



RESEARCH ARTICLE

Computational Pharmacogenetics of P-Glycoprotein Mediated Antiepileptic Drug Resistance

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Received: April 21, 2018

Revised: July 12, 2018

Accepted: July 27, 2018

Abstract:**Background:**

The treatment of epilepsy using antiepileptogenic drugs is complicated by drug resistance, resulting in treatment failure in more than one-third of cases. Human P-glycoprotein (hPGP; *MDR1*) is a known epileptogenic mediator.

Methods:

Given that experimental investigations have suggested a role for pharmacogenetics in this treatment failure, it would be of interest to study hPGP polymorphisms that might contribute to the emergence of drug resistance. Changes in protein functional activity could result from mutations as well as altered abundance. Bioinformatics approaches were used to assess and rank the functional impact of 20 missense *MDR1* polymorphisms and the top five were selected. The structures of the wildtype and variant hPGP were modelled based on the mouse PGP structure. Docking studies of the wildtype and variant hPGP with four standard anti-epileptic drugs were carried out.

Results:

Our results revealed that the drug binding site with respect to the wildtype protein was uniform. However, the variant hPGP proteins displayed a repertoire of binding sites with stronger binding affinities towards the drug.

Conclusion:

Our studies indicated that specific polymorphisms in *MDR1* could drive conformational changes of PGP structure, facilitating altered contacts with drug-substrates and thus modifying their bioavailability. This suggests that *MDR1* polymorphisms could actively contribute to the emergence of pharmaco-resistance in antiepileptic therapy.

Keywords: P-glycoprotein, Drug-resistant epilepsy, *MDR1* polymorphism, Structure-activity relationship, Docking, Homology modelling, Differential ligand affinity, Bioavailability.

1. INTRODUCTION

PGP (HGNC nomenclature: ABCB1) is a key transmembrane protein conserved from bacteria to man, and its function is to protect the organism from toxic xenobiotics. PGP has turned out to be a critical player in multiple drug resistance phenomena. Here, we are interested in its role in antiepileptic drug resistance. Epilepsy is a chronic neurological condition affecting more than 50 million people worldwide and 1-2% of the population [1]. The recurring limitation in the treatment protocol of epilepsy is the failure of drug-response in more than one-third of cases. This is the case with the > 30 FDA-approved drugs for epilepsy. PGP is an ATP-coupled efflux pump documented as an

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epileptogenic mediator [2]. It is known to be highly expressed in the blood-brain barrier, which is pharmacologically crucial for the bioavailability of drugs acting on the central nervous system [3]. The experimental evidence for the role of PGP polymorphisms in antiepileptic drug resistance has not been conclusive so far [4 - 10]. There is evidence for its cognate role in antidepressant therapy [11]. There are at least two mechanisms by which PGP could mediate refractory epilepsy. First, elevated levels of PGP protein might be linked with the low intracellular drug concentration in cortical cells observed in epilepsy treatment. Elevated abundance could result from multiple mechanisms, for example, promoter sequence mutations, decrease in methylation-mediated epigenetic silencing, change in miRNA-mediated transcript abundance, and decrease the rate of protein degradation. PGP is well-known for its broad substrate specificity, and greater PGP abundance would result in phenotypic drug resistance due to effective drug extrusion. Alternatively, a gain-of-function mutation or changes in protein folding might enhance its functional activity, resulting in the same phenotype, *i.e.*, hyperactive PGP leading to pharmacoresistant epilepsy (Fig. 1)[12, 13].

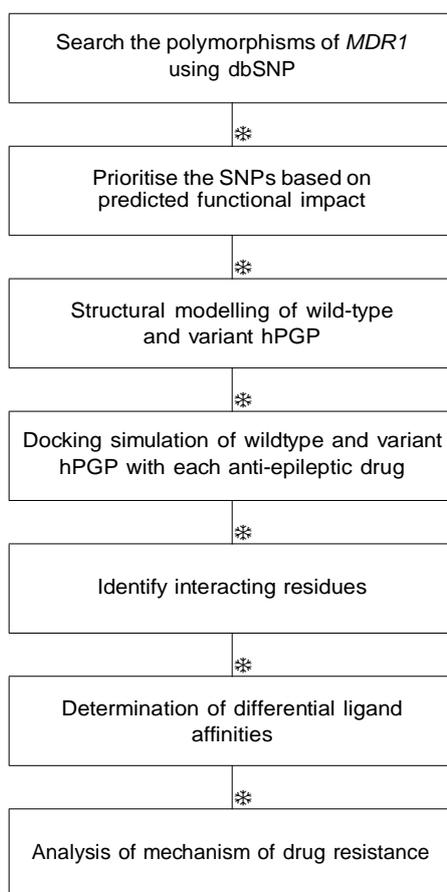


Fig. (1). Methodology for in silico study of *MDR1* polymorphisms in pharmacoresistant epilepsy.

