as tariquidar (XR9576), zosuquidar (LY335979), laniquidar (R101933), and ONT-093 have high potency and specificity for MDR1. [3] The pharmacokinetic studies to date have shown no significant interactions with CYP450 3A4 drug metabolism and no clinically significant drug interactions with common chemotherapy agents. [3] The prevalence of MDR1 expression in several tissues is proving to be a major issue when considering side effects of potential inhibitors. It is for this reason that most MDR1 inhibitors have had sub-optimal results in clinical trials.

4- Isoxazolyl-1,4-dihydropyridines (IDHP) have been known to bind L-type voltage gated calcium channels, and dihydropyridines (DHPs) have been in general medical practice for use as anti-arrhythmic agents for decades,[4] and as such the DHPs has been recognized as a privileged scaffold found in medical chemistry.[5] More recently however, the DHPs scaffold has been recognized as a substructure to design around to produce MDRR's.[6-11] Given that the clinically used DHP such as nicardipine and niguldipine are known ligands for MDR1. [10-12] We constructed a common pharmacophore model (**Fig. 3-2**) to visualize common structure features to direct synthetic efforts towards more valid analogs. **Figure 3-2** shows clinically used 1,4-dihydropyridine nicardipine (cyan), niguldipine (pink).

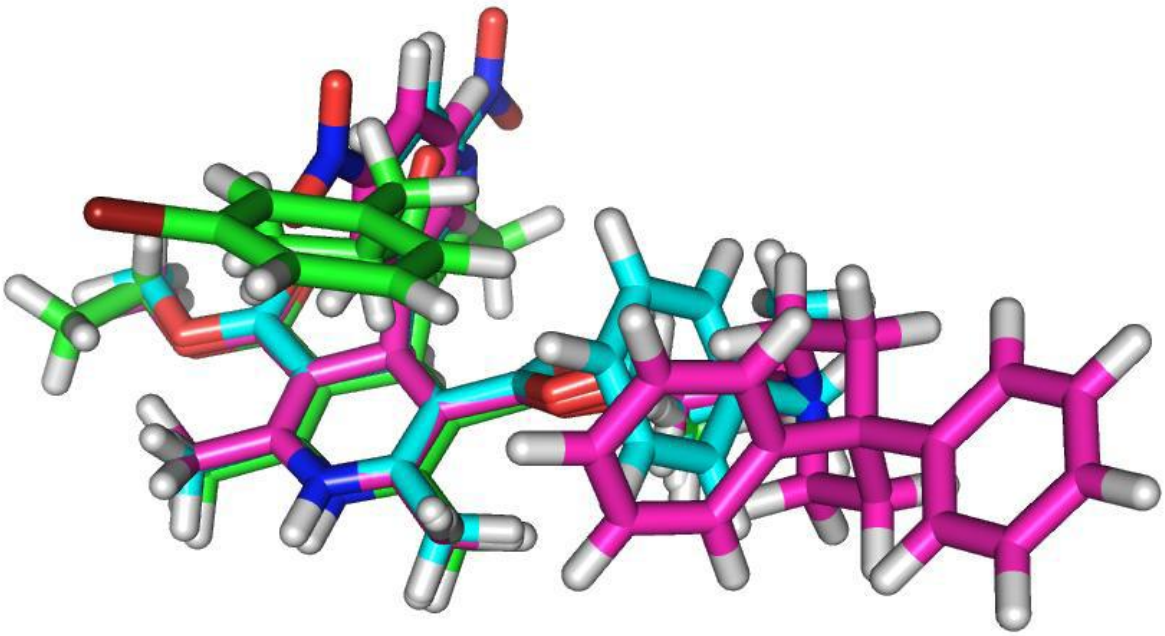

Figure 3-2: The overlapping pharmacophore of nicardipine(cyan), niguldipine(pink) and novel IDHP(green)

The spatial conservation of the function groups is apparent, the most apparent difference being the conformationally unique structure that the isoxazole moiety imparts to the IDHP. The IDHP allows for more novel and structurally divergent compounds to expand the library of compounds which have previously been studied, which in turn could potentially allow for corresponding more divergent and novel pharmacology.

