In silico exploration of phenytoin binding site in two catalytic states of human P-glycoprotein models

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P-glycoprotein (P-gp), an ATP-dependant efflux pump transports a wide range of substrates across cellular membranes. Earlier studies have identified drug efflux due to the over-expression of P-gp as one of the causes for the resistance of phenytoin, an anti-epileptic drug (AED). While no clear evidence exists on the specific characteristics of phenytoin association with the human P-gp, this study employed structure-based computational approaches to identify its binding site and the underlying interactions. The identified site was validated with that of rhodamine, a widely accepted reference and an experimental probe. Further, an in silico proof-of-concept for phenytoin interactions and its decreased binding affinity with the closed-state of human P-gp model was provided in comparison with other AEDs. This is the first report to provide insights into the phenytoin binding site and possibly better explain its efflux by P-gp.

Keywords: P-Glycoprotein, Multidrug resistance, Phenytoin, Efflux, Drug binding site, In silico.
considering the ionized states of the drug, (ii) compare the nature of interactions of phenytoin with other AEDs, and (iii) analyze their binding with the homology models of the two predominant catalytic states of human P-gp. Thus, we aim to provide an in silico proof-of-concept for the binding of phenytoin to P-gp, which can possibly validate the resistance of phenytoin according to the transporter hypothesis.

Materials and Methods

Homology modeling

The protein sequence (FASTA) and its annotation of human P-gp were retrieved from the SwissProt database (ID: P08183). The PDB structures 3G5U and 2HYD were chosen as templates for the inward-facing and outward-facing conformation models of human P-gp, respectively and submitted to the Itasser online server for modeling. A Sav1866-human P-gp pair-wise alignment based on Stockner et al was generated by the EMBOSS-Needle program for modeling the outward-facing conformation. The generated models were assessed by the scores provided by the Itasser server itself. After a series of structural refinements by the ModRefiner server, the models were structurally validated by the PROCHECK, ProSA and Qmean6 scores. The MolProbity server checked the correct assignment of Asn, Gln and His flips in the models.

Docking

The SMILES notation from the PubChem database for rhodamine123 (CID: 65218), carbamazepine (CID: 2554) and levetiracetam (CID: 5284583) was used to generate their PDB files using the Open-Babel software. The 3D structure of phenytoin in PDB format was downloaded from the DrugBank (ID: DB00252). The ionization state of phenytoin at physiological pH was calculated using the Henderson-Hasselbach equation. Accordingly, the molecule was ionized by addition of charges to the amide nitrogen in all possible combinations and converted to pdb format (Fig. 1).

Autodock Vina was used for the docking. All PDB files of the modeled protein structures and the ligands were converted into PDBQT files using the AutoDockTools package after addition of necessary charges and hydrogens atoms. Based on the default optimization parameters, a uniform grid box of 28 Å × 28 Å × 28 Å with a spacing of 1 Å was positioned at the hypothesized common binding region. Pymol v.1.3 was used for visualization. The best conformer was identified and analyzed for hydrogen bonds and hydrophobic interactions with the models by the LigPlus software.

Results and Discussion

Homology modeling

The transporter hypothesis has been extensively studied and known to be one of the mechanisms responsible for the resistance of phenytoin. This prompted us to study interactions of phenytoin with the two predominant catalytic states of P-gp to understand its efflux mechanism better. The 3D structure, 3G5U represents the inward-facing, closed conformation of P-gp. Due to the good sequence identity of approximately 87% of murine P-gp with human P-gp, the 3G5U was chosen as template for the above-mentioned conformation. Itasser generated five models for this conformation and the model with the highest C-score was chosen for this study (Fig. 2, panel A). The latter will be referred as ‘3G5U model’ in the rest of the manuscript.

The sequence alignment of Sav1866 and human P-gp showed that 29.9% of the regions were identical and 48.1% similar. Since the resolution of 2HYD is the highest among all experimentally determined 3D structures of the ABC transporter family, it can be considered as the most appropriate template to model the outward-facing, open conformation of human P-gp. Itasser generated three models based on this alignment, of which the model having the required conformation and a good C-score (Fig. 2, panel B) was chosen for this study (henceforth referred as ‘2HYD model’).
Despite low resolution of one of the template structures and low sequence identity of other, the promising results in the structural validation supported the good quality of both models in our study. After energy minimization, both models were validated structurally and the scores obtained are listed in Table 1 with their detailed interpretations. These scores implied that the chosen models had good stereochemistry. The residues among disallowed regions of the Ramachandran plot in both models were neglected, since they were found not to be part of the hypothesized common drug-binding pocket. Moreover, these residues were found in the loop regions of the models and not among those representing the helix or sheet structures. The models also gave acceptable ProSA Z-scores and good energy profiles with negligible erroneous regions. The Qmean6 scores implied that the models had a good degree of nativeness with reference to the existing structures. Further, MolProbity Ramachandran analysis showed that outliers present in the models were negligible. The Asn, Gln and His flips were identified and corrected in both models by MolProbity. Since binding of substrates to P-gp is related to the number and strength of hydrogen bonds formed between them, the correct assignment of these flips in both models were checked. This would also favor correct formation of hydrogen bonds and avoid in-correct van der Waals clashes. Thus, warranting both models for our structure-based studies.