PGP consists of two homologous halves, each consisting of a transmembrane (TM) domain with six alpha helices and a Nucleotide-Binding Domain (NBD) [14]. A large, hydrophobic and polyspecific drug-binding pocket resides in the inverted V-shaped internal cavity formed by the transmembrane domains [15]. It is clear that the key to epilepsy treatment would involve control of the epileptogenic mediator proteins, including hPGP. Structural studies of hPGP might enhance our current understanding of the role of PGP in drug resistance mechanisms, and provide any evidence of the relationship between specific *MDR1* haplotypes and altered drug pharmacokinetics. Given that little information is available on the pharmacology of missense polymorphisms of *MDR1*, analysis of the role of gain-of-function mutations in PGP would be valuable. Earlier bioinformatic studies of susceptible genetic Loci have identified and analyzed key polymorphisms specific to pathological phenotypes, for *e.g.*, polymorphisms associated with methylenetetrahydrofolate reductase (MTHFR) gene [16 - 21]. Genetic polymorphisms are known to influence susceptibility to epilepsy (for *e.g.*, BDNF [22, 23]). Here, we have attempted an in silico study of PGP polymorphisms and their structure-activity relationships with respect to drug-substrates to obtain a comprehensive picture of PGP role in the

emergence of antiepileptic drug insensitivity. PGP-mediated processes are also the major contributors to the emergence of drug resistance in cancer therapy and other conditions. Our results could extend to examining the role of P-glycoprotein in generalized drug-resistance in multiple conditions [24].

2. MATERIALS AND METHODS

2.1. Polymorphism Analysis

The hPGP sequence was retrieved from UniProt (acc. no. P08183). A PSI-BLAST search was performed using hPGP as query and target database as Vertebrates, with an E-value of 0.001 until convergence [25]. We selected top 5000 sequences from this result and clustered for redundancy at 40% sequence identity [26]. Multiple alignment of all the hits was performed using ClustalX [27] and manually edited (available in Supporting Information). The dbSNP was used to identify hPGP SNPs with the search term: “human [orgn] AND missense AND PGP”. The hits were assessed for the functional impact of polymorphisms using the curated multiple alignments obtained above. Three different tools were used: SIFT [28], PolyPhen2 [29], and PhD SNP [30]. Consensus of these predictions was used to evaluate the functionally important SNPs.

2.2. Homology Modeling

The template structures were retrieved using a Blast search of hPGP against the PDB database [31]. ClustalX was used to align the template and hPGP (*i.e.*, target). Modeller9 was used for modelling and energy-minimisation [32]. For each target, five separate models were generated and the model with the least DOPE (discrete optimized potential energy) score was chosen as the best model. The structure of a variant protein could be obtained by modeling in the mutation on the wild-type structure, however, this would not model any global effects due to the mutation. In order to fully account for the effects of the mutation, we modelled the variant proteins independently of the wildtype protein. Molprobit was used to validate the models obtained [33].

2.3. Protein and Ligand Preparation

Autodock 4.2 suite of tools was used for carrying out the docking simulations of hPGP variants and anti-epileptic drugs [34, 35]. Hydrogen atoms missing in the protein were added. This was followed by the addition of partial charges to the atoms. The protein was then converted to PDBQT format. The SMILES notation of the drugs of interest was retrieved from Pubchem [36]. The PDB co-ordinates of the drugs of interest were generated from their SMILES representation using OpenBabel [37]. To generate the conformers of each drug, we used MGLtools by calculating the number of bond torsions in the 3D structure. The ligand was then converted to the PDBQT format as well using AutoDock Tools. Target affinity maps for each atom type in the ligand were generated by autogrid by defining a uniform grid box centered in the hPGP internal cavity. This procedure was repeated for each target-ligand pair, for a total of $6 \times 4 = 24$ times.

2.4. Docking

We employed the Lamarckian genetic algorithm with default parameters for docking search, with 2,500,000 cycles per run, and 10 runs per receptor-ligand pair. The binding mode with the least binding energy was defined as the best pose. The ten poses obtained for each receptor-ligand pair were clustered at 2.0Å r.m.s. to validate the convergence to the best pose. The docked complex was then loaded in PDBQT format, converted to PDB coordinates using OpenBabel, and finally visualized using Rasmol2.7 [38]. The differential affinity of the variant for a given ligand relative to the wildtype was estimated as the difference in the binding energies, *i.e.* $\Delta\Delta G_{\text{mut}} = \Delta G_{\text{bind,mut}} - \Delta G_{\text{bind,wt}}$.