To further study the effect of IDHPs as MDRRs on MDR1 and to aid in further analog development a binding box for IDHP was needed as a working hypothesis. Given that MDR1 has a large number of potential substrates we chose to focus our efforts on the initial characterization of the IDHP binding site. Shown in **Figure 3-3** is a space filling model of MDR-1. The binding sites of interest are known as the DHP binding site resides near the NBD. It's shown below (**Fig. 3-3**) that the yellow spheres represent how ATP interacts with the NDB, this gives the general areas in which the putative IDHP binding site is located.

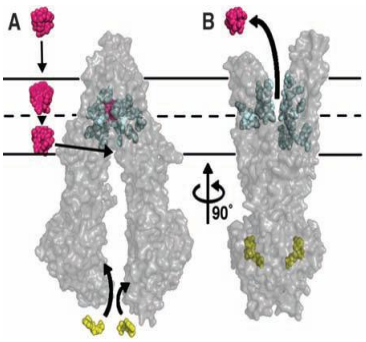

Figure. 3-3: Model of substrate binding sites of MDR-1. The Rhodamine binding site (cyan) and the alternating axels motion of the protein is shown. The horizontal bar represents the approximate position of the lipid bi-layer Reprinted with permission from the publisher.

Shown in **Figure 3-4** is a ribbon diagram of MDR-1, the NBD's are labeled below as NBD1 and NBD2.

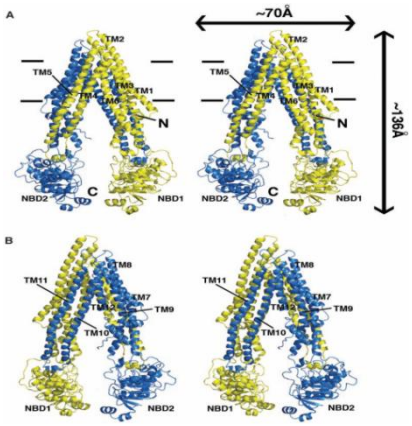

Figure. 3-4: Ribbon Diagram of P-gp. (A) Front and (B) Back stereo view of P-gp. The N- and C-terminal of each half of the molecule is colored yellow and blue respectfully. The horizontal bar represents the approximant position of the lipid bi-layer. Reprinted with permission from the publisher

The characterization of the DHP site was first attempted by using domain mapping experiments. [13] Domain mapping studies in combination with immunoprecipitation were utilized to identify the DHP binding site.[14] Other studies [15] have been performed with photo labeled DHP derivatives, the studies showed binding at two major regions, one in each half of the protein [16]. This suggested that the DHP derivatives bind to both NBD.

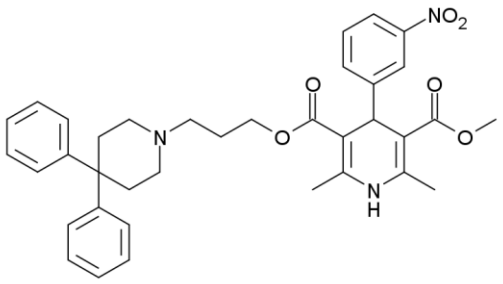

Figure 3-5: Niguldipine

To further refine the DHP binding site a niguldipine (**Fig.3-5**) was chemically modified. The study that we are currently using as a starting point used a photoaffinity label which replacing the nitro group seen in niguldipine (**Fig. 3-5**) with an azido group. Given the previous efforts to identify MDR1 binding sites a mass spectrometric (MS) approach was utilized in this study.