<table>
<thead>
<tr>
<th>Validation tool</th>
<th>Parameter</th>
<th>3G5U model</th>
<th>2HYD model</th>
</tr>
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<tbody>
<tr>
<td>ITasser</td>
<td>C-score</td>
<td>0.06</td>
<td>-0.05</td>
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<tr>
<td>PROCHECK</td>
<td>Core regions</td>
<td>99%</td>
<td>98.70%</td>
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<td></td>
<td>Most favored regions</td>
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<td></td>
<td>Disallowed regions</td>
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<td>1.2%</td>
</tr>
<tr>
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<td>-10.05</td>
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<tr>
<td>Qmean6</td>
<td>Raw score</td>
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<tr>
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<td>96%</td>
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<tr>
<td></td>
<td>(1242/1278)</td>
<td>(1130/1177)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Favored</td>
<td>99.8%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(1276/1278)</td>
<td>(1177/1177)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Outliers</td>
<td>PRO709 and LYS1102</td>
<td>NIL</td>
</tr>
</tbody>
</table>

a A significant C-score lies in the range of [-5,2]

b A good quality model would be expected to have over 90% in the most favoured regions

c The quality estimate ranges between 0 and 1 with higher values for better models

d Models of low quality are expected to have strongly negative QMEAN Z-scores

Docking
Identification of binding site

Apart from its conventional use to virtually screen potential drug candidates, docking also helps to gain insights on a complex phenomenon, such as efflux by P-gp and its specific association with each substrate. Since the TMHs form the entry portals for the drug (TMHs 2 and 11 on one end and TMHs 5 and 8 at the other) and the drug binding pocket (residues of TMHs 4, 5, 6 from TMD1 and 10, 11 and 12 from TMD2), the binding box was positioned around the TM region in both the models used for docking.

Rhodamine123 (a prototypical P-gp substrate used in this study as a reference, as it is conventionally used by others researchers in the field) was docked with the 3G5U model. Upon visualization of the docked rhodamine, it can be observed from Fig. 3 that along with TMHs 5, 6 and 12 as reported, the TMHs 1 and 7 also contributed to its binding site. It was significant to note that the same set of TMHs with some common residues formed the binding site of phenytoin as seen in Fig. 4. Of all the residues that contributed to this binding site, Phe343, Ile340, Val981 and Val982 have been reported in the in vitro binding studies. The phenytoin binding site overlapped with that of rhodamine, in consistence with earlier reports on the hypothesized common drug binding pocket and, therefore, could be considered as the probable binding region of phenytoin.
The LigPlus analysis also showed that both rhodamine 123 and phenytoin formed hydrogen bonds with Trp315 and Tyr307, respectively (Fig. 4). The benzyl rings of rhodamine and phenyl and benzyl rings of phenytoin exhibited hydrophobic interactions with the surrounding residues of the binding site, implying its hydrophobic nature. These interactions could be validated through site-directed mutagenesis-based and/or photoaffinity labeling studies.

The reported similarity in the pharmacological action of the tricyclic anti-convulsants, carbamazepine and phenytoin may be attributed to the common pharmacophore composed of the aromatic ring and a central amide group. Carbamazepine possesses two aromatic rings positioned on either sides of the iminostilbene ring, of which an amide group is part of the carbamyl. Levetiracetam is an adjunct, new generation AED which is administered for partial and generalized seizures like the other two AEDs taken in our study. Figure 5 shows that carbamazepine and levetiracetam did not form any hydrogen bonds with the 3G5U model unlike phenytoin. This might be due to the presence of only one H bond donor in both carbamazepine and levetiracetam, unlike phenytoin which has two H bond donors.

An earlier QSAR study has also reported that for a compound to be P-gp’s substrate, it should possess a minimum of 2 H bond donors and obey the spatial separation. To a greater extent, our *in silico* identification substantiated the reported difference in the interaction pattern among the AEDs. This might also explain the relatively lesser involvement of the human P-gp in the efflux for levetiracetam in comparison with the other membrane-bound transporters. Furthermore, this was also significant since the binding energy values reported in Table 2 only indicated the affinity and not the strength or nature of the interactions. In addition, the above interactions of phenytoin could be affirmed to its hydrophobicity that would imply its specificity to be effluxed by P-gp.

