

Structural Mapping of Inhibitor Binding Sites on P-glycoprotein: Mechanism of Inhibition of P-glycoprotein by Herbal Isoflavones

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Running title: Binding of isoflavones to P-glycoprotein

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Abbreviations

MDR, multi drug resistance; BCRP, breast cancer resistance protein; Pgp, P-glycoprotein; ABC, ATP binding cassette; TMD, transmembrane domain; NMD, nucleotide binding domain

ABSTRACT

Aims: To find out detailed atomic knowledge of inhibitors binding sites on P-glycoprotein (Pgp) so that information can be utilised for designing of stronger inhibitor molecules with therapeutic interests.

Study Design: Pgp is a transmembrane molecular pump responsible for multi-drug resistance in metastatic tumours, which removes toxins by exporting a variety of structurally unrelated compounds outside the cells. That forms Pgp a promising target for designing anti cancer supplementary therapeutic molecules. Isoflavones are chemical compounds present in soyabean and other herbal extracts. The idea was to characterize the isoflavones binding sites on Pgp to find hotspots and eventually prove useful in designing compounds with higher affinity for Pgp.

Place and duration: School of Biotechnology, Gautam Buddha University, Greater Noida, between February 2012 and December 2012.

Methodology: The biochemical nature of binding of isoflavones to Pgp has been previously reported, but the atomic details of their interactions were not understood so far. Therefore to achieve the objectives we have used *in silico* methods to study binding of two types of isoflavones called aglycones and glycosides to Pgp. The docking studies of isoflavones on Pgp was performed by Grid-Based Ligand Docking with Energetic (GLIDE).

Results: Isoflavones binds at two slightly distinct sites perpendicular to each other, present in the large hydrophobic channel of Pgp. Three isoflavones bind to site 1, whereas eight isoflavones bind to Site 2 by forming van der Waals and H-bonded interactions. Both the binding sites are highly hydrophobic in nature and are mainly

contributed by side chain of non polar residues present on 12 transmembrane α -helices. Site 1 has minimum dimension as small as 7.5Å and maximum as 22Å whereas Site 2 is wider and deeper than Site 1. One wall of the site 2 is formed by polar amino acid residues of the helix H12, which makes several hydrogen bonds with ligands.

Conclusions: Structure analysis revealed that addition of polar group to hydrophobic ligand may enhance its binding affinity for Pgp, which can be used for designing potent ligand to inhibit Pgp to find lead compounds for drug design against Pgp.

Key words

Multidrug resistance, Cancer, Isoflavones, Molecular Docking, Ligand

1. INTRODUCTION

Toxic chemotherapy is used for treatment of cancer. Multi drug resistance (MDR) is the most common phenomena occurring in metastatic cancerous cells. Therefore, MDR has been major concern for cancer biologist since long time. MDR is generally conferred by efflux of drug compounds by transmembrane proteins in an ATP dependent manner [1]. These transporters belong to Breast Cancer Resistance Protein (BCRP) and p-glycoprotein (Pgp) classes. BCRP is an efflux transporter encoded by ATP-binding Cassette (ABC) gene 2 [2]. It is expressed in various tissues of the human body including the apical side of the intestinal lumen, bile canaliculus in liver hepatocytes and capillaries in the blood brain barrier [3]. BCRP and Pgp both share similar functions and have remarkable structural fold similarity. These proteins belong to ABC transporter family and involved in ATP dependent active transportation of drugs and xenobiotic compounds [4]. Pgp is mostly studied among these MDR proteins. It has a very broad

specificity to compounds ranging from 330Da to 4000Da molecular weights [5, 6]. It binds mainly to hydrophobic compounds and expels them outside hence also termed as “hydrophobic vacuum cleaner” of the cell [7].

Several structures of bacterial ABC transporters have been determined [8-13] although only low resolution structure of mouse Pgp is available [14-16]. The structure of mouse Pgp has been solved at 3.8Å resolution. Mouse Pgp serves as a good model as it has 87% sequence similarity with human form. Pgp is a 170 kDa transmembrane protein, expressed in various resistant tumour cell membranes. Because it is involved in MDR of tumour cells, designing of inhibitors to Pgp may have great therapeutic value.

Isoflavones and flavonoids are groups of polyphenolic compounds derived from plant extracts. These are derived from flowers, fruits, vegetables, nuts and stems and form an abundant component of human food [17-20]. Isoflavones are the largest group of natural isoflavonoids, present in mostly leguminous plants. The two major categories of isoflavones are aglycones and glycosides depending on presence of a glucose substitution. These compounds have a wide biochemical and pharmacological effect on human health such as anti carcinogenic, antiviral and anti-inflammatory. These active plant compounds have also been found inhibiting Pgp proteins and contributing to decrease MDR in tumour cells [21-24]. Several successful efforts have been made to understand the interactions of flavonoids with Pgp and BCRP [21, 22], whereas the details of atomic interactions of isoflavones with these targets are not understood. Such knowledge can provide further scope of designing potent inhibitors of Pgp and BCRP proteins, which can have a good therapeutic potential. As the crystal structure of BCRP is not yet available to best of our knowledge in the databank, therefore we decided to

study interactions of isoflavones with available Pgp structure. Isoflavones binding studies may play a vital role in finding hotspots in Pgp and eventually may be proved useful in designing compounds with higher affinity to the protein, which can be used along with anti cancer chemotherapeutic drugs. This manuscript provides details of interactions between 11 isoflavones varying in their chemical nature with Pgp proteins, which can help in understanding the precise sites and groups involved in recognition of inhibitor by Pgp protein.