The first step that was taken to identify the binding site of niguldipine, was to photolyze the modified DHP in the presence of MDR1. The protein was then solubilized in detergent. The solubilized protein was then identified via PAGE-SDS gels and purified via lectin affinity chromatography.[13] For the identification of the protein, Western Blot analysis was carried out using monoclonal anti-MDR1 antibody C219. [13] The identified protein was then digested via trypten, but trypten peptides can cleave at 179 possible sites on the remaining protein so further purification was needed. The protein was then further purified via HPLC. Three runs on the HPLC shows peaks corresponding to the niguldipine bound protein, and these fractions where then pooled. The pooled samples were then analyzed via Matrix-assisted laser desorption/ionization (MALDI). [13] The sequence containing the DHP bound protein was isolated followed by Edman degradation and MS analysis to identify the sequence. Edman degradation allowed for the sequential cleavage of the N-terminal amino acids until the bound niguldipine sequence is

encountered. Edman coupling combined with MS can then reveal the maximum number of amino acids that compose the labeled site. The final localization of the niguldipine binding site in MDR1 was found to be correspond to the sequence 468-527,[13] this is in agreement with the previous immunoprecipitation experiments that showed the dexniguldipine binding site in the N-terminal or the cytoplasmic half of MDR1.[17] In other proposed structural model of MDR1 the niguldipine binding site is also in the cytoplasm assigned to the sequence 491-526. [18] The results suggest that this region is near the N-terminal NBD this indicates that the chemo sensitizer binding site and ATP binding domains interacts with each other. It is known that the drug binding site and ATP hydrolysis are coupled. [19] Observing that both sequences are closely related to one another is evidence that this is the correct binding sequence.

Various studies have attempted to locate the drug-binding sites and key residues responsible for the interaction with ligands. Studies of ligand–ligand interactions on MDR1 revealed that some ligands interact with the transporter as single molecules, whereas others interact as pairs.[20]

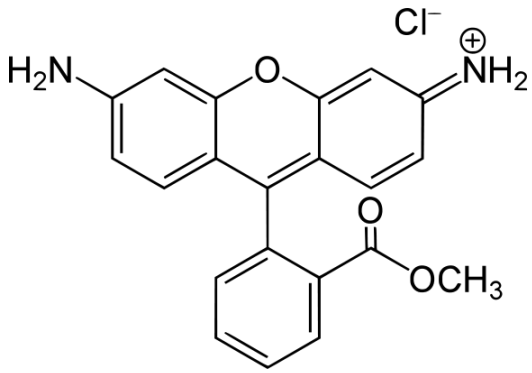

Figure 3-6: Rhodamine 123

As mentioned above MDR1 contains multiple sites, distinct sites for transport of rhodamine 123 (R-site) in addition to a modulatory site for prazosin and

progesterone.[22][30] Binding of ligands at one of these transporters site enhances the interaction at the others. [22] Equilibrium binding studies on MDR1 provided evidence for three sites for transported ligands (vinblastine, pacitaxel, and Rhodamine), which can interact with ligand in the absence of externally added nucleotides, in addition to a modulatory site for niguldipine/GF120918.[24] The observation that there could be similarities in both the R-site and the DHP binding site, which could allow for this cooperation and communication to occur that, could cause the conformational changes and allow MDR1 to function. Given the previous research on the binding of DHP a computer model was used to produce a refined ligand binding box.

3.2 Computer Modeling/Virtual Docking

To provide testable hypotheses for binding at the DHP binding site, computer modeling was employed. A MDR1 human crystallographic structure doesn't currently exist, so a homology model was constructed from the published X-ray crystal structure Mus musculus (pdb accession number: 3G5U) a close homolog of the human ABC transporter MDR1. A sequence homology was then performed between the known human MDR1 sequence and the 3G5U followed by threading of the known human MDR-1 sequence into the published X-ray crystal structure. As explained above the binding site for the DHP has been broadly defined as the resides 468-527.To avoid any bias in further defining the DHP binding site, the MDR1 human homology model was submitted as the entire MDR1 transporter to the Q-site finder online server. [37] This software displayed ten different binding sites on the MDR1 homology model. The program binds hydrophobic probes to the MDR1 homology model and finds clusters of probes with the most favorable binding energy. These clusters are placed in rank order of the likelihood of

being a binding site according to the sum total binding energies for each cluster. The first binding cluster was bound in the rhodamine binding site or R-site, the second and third sites were located inside the NBD. With the characterization of the niguldipine binding site in place from the photoaffinity binding site study, [13] combined with the output of the Q-site finder gave a tentative binding site. The overlap of the two sites is considered a possible binding location for DHP. [39] **Figure 3-7** shows the photoaffinity study in red and the Q-site finder result shown in blue