**Role of ionization in binding of phenytoin**

The degree of ionization of phenytoin, depending on its pI (8.33) and pH of microenvironment can be calculated using the Henderson Hasselbach

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**Fig. 3**—Overlap of phenytoin (green) binding site with rhodamine123 (magenta) [The zoomed image on the right shows the involvement of the same TMHs in their binding (TMHs are colored as same as in Fig. 2)]

**Fig. 4**—The Ligplus images showing the hydrogen bond and hydrophobic interactions of rhodamine (Rho) and phenytoin (Ph) with 3G5U [The hydrogen bonds represented as a dashed line and hydrophobic interactions as an arc with spokes radiating towards the compound. The common residues forming the sites can be observed]
equation\textsuperscript{38}. Since phenytoin is hydrophobic and has a high ability to bind to serum proteins\textsuperscript{39}, its ionization state will affect binding to both target and non-target proteins, which in turn would affect its pharmacodynamics. Hence in order to exclude the possibility of this property affecting the results, we had taken into account all the three possible forms of ionization of the amide groups, even though at physiological pH 7.4 and in cerebrospinal fluid (pH 7.3), the majority of the drug would be unionized.

Upon docking, it was observed that all the three ionization states had similar binding affinities to that of the un-ionized phenytoin as shown in Table 2. The similar binding energies of the ionized and un-ionized forms of phenytoin indicated that ionization of phenytoin was not a major factor for its binding with P-gp. Figure 6 shows the same kind of interactions by pht1 and pht3. Especially in both cases, a hydrogen bond with Tyr307 and similar hydrophobic interactions by the surrounding residues were observed. The ionized amide group served as hydrogen bond acceptor in both the ionized states pht1 and pht3, unlike the un-ionized form, where the carbonyl oxygen acted as a hydrogen bond acceptor. Further, it was observed that despite the presence of two ionized groups in pht3, only N1 of the amide group interacted. The pht2, where the 2nd nitrogen atom was ionized did not show hydrogen bonds in any of the conformations. In all cases, the aromatic rings underwent aromatic-aromatic interactions. Since one aromatic ring and one amide group of the hydantoin form the phenytoin pharmacophore\textsuperscript{37}, differences observed in the interactions of three ionized states might be due to the significant role of this amide group.

<table>
<thead>
<tr>
<th>Compound</th>
<th>3G5U model</th>
<th>2HYD model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>-9.1</td>
<td>-7.2</td>
</tr>
<tr>
<td>Pht1</td>
<td>-8.2</td>
<td>-7.0</td>
</tr>
<tr>
<td>Pht1</td>
<td>-8.0</td>
<td>-7.0</td>
</tr>
<tr>
<td>Pht2</td>
<td>-8.4</td>
<td>-7.0</td>
</tr>
<tr>
<td>Pht3</td>
<td>-8.2</td>
<td>-7.0</td>
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<tr>
<td>CBZ</td>
<td>-8.9</td>
<td>-7.5</td>
</tr>
<tr>
<td>LEV</td>
<td>-5.6</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

Table 2—The binding energies (KCal/mol) of all the docked compounds with the two human P-gp models

Binding with two catalytic states of P-gp

The conformational changes in P-gp during drug translocation pathway influence the affinity of substrates, in turn causing its efflux\textsuperscript{5}. The underlying mechanism of phenytoin efflux could be comprehended better, if interactions during such changes are known. Since within the common drug-binding pocket, allosteric communications shift the binding site alternatively between low- and high-affinity\textsuperscript{40}, we considered these two conformations of the human P-gp. The observed difference in the values of binding energies of docked compounds (Table 2) between the two models implied differences in their binding affinities. The higher binding affinity of compounds to the 3G5U model and lower binding affinity to the 2HYD model might be due to the binding of ligand to a high affinity site in the in-ward facing conformation of P-gp, which is a competent state to bind drugs\textsuperscript{9}.

The visualization of phenytoin binding site in 2HYD model identified that residues of TMHs 4, 5, 6 and 7 formed the site (Fig. 7). The differences in the involvement of TMHs 11 and 12 were observed with
phenytoin binding sites in both our models. TMH 12 was found to be involved in phenytoin binding site with 3G5U model, but not with 2HYD model, while TMH11 was involved in the 2HYD model, but not in the 3G5U model. This might be because TMH12 rotates and/or moves laterally with respect to TMH6 upon ATP hydrolysis as reported. Due to this rearrangement, the conformational change from in-ward to outward takes place, causing an opening to the extra-cellular space. This shifts the drug to a low affinity site resulting in its efflux, thus validating our approach to identify the phenytoin binding region in the models of two predominant catalytic states of the human P-gp.

Conclusions
Our study has evaluated for the first time the interactions of phenytoin with models of two conformational states of P-gp using a systematic combination of in silico methods. The identified phenytoin binding region is validated with experimentally known rhodamine and the hypothesized common drug binding pocket and is found to have distinct and overlapping residues. The absence of hydrogen bond formation by AEDs that are non-substrates differentiates them remarkably from phenytoin. The underlying interactions of phenytoin and its ionization states have been analyzed and its relative affinity with the two conformational models have been documented. These findings provide a sound in silico basis for validating other AEDs that may be substrates of this multi-drug transporter. Also, this methodology can be extended for screening and identifying other potential P-gp substrates as well as allosteric modulators, subsequent to which validation can be done using in vitro binding studies. Furthermore, this study has added new information to the existing QSAR data to provide a better explanation of the drug translocation hypothesis.

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