2. METHODS

2.1 Evidence of interactions between isoflavones and Pgp

BCRP and Pgp have similar structure and involved in same function of transporting xenobiotic molecules outside the cells and responsible for MDR in cancerous cells [25]. Earlier in vivo and in vitro studies have indicated that flavones and isoflavones inhibit BCRP and Pgp mediated exotransport. The BCRP and Pgp were over expressed and effects of flavones and isoflavones were investigated by monitoring exotransport of various known substrates and compounds [23, 26-30]. The binding pattern of isoflavones to BCRP and Pgp was unknown so far. Since the three dimensional crystal structures are available only for mouse Pgp, we performed *in silico* studies to identify interactions between isoflavones and Pgp. The sequence alignment of mouse Pgp with human Pgp revealed 87% overall sequence identity and almost 100% identity with the ligand binding site.

2.2 Docking isoflavones on Pgp

The atomic coordinates of 11 isoflavones (Diadzein, Daidzin, Formononetin, Ononin, Genistein, genistin, Biochanin A, Sissotrin, Coumestrol, Coumestrin, Glycitein) were generated using structures described earlier [30]. 3-D atomic coordinates for isoflavone structures were generated using LIGPREP with the correct chiralities of atoms. The resulting geometries were optimized by molecular mechanics using IMPACT in a dynamic environment using standard TIP4P water model. Energy minimization was done using Polak-Ribier conjugate gradient algorithm using Optimized Potentials for Liquid Simulations 2005 (OPLS 2005) force field. RMS gradient of 0.01 was used as the convergence threshold. Conformational models of all the ligands were generated. The ligands were prepared using LIGPREP [31]. The structures with correct bond length, bond angles, and conformation, stereochemistry and ionization states were selected for docking studies.. The selected isoflavones belong to two categories one containing glucose substitution and another without glucose, called glycosides and aglycones respectively. All the isoflavones except coumestrol and coumestrin have identical basic structure, which have variations in groups substitute at C7, C8, C9 and C14 positions on the nucleus structure (Table 1).

To understand interactions between isoflavones and Pgp, docking studies were performed using GLIDE [32]. Atomic coordinates of the native receptor were extracted from protein data bank (PDB: 3G5U). The submitted coordinates were corresponding to a homo dimer of PgP. Monomer (Chain A) of the Pgp was used for study. Each monomer contained 1271 amino acid residues and 18521 non hydrogen atoms. A structure of Pgp complex with a cyclic peptide was also available as PDB code 3G60 in

protein databank. Pre-processing of the native receptor structure was done by assigning correct bond order, addition of hydrogen atoms, capping uncapped termini, adjusting bonds and formal charges for groups and correcting mislabelled elements. All docking studies were carried out using extra precision (XP) method of GLIDE (Grid-Based Ligand Docking with Energetic) which examines the complementarities of ligand-receptor interactions using a grid-based method based on the empirical ChemScore function for flexible ligand docking. The centroid of selected active site residues (using PDB: 3G60) were used for receptor grid generation for docking as well as blind docking. 1000 docked poses per compound were generated for the different conformations of the compounds. The affinity grid maps centred on the peptide binding site had dimensions of $80 \times 80 \times 80 \text{ \AA}$ with $0.375 \text{ \AA}$ spacing between grid points. The binding free energy of the receptor-ligand binding (ΔG_{bind}) is calculated by taking the difference between the free energies of the receptor-ligand complex ($\Delta G_{\text{complex}}$) and the unbound receptor ($\Delta G_{\text{receptor}}$) and ligand (ΔG_{ligand}).

3. RESULTS AND DISCUSSIONS

3.1 Structure of the Pgp model

Pgp is a transmembrane pump which channelizes drugs and other xenobiotic compounds outside the cell and is responsible for MDR. Low resolution crystal structure is only reported for mouse Pgp [16]. The PDB coordinates were available for native Pgp (PDB: 3G5U) and complex with a cyclic hexapeptide inhibitor (PDB: 3G61) in protein data bank. The native structure of Pgp was determined at $3.8 \text{ \AA}$ resolution. The structure shows a dimer having 1284 amino acid in each monomeric unit. Each

half contains six membrane spanning domains in form of transmembrane domain (TMD), followed by a nucleotide binding domain (NMD). Pgp has a helical bundle rich fold with 56% α -helical and 7% β -strand secondary structure content. The TMD is formed by 12 long α -helices.