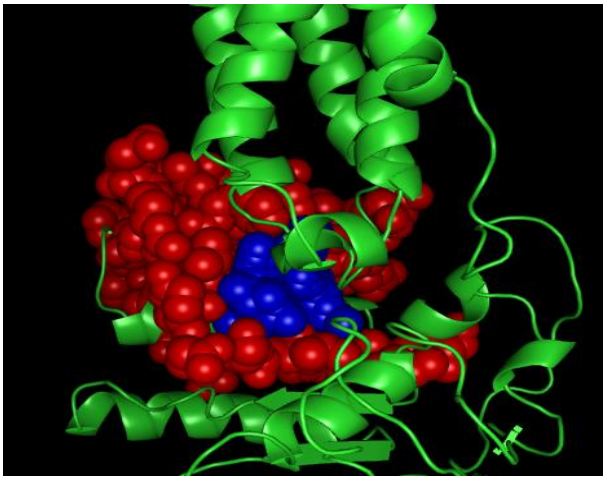

Figure 3-7: Highlight of the both the photoaffinty binding site (red) and the Q-site predicted binding site (Blue)

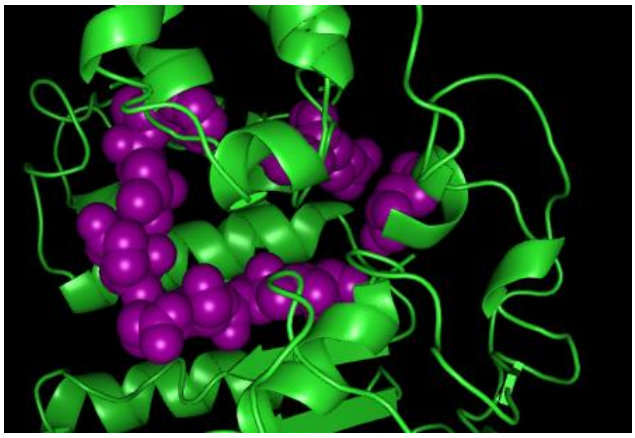

Figure 3-8: The overlapping amino acid sequence (purple) from the photoaffinity binding site and the Q-site binding site

The overlapping amino acids from the photoaffinity and the Q-site finder are shown in purple in **Figure 3-8**. Given the obvious shape and location of overlapping amino acids, this defined site can be thought of as a putative binding site for IDHP. With a binding site in place IDHPs were then used to validate the *in silio* model. A library of IDHPs was then studied to validate the binding site, shown in **Table 3-1**.

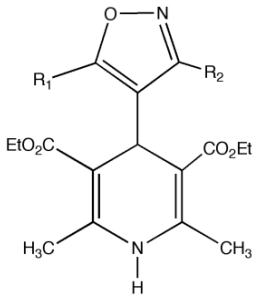

Figure 3-9: General Structure of IDHP used for table 1

IDHP	R_1	R_2	MDR1 (% inh.)
1a	CH_3	C_6H_5	48.9
1b	CH_3	o-MeO-C_6H_4	32.8

1c	CH_3	2-MeO-5-Cl-C_6H_3	15.0
1d	CH_3	o-Cl-C_6H_4	10.9
1e	CH_3	m-Cl-C_6H_4	26.8
1f	CH_3	p-Cl-C_6H_4	11.7
1g	i-Pr	C_6H_5	38.4
1h	$C_6H_5CH_2CH_2$	C_6H_5	27.6
1i	p-Biphenyl-CH_2CH_2	CH_3	18.6
1j	1-naphthyl CH_2CH_2	CH_3	19.0
1k	m-Br-$C_6H_5CH_2$ $(CH_3)CH$	CH_3	61.2
1l	1-naphthyl $CH_2(CH_3)CH$	CH_3	38.3