3. RESULTS

Nearly 500 hPGP SNPs were retrieved, however most of these were unannotated, and we obtained a set of 20 hPGP SNPs for further study, none of whose functional effects were known in the literature (Table 1). The results of our assessment of functional impact by various approaches are summarised in Table 2. Most of the SNPs were determined to be neutral, not disease-causing or deleterious. Five SNPs were predicted to be functionally important by at least one of the tools, as shown in Table 2.

Table 1. Missense SNPs of human PGP and their location. SNPs are represented in the usual convention: wildtype aminoacid followed by position followed by replacement aminoacid.

No.	rsid	SNP	Location
1	rs28381804	F17L	N-terminal domain
2	rs9282564	N21D	N-terminal domain
3	rs1202183	N44S	TM1
4	rs9282565	A80E	Linker between TM1 and TM2
5	<i>rs1128501</i>	G185V	TM3
6	rs36008564	I261V	Linker between TM4 and TM5
7	rs2229109	S400N	NBD1
8	<i>rs28381902</i>	E566K	NBD1
9	<i>rs28381914</i>	R593C	NBD1
10	rs2235036	A599T	NBD1
11	rs35023033	R669C	NBD1
12	rs2235039	V801M	Linker between TM8 and TM9
13	rs2032581	I829V	TM9
14	rs28381967	I836V	TM9
15	rs2032582	S893A	Linker between TM11 and TM12
16	<i>rs72552784</i>	A999T	NBD2
17	rs28401798	P1051A	NBD2
18	<i>rs55852620</i>	Q1107P	NBD2
19	rs2229107	S1141T	NBD2
20	rs28364274	V1251I	NBD2

Table 2. Topranked polymorphisms based on consensus prediction of functional impact.

No.	SNP	PolyPhen2 Prediction	PolyPhen2 Probability	SIFT Prediction	SIFT Score	phdsnp Prediction	phdsnp Reliability
1	G185V	Prob. Damaging	1	Damaging	1	Disease	8
2	R593C	benign	0.392	Damaging	1	Disease	6
3	E566K	Prob. Damaging	1	Damaging	0.88	Disease	6
4	Q1107P	Prob. Damaging	0.962	tolerated	0.19	Disease	6
5	A999T	Poss. Damaging	0.465	tolerated	1	Neutral	7

Table 3 provides the representative structures of P-glycoprotein in the PDB. Of these homologous hits, the mouse structures cover the full length of the hPGP. Some mouse structures co-crystallised with a ligand might not be representative of the native PGP conformation. When 4Q9H was superimposed with the 3G5U structure, it was observed that the register of the C-terminal half of the 'inverted-V' of 4Q9H was displaced relative to that of 3G5U (Fig. 2), which rendered 4Q9H unsuitable for modeling the full hPGP structure. The alignment between the hPGP and 3G5U is very good, showing > 87.5 % sequence identity and good sequence coverage (Fig. 3). 3G5U was used as the template for homology modeling. The target structures of the hPGP wildtype and the five variants were independently modeled and energy- minimised five times each, and the best model was used for further studies. All the modelled structures are available in the Supporting Information and their mutual rms deviations are shown in Table 4.

Table 3. Crystal structures of PGP homologues.

pdbid	Organism	Chains	Length	Res. A	#TM helices
3G5U	<i>Mus musculus</i>	A,B	1284	3.80	12
4Q9H	<i>Mus musculus</i>	A	1284	3.40	12
4KSB	<i>Mus musculus</i>	A	1284	3.80	12
3G61	<i>Mus musculus</i>	A,B	1284	4.35	12
3G60	<i>Mus musculus</i>	A,B	1284	4.40	12
4KSC	<i>Mus musculus</i>	A	1284	4.00	12
4LSG	<i>Mus musculus</i>	A,B	1284	3.80	12
4M1M	<i>Mus musculus</i>	A,B	1282	3.80	12

(Table 3) contd.....

pdbid	Organism	Chains	Length	Res. A	#TM helices
2HYD	Staphylococcus aureus	A,B	578	3.00	6
3B5Z	Salmonella enterica	A,B,C,D	582	4.20	5
3WME	Cyanidioschyzon merolae	A	612	2.75	3

Table 4. Mutual rms deviation of the template and modelled structures (in Å). Variant numbering corresponds to the order in Table 2.