3.2 The ligand binding channel

The structure of Pgp is a dimer, comprising of two identical monomeric units, containing 1271 amino acids in each. The structure is V shape, having ATP binding domain on both ends. The membrane spanning region is made up of 12 long alpha helices (H1: 44-87, H2: 93-158, H3: 166-205, H4:210-249, H5: 266-317, H6: 327-365, H7: 708-736, H8: 743-794, H9: 807-847, H10: 852-904, H11:909-957 and H12: 968-1009). Each consecutive α -helices are connected through flexible loop regions which are capable of making the helices move apart while performing its functions. The wall of the ligand binding site is formed by the contribution of residues on these 12 α -helices. Furthermore, hydrophobic stacking of these α -helices makes them more amenable to opening and closing of the interstitial space. The ligand binding site is present in form of a wide and long hydrophobic channel. The channel is formed by mainly hydrophobic side chains. Phe amino acid is most abundant in the channel. The major hydrophobic amino acids involved in the formation of ligand binding hydrophobic channel are Leu 64, Met 68, Phe 71, Tyr 110, Tyr 111, Tyr 113, Ile 117, Val 121, Phe 190, Phe 299, Leu 300, Tyr 303, Tyr 306, Phe 310, Trp 311, Leu 328, Phe 331, Phe 332, Ile 336, Phe 339, Phe 724, Phe 728, Val 731, Val 732, Leu 757, Leu 758, Phe 766, Phe 833, Phe 938, Met 945, Tyr 949, Phe 953, Val 970, Leu 971, Phe 974, Val 978, Phe 990 (Fig. 1). The

remaining part of sequence forms two ATP binding domains which are α - β bundle. ATP binding domains are located from 378-626 and 1018-1271 amino acid residues. The cavity formed is very big in size and has a total volume of approximately 6000Å³ [16].

3.3 Binding of Isoflavones

The hydrophobic channel of Pgp is very wide and is responsible for binding of a wide variety of hydrophobic ligands. H3 and H9 helices are present on the outer face of the structure and seems to be playing no direct role in ligand binding, whereas remaining nine helices have protruding-in side chains of amino acids, responsible for formation of hydrophobic cavity. In present study, isoflavones occupy two close sites in the hydrophobic cavity, which are formed by six helices, H4, H5, H6, H7, H8 and H12. Due to the presence of structural and chemical variations, isoflavones bind Pgp at two slightly distinct places in the hydrophobic channel, called Site 1 and Site 2 for ease of description in the manuscript. Sissotrin, coumestrin and formononetin bind to Site 1 whereas biochanin A, genistein, genistin, diadzein, diadzin, glycitein, coumestrol and ononin bind to Site 2 in the wide hydrophobic channel. The isoflavones binds Pgp and BCRP in similar pattern, glycosides isoflavones have higher binding affinity to both Pgp and BCRP than the aglycones ones as indicated by their docking scores and low IC50 values, which can be attributed to their polar nature by presence of glucose moiety (Table 1). This implies that presence of polar groups in a hydrophobic ligand may contribute to its higher binding affinity to Pgp or BCRP.

Site 1: Binding of sissotrin, coumestrin and formononetin: These three compounds bind to Pgp with almost identical manner at a common site. This site is formed by mainly

hydrophobic side chains of residues Leu 300, Tyr 303, Phe 332, Leu 335, Phe 339, Phe 724, Phe 766, Phe 974, Leu 978 and Phe 990. Sissotrin, coumestrin and formononetin are stacked in this hydrophobic cavity through van der Waals interactions. Along with these hydrophobic forces, sissotrin and coumestrin shared common hydrogen bonds with the amide side group of Asn 717 and hydroxyl side group of Ser 989 whereas, formononetin made a hydrogen bond with the side chain of Tyr 303. In addition coumestrin also makes one hydrogen bond with the side amide group of Asn 721 (Fig. 2a).

Site 2: binding of biochanin A, genistein, daidzein, daidzin, genistin, glycitein, coumestrol and ononin. Site 2 is located slightly overlapping and almost perpendicular to the site 1. Site2 binds the above mentioned seven isoflavones in the hydrophobic cavity. This site is formed by residues Phe 299, Tyr 303, Tyr 306, Phe 310, Leu 335, Ala 338, Phe 339, Phe 766. Phe 299 and Phe 339 provide stacking interactions from two sides of the aromatic rings in ligands. One of the side of the site 2 is made up of polar face of the helix H12 consisting of exposed side chain of Gln 721, carbonyl groups of Met 982 and Gly 985, hydroxyl side chain of Ser 989 and amide side group of Gln 986. OH groups of ononin, daidzein and genistin interacted with the polar surface of the site 2 through hydrogen bonding interactions. Daidzein and genistin interacted with the side chain of Gln 986 through H-bonding whereas ononin makes hydrogen bonds with hydroxyl group of Ser 989 and carbonyl oxygen of Gly 985 residues (Fig. 2b).