Table 3-1: IDHP activity at MDR-1

MDR1 screening and the establishment of MDR1 inhibition activity was performed by the Psychoactive Drug Screening Program (PDSP) of the NIMH. The assay was performed using live Caco-2 cells, a cell line derived from human colonic epithelium cell that express MDR-1.[39] The assay uses Calcein-AM which passively diffuses in to the cell, after which it is hydrolyzed turning the compound fluorescent and adding a negative charge thus trapping the compound in the cell. Calcien-AM can be effluxed out of the cell via MDR-1, thus MDR-1 inhibition is a function of the fluorescencet that is observed. Flourescence is measured using a FlexStation II fluorimeter in a 96 well plate after preincubation with the given IDHP (50μM for 30min). After which calcein-AM was then added to a final concentration of 150 μM. Fluorescence was then monitored over 4 minutes, with each assay performed in quadruplicate, with 25 μM cyclosporine used as a

control. The value of untreated control cells where taken as 0% inhibition and the slope of the fluorescene is normalized taking the value of cyclosporine at 100%.[40]

With the binding data in hand ligand structures were drawn and energy minimized (Powell method, 0.01 kcal/mol*A gradient termination, MMFF94s force field, MMFF94 charges, 1000 maximum iterations) using the Sybyl modeling program (Tripos, St. Louis, MO, USA). Structures were virtually docked into an *in silico*-activated virtual dockings of energy minimized ligands to the MDR1 human homology model were performed using the program GOLD (Cambridge Crystallographic Data Center, Cambridge, UK) and scored using GOLD Score with default settings. Ligand-receptor ensemble structures were each obtained by merging the highest ranked docking output ligand orientation structures with the input MDR-1 human homology model structure using the SYBYL software package (Tripos, St. Louis, MO), followed by energy minimization, molecular dynamics, and a final energy minimization simulation. Aggregates for molecular dynamics and minimization simulations were defined as residues more than 6 Å from the ligand. Binding was localized around the SER 475, one of the amino acids at the interface of the overlapping photoaffinty binding and the Q-site finder amino acids (ARG 467,ILE 470, GLY 471, VAL 472, VAL 473, SER 475, GLN 474, GLU 476, PRO 477, VAL 478, LEU 478, PHE 480, TRY 490, GLU 491). From this analysis three binding site cohorts were defined from the library of compounds.

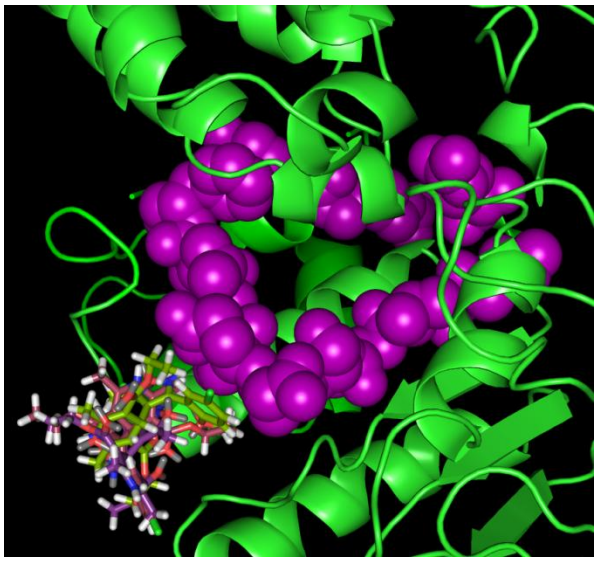

Figure 3-10: The low affinity cohort compounds 1c, 1f, and 1d

Figure 3-10 shows what has been termed the low affinity cohort where compounds 1c, 1f and 1d bound all of the associated compounds that where bound in this location never achieved more than 15% MDR-1 inhibition.