–	3g5u	wt	Mut1	Mut2	Mut3	Mut4
wt	4.51	–	–	–	–	–
Mut1	4.24	0.53	–	–	–	–
Mut2	4.43	0.62	1.31	–	–	–
Mut3	4.32	0.71	1.19	1.33	–	–
Mut4	4.33	0.43	0.97	0.81	0.59	–
Mut5	4.63	0.49	0.71	1.04	0.48	0.44

Phenobarbital was first used as an antiseizure drug in 1912, followed by phenytoin. Today, more than 30 drugs are FDA-approved in the treatment of epilepsy, yet all of them face pharmacoresistance and more than one-third of epilepsy cases remain untreatable. In addition to phenobarbital and phenytoin, two other common antiepileptic medications, namely valproate and carbamazepine, were included in the set of ligands studied (Table 5).

Table 5. Anti-epileptic drugs.

No.	Drug	Pubchemid	Smiles Notation
1	Valproate	3121	<chem>CCCC(CCC)C(=O)O</chem>
2	Phenytoin	1775	<chem>C1=CC=C(C=C1)C2(C(=O)NC(=O)N2)C3=CC=CC=C3</chem>
3	Carbamazepine	2554	<chem>C1=CC=C2C(=C1)C=CC3=CC=CC=C3N2C(=O)N</chem>
4	Phenobarbital	4763	<chem>CCC1(C(=O)NC(=O)NC1=O)C2=CC=CC=C2</chem>

Docking between each hPGP protein (wildtype + 5 variants) and ligand was carried out. Ten docking runs were performed per receptor-ligand pair. Each run provides one low-energy docked conformation of the respective receptor-ligand pair. The site corresponding to the least-energy binding mode was taken as the binding site of the ligand with the receptor. To ascertain convergence to the lowest-energy binding mode, the ten runs of each receptor-ligand binding conformations were clustered at 2.0Å r.m.s. The lowest-energy binding modes showed good convergence. Of the 24 receptor-ligand pairs, 21 had energy-histograms showing the least binding energy (± 0.2 kcal/mol) as the most probable conformation and the least binding energies of the rest were within 1 kcal/mol of the binding energies of the most probable conformation. This provided confidence that the docking procedure resulted in convergence to the optimum receptor-ligand conformation. The structures of the receptor-drug complexes, as well as the best poses (defined as within 4.5Å of the ligand), are available in the Supporting Information. A comparison of the best poses between the wildtype and one of the variants is illustrated in Fig. (4). The groups of contacting residues specific to each docked complex are shown in Table 6. These residues were defined at a contact distance of < 4.5Å from the drug in the bound conformation. The summary of the docking analysis is presented in Table 7.

Table 6. Contacting residues of the receptor within 4.5Å of the ligand in the best pose of each docked ligand-receptor pair. Residue numbering follows the UniProt human PGP entry P08183.

Receptor	Ligand	Interacting Residues
Wildtype	Drug1	Leu244,Tyr247,Arg286,Ile289,Lys290,Ile293,Ala823,Lys826
Wildtype	Drug2	Gly141,Ile144,His145,Arg148,Val179,Ser180,Asn183,Asp886,Leu890,Leu924,Pro927,Tyr928,Ser931,Lys934
Wildtype	Drug3	Gly141,Ile144,His145,Arg148,Val179,Ser180,Asn183,Asp886,Leu890,Leu924,Pro927,Tyr928,Ser931,Lys934
Wildtype	Drug4	Met701,Asn704,Leu705,Trp708,Ile829,Arg832,Leu833
G185V	Drug1	Thr422,Leu554,Gln556,Ala557,Thr558,Gln570,Leu573,Asp574,Arg577,Val584,Thr591,Val592,Asn594,Ala595
G185V	Drug2	Ile199,Phe303,Tyr307,Asn721,Leu724,Gln725,Ser766,Gln838,Met986,Ala987,Gln990,Val991,Phe994
G185V	Drug3	Ile199,Phe303,Tyr307,Asn721,Leu724,Gln725,Ser766,Gln838,Ala987,Gln990,Val991,Phe994
G185V	Drug4	Lys291,Ala292,Asn296,Gln773,Phe777,Gly778,Glu782,Lys826,Ser831,Phe994,Ala995,Pro996
R593C	Drug1	Leu244,Tyr247,Lys285,Arg286,Ile289,Lys290,Ile293,Thr785,Lys826
R593C	Drug2	Thr627,Met628,Gln629,Thr630,Leu688,Phe804,Pro807,Lys808,Asn809,Thr810,Thr811

(Table 6) contd....

Receptor	Ligand	Interacting Residues
R593C	Drug3	Gln132,Val133,Trp136,Ile190,Glu875,Leu879,Phe938,Thr941,Phe942
R593C	Drug4	Gln132,Val133,Trp136,Ile190,Glu875,Leu879,Phe938,