3.4 Comparison with the binding of cyclic peptide inhibitor

The inner faces of hydrophobic channel in Pgp have a gradient of polarity and hydrophobicity. The upper side (towards ATP binding domains and cytosolic face) has charge and polar residues. The polarity decreases and hydrophobicity increases as ligands go deeper in the channel (Fig. 3). Isoflavones which occupy identical spaces belongs to two categories, category 1 which includes sissotrin, coumestrin and formononetin bind to Site 1 whereas biochanin A, genistein, daidzein, daidzin, genistin glycitein, coumestrol and ononin belong to category 2 which bind to Site 2 on Pgp structure. Pgp complex with a cyclic peptide has also been determined and deposited to the database with accession code PDB: 3G60 [16]. When compared it was observed that cyclic hexa peptide (AV)₃ binds at the site 2 to the Pgp and therefore share the binding space with biochanin A, genistein, daidzein, daidzin, genistin glycitein, coumestrol and ononin isoflavones. The peptide has bound slightly deeper in the site 2 of the hydrophobic cavity which may be attributed to its highly hydrophobic nature. As the cavity have more hydrophobic residues in the bottom to prefer similar compounds (Fig. 3).

4. CONCLUSIONS

Pgp contains a wide cavity for binding to relatively hydrophobic and aromatic ligand molecules. The hydrophobic cavity is formed by contribution of side chains of mainly Phe, Leu and Ile residues from six α -helices in the Pgp structure. Isoflavones recognizes two slightly distinct sites on Pgp called site 1 and site 2. Sissotrin, coumestrin and formononetin recognize interacting groups in site 1 whereas biochanin

A, genistein, daidzein, daidzin, genistin glycitein, coumestrol and ononin bind to site 2 in the wide hydrophobic channel present in Pgp. Site 1 and site 2 are located differently in the hydrophobic channel and also contain a common space as well (Fig. 4). Both the sites have hydrophobic amino acids to form van der Waals interactions with the ligands. In addition to hydrophobic residues, site 2 also contains one polar face from helix H2, which is responsible for forming several hydrogen bonded interactions with the ligands. A polar gradient is present in the hydrophobic channel of Pgp. The upper region of the channel is very polar and charged whereas the hydrophobic environment starts increasing towards bottom of the channel (Fig. 4). Site 1 has minimum dimension as small as 7.5Å and maximum as 22Å whereas site 2 is wider and deeper than Site 1. Therefore, Pgp is capable of binding a variety of substrates, although hydrophobic compounds bind deeper in the channel whereas relatively polar compounds bind towards top of the channel. Hence, the cyclic (AV)₃ peptide being highly hydrophobic binds in the deeper region of site 2 in comparison to the isoflavones. These features can be utilised to construct compounds which have hydrophobic and polar groups and can occupy both the sites form stronger attractive interactions. As the movement of helices are responsible for transporting drugs outside the cell, bigger compounds which can occupy most of the area of the channel including site 1 and 2 may form an effective inhibitor to Pgp. In addition, presence of polar groups in the ligand may enhance its binding affinity for Pgp.

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372 **Table 1: Structure and binding of isoflavones**

Name	Substitution position				Docking score	H-bonded residues	Residues involved in van der Waals interactions	Buried surface area (Å ²)	pIC50 for BCRP [30]
	7	8	9	14					
Genistein	OH	H	OH	OH	-7.3	Gly 222 (NH)	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	239.2	6.6
Genistin	OH	H	Glc	OH	-7.5	Ser 989 (OH) Gly 985 (O) Gln 986 (NH2)	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	337.1	5.4
Biochanin A	OH	H	OH	OCH ₃	-7.2	Gly 222 (NH)	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	252.5	6.3
Sissotrin	OH	H	Glc	OCH ₃	-7.6	Gln 721 NH2 Met 982 (O) Ser 989 (OH) Asn 717 (CO, NH2)	Leu 300, Tyr 303, Phe 724, Phe 332, Phe 766, Phe 833, Phe 974, Val 978, Phe 990	373.1	5.6
Glycitein	H	OCH ₃	OH	OH	-7.4	-	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	267.1	6.0
Daidzein	H	H	OH	OH	-7.3	-	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	237.4	5.6
Daidzin	H	H	Glc	OH	-7.9	Met 982 (O), Gln 986 (NH2) Ile 302 (O)	Ala 225, Phe 299, Leu 300, Tyr 303, Leu 335, Ala 338, Phe 339,	331.3	4.5
Formononetin	H	H	OH	OCH ₃	-4.9	Tyr 303 (OH)	Leu 300, Tyr 303, Phe 724, Phe 766, Phe 833, Phe 990	270.1	5.7
Ononin	H	H	Glc	OCH ₃	-7.7	Gly 222 (NH) Ser 989 (OG) Ser 989 (OG), Gly 985 (O)	Leu 221, Phe 299, Ile 302, Phe 303, Ala 338, Phe 339	342.2	4.9
Coumestrol	-	-	OH	OH	-5.8	Ser 989 (OH) Asn 717 (NH2)	Leu 300, Tyr 303, Leu 758, Phe 766, Phe 833,	214.6	7.2
Coumestrin	-	-	Glc	OH	-7.7	Ser 989 (OH), Gly 985 (O) Gln 717(NH2)	Leu 300, Tyr 303, Phe 724, Phe 332, Phe 766, Phe 833, Phe 974, Val 978, Phe 990	371.8	5.4

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Legends to figures

Figure 1: interior of the hydrophobic channel of Pgp. Hydrophobic residues forming channel are shown in ball and stick model. The figure was drawn using PyMol [33].