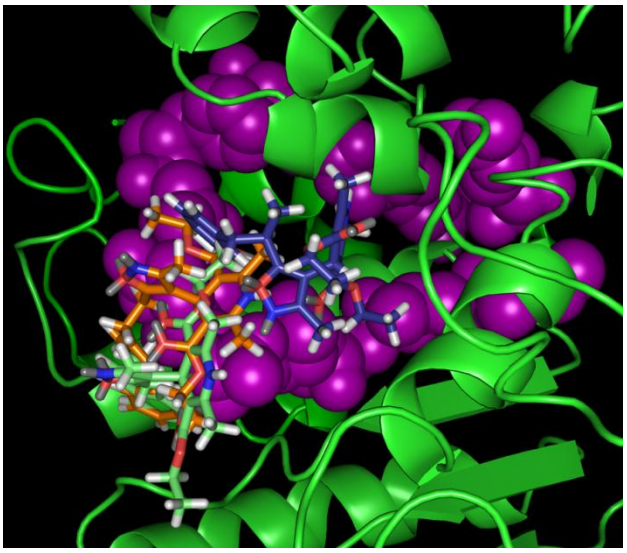

Figure 3-11: The medium affinity cohort compounds 1i, 1j and 1l

Figure 3-11 shows what has been termed the medium affinity cohort where compounds 1i, 1j and 1l bound. Compounds 1i and 1j where bound in the same location

adjacent to the binding box and never achieved more than 19% MDR-1 inhibition. 1l on the other hand, bound with a portion of the structure inside the defined binding box, or the high affinity cohort, this would explain the abnormally high 38% MDR-1 inhibition that was observed for this binding location.

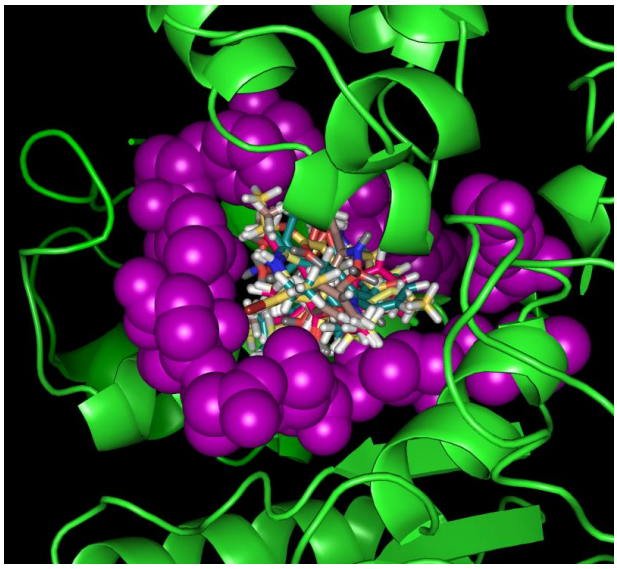

Figure 3-12: The high affinity cohort, also considered the binding site for IDHP

Figure 3-12 shows what have been termed the high affinity cohort where compounds 1a, 1b, 1e, 1k, 1g, 1h bound. Compounds that bound in this location had robust MDR-1 activity with a range varying from 27% to 61% MDR1 inhibition. Most notably, compound 1k the most active compound in this series is bound here validating the computational model as a predictive tool. To aide in further development of second generation MDRRs, 1k was selected and all interactions were examined in a 6 Å radius around the IDHP structure. The binding box interactions were then divided into regions to classify the overall interactions that allow for robust binding, the purple amino acids that are shown represent the overlapping amino acids from the photoaffinity study and Q-site.