Figure 2: Ball and stick representation of ligand interacting residues of hydrophobic channel. Sissoterin (blue), coumestrin (green) and formentin (magenta) are also drawn in site 1 (a) and biochanin A (magenta), genistein (green), daidezein (pink), daidezin (grey), genistin (dark yellow), glycitein (blue), coumestrol and ononine (pink) are drawn in site 2 (b).

Figure 3: Top view of the hydrophobic channel showing electrostatic potential of the inner side of the cavity. Isoflavones binding at site 1 (magenta) and site 2 (cyan) are also shown. Cyclic peptide inhibitor [16] is also drawn in yellow colour ball and stick model.

Figure 4: Site 1 and site 2 are shown. Key residues interacting with ligands at respective sites are also drawn in ball and stick model.

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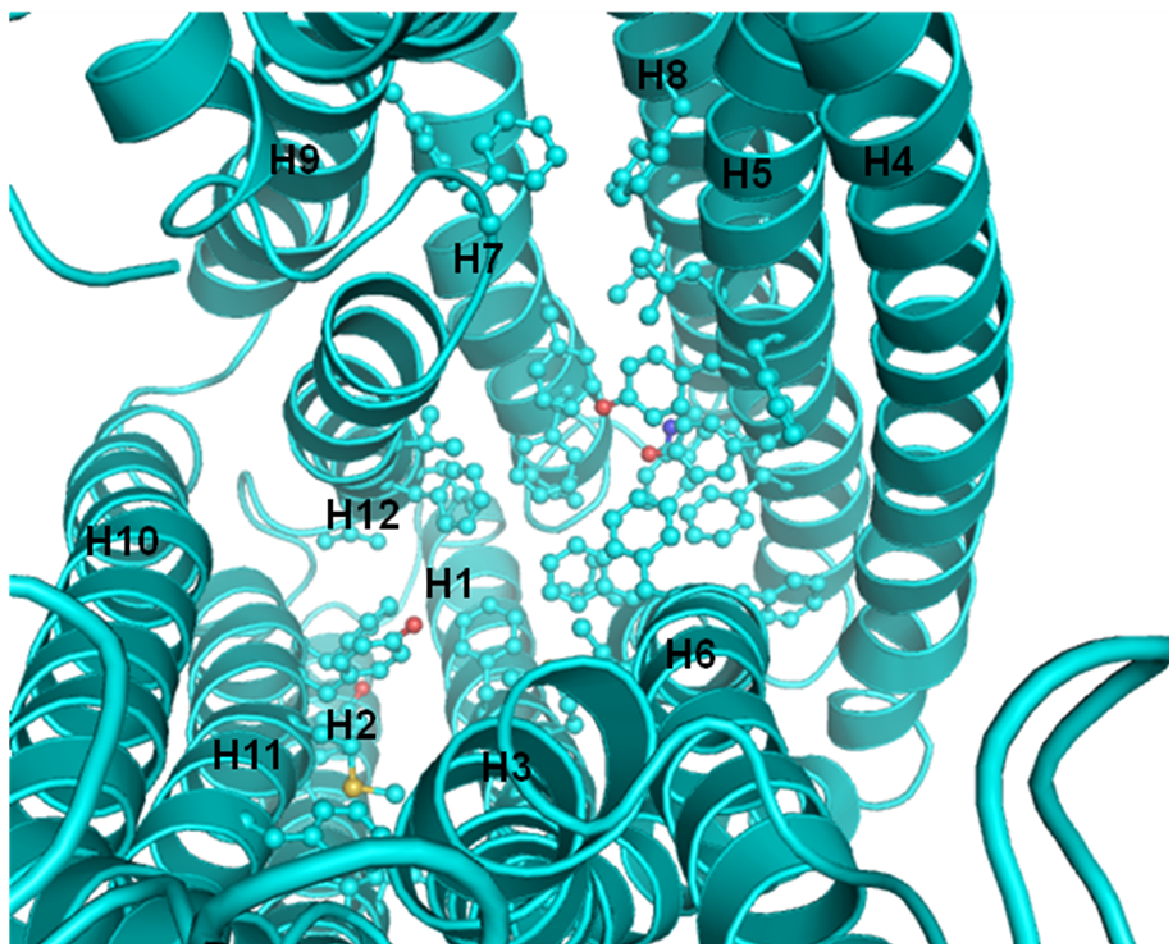


Figure 1

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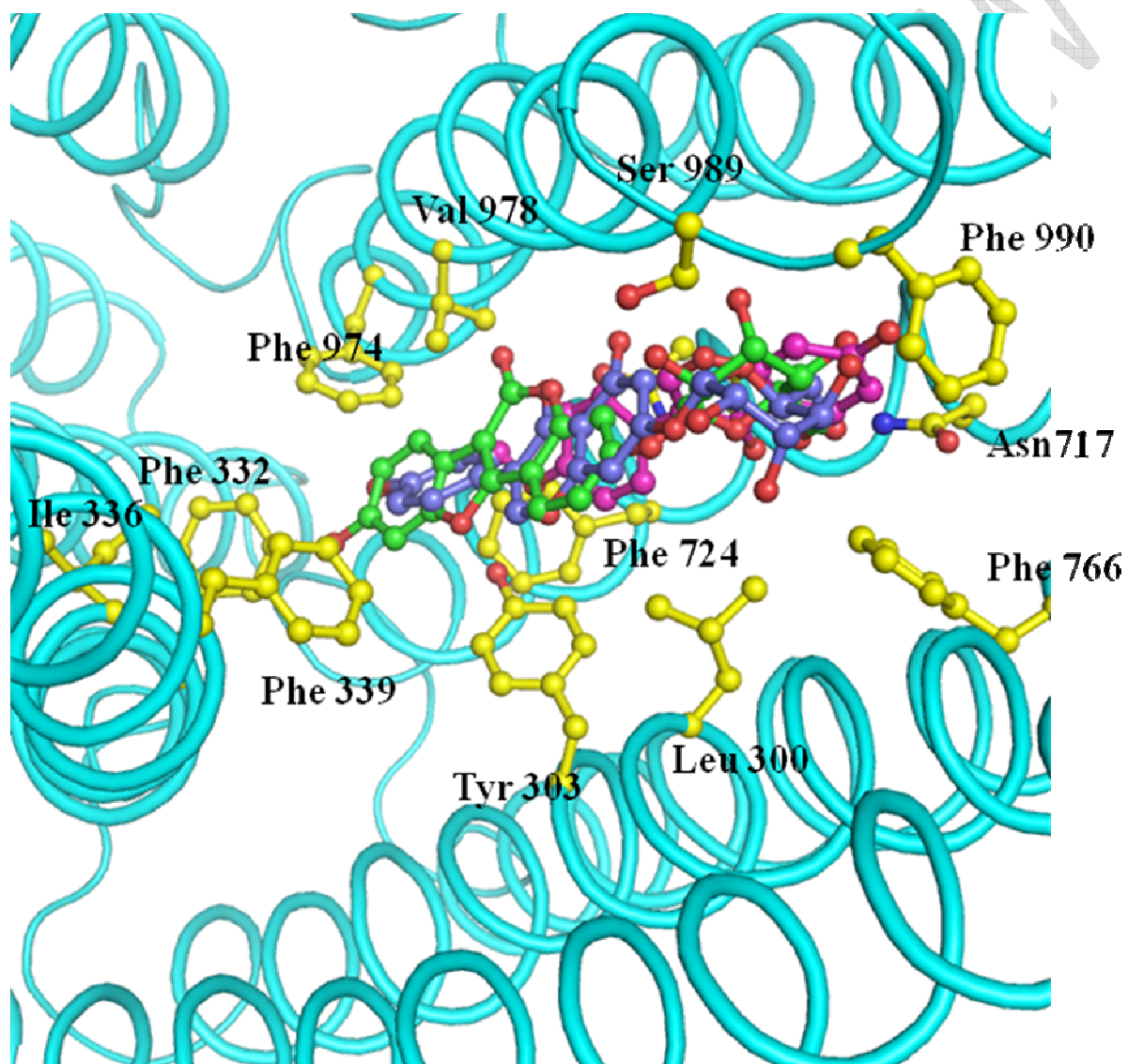