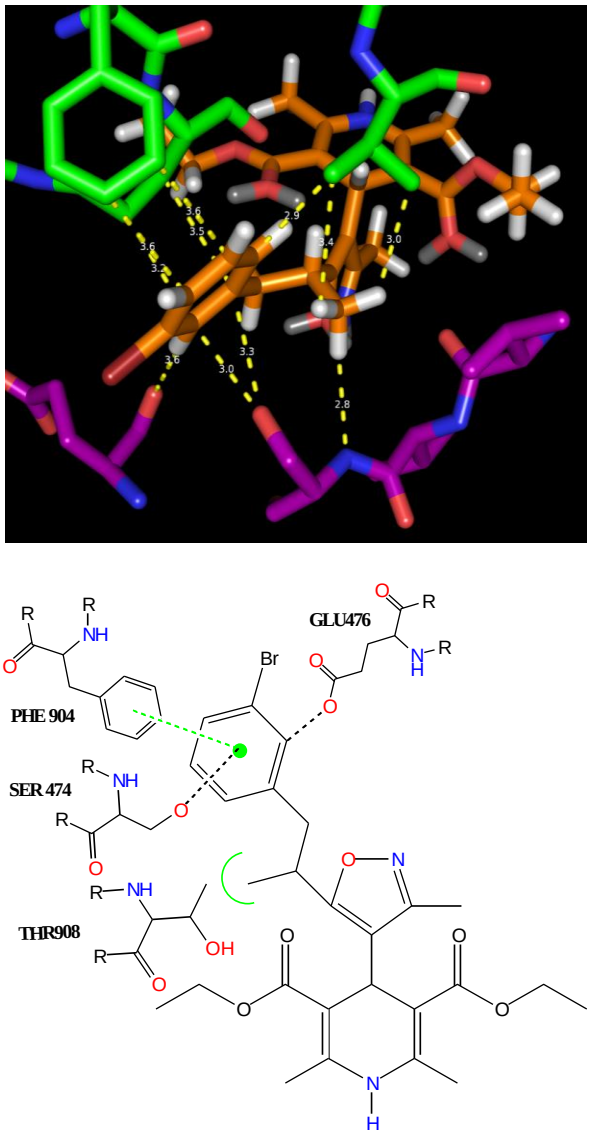

Figure 3-13: Region 1 of compound 1k

Figure 3-13 shows region 1 of compound 1k the majority of the interactions are liphophilic from Phe 904, Arg 905 and Val 908. There is also polar interaction that where are observed with the π cloud of the phenyl ring with residues Glu 476, Ser 474, and Val 472.