Figure 2a

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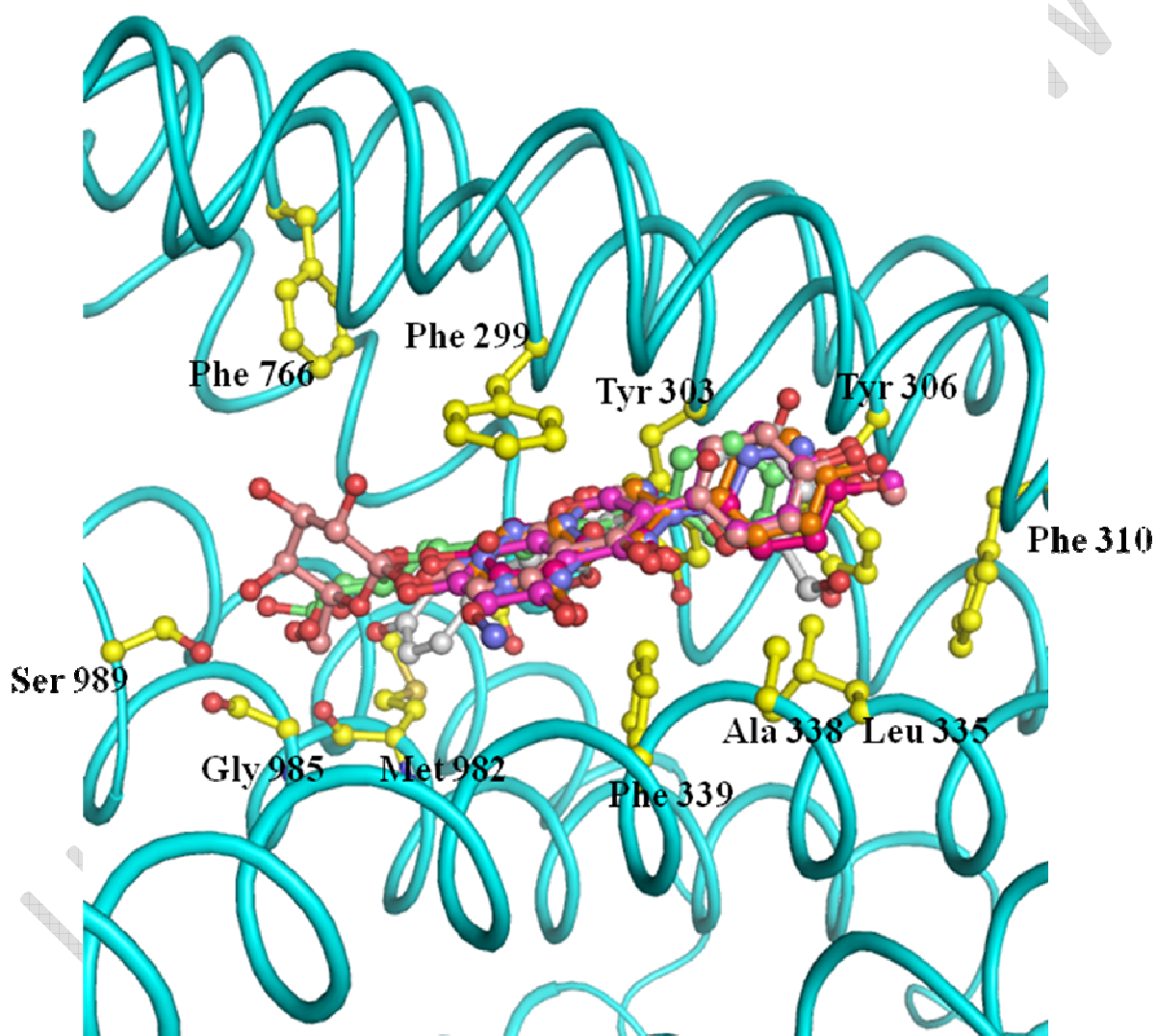


Figure 2b

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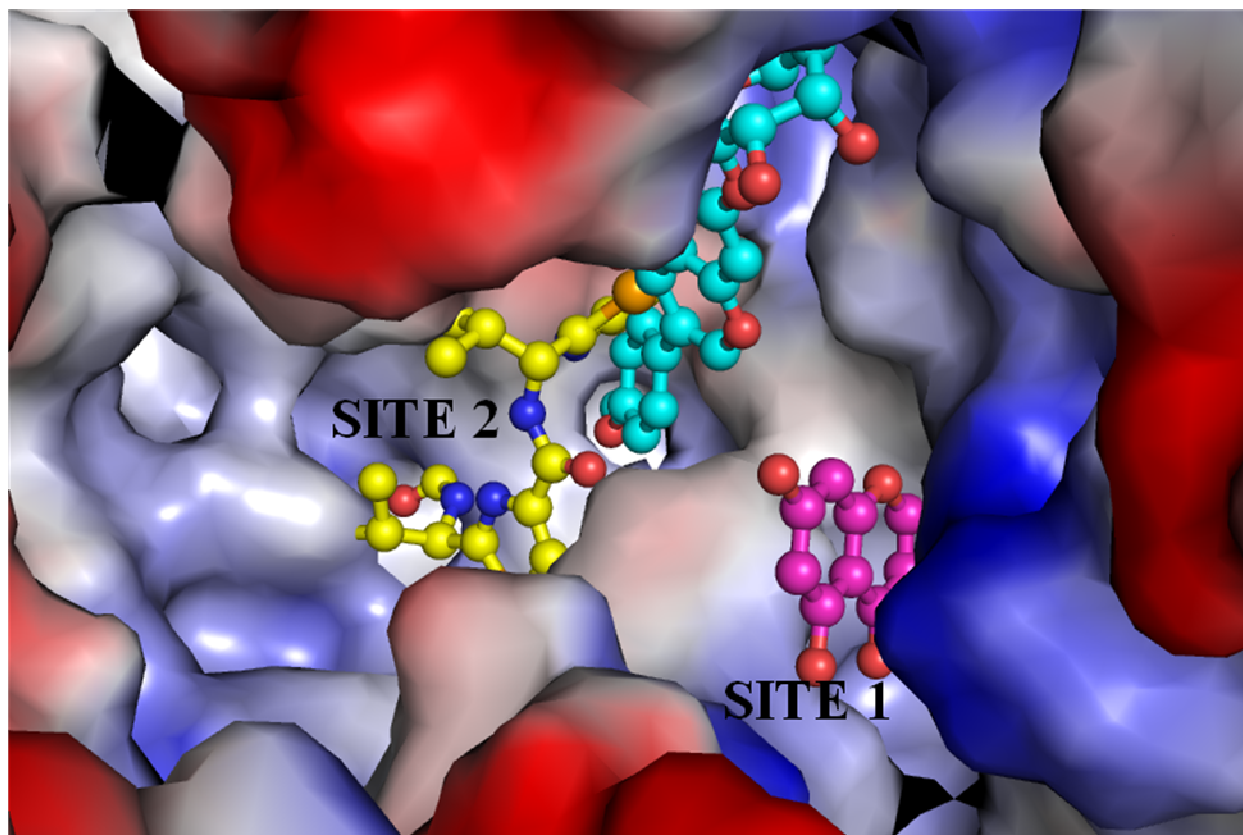


Figure 3

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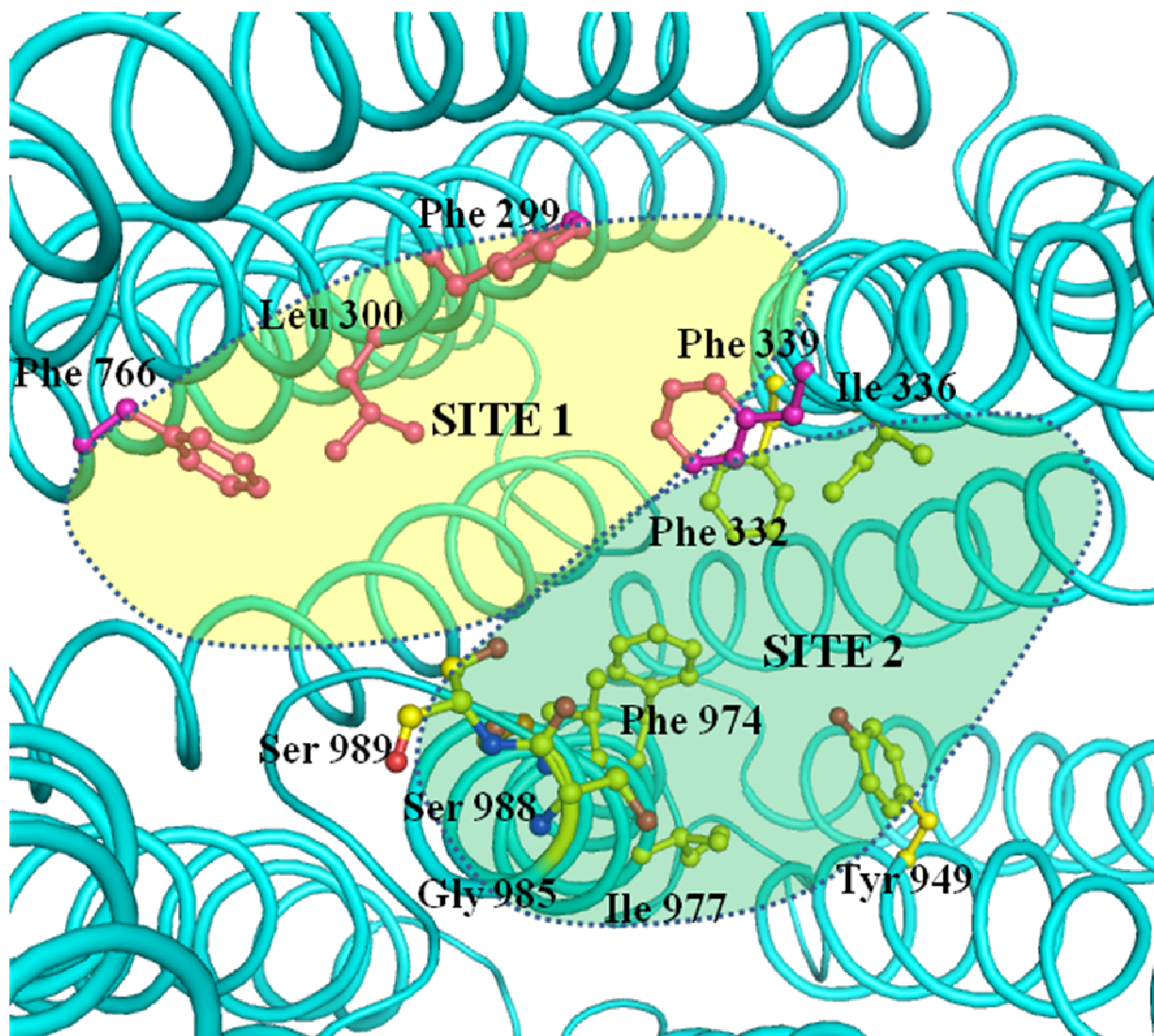


Figure 4

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