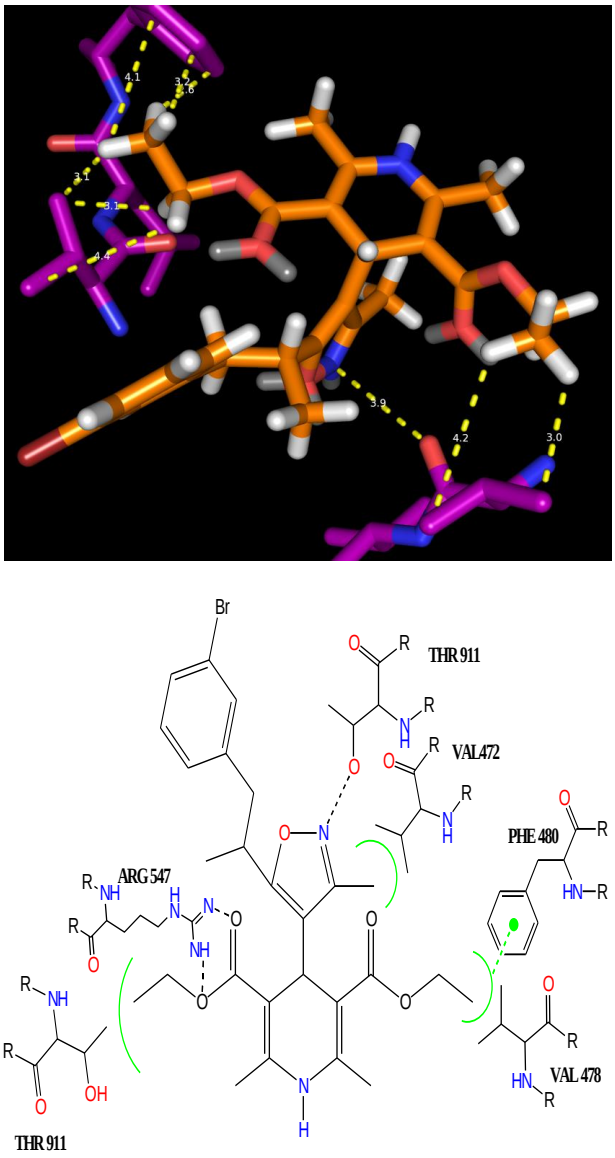

Figure 3-14: Region 2 of Compound 1k

Figure 3-14 show region 2 of compound 1k there are liphophilic interactions on both the methyl groups by residues Phe 480, Val 478 and a polar interaction with Val 472 with the isoxazolyl.

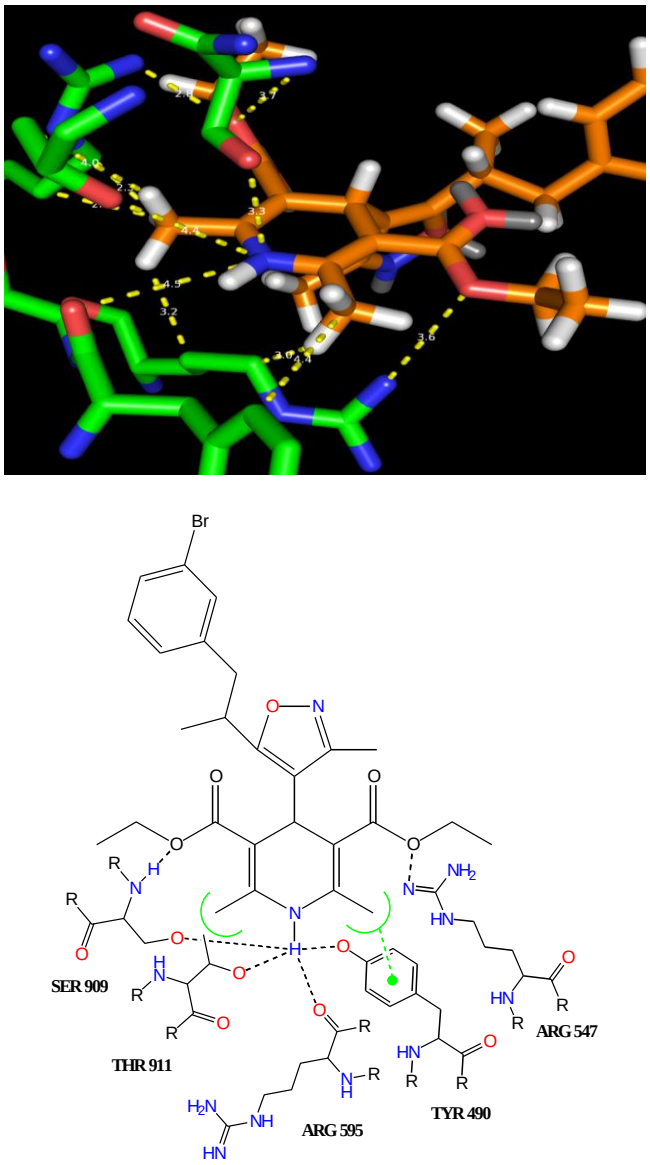

Figure 3-15: Region 3 of Compound 1k

Figure 3-15 shows region 3 of compound 1k there are multiple interactions with the DHP nitrogen with the residues Ser 909, Thr 911 and Tyr 490, with additional polar interactions involving Ser 909, Arg 547 and Arg 543. This region also highlights a key anchoring point for the compound, the 1, 4-dihydropyridine substructure has been shown to be key in binding MDR1. Further analog development will be focused on conserving the 1,4-dihydropyridines substructure while altering other key structural factors in the

series. The isoxazolyl has minor anchoring interactions but the spatial arrangement that is allowed via the inclusion of the isoxazolyl is novel and as such will be conserved in later analog development. The future IDHP analog development will focus on the modification of groups adjacent to the isoxazolyl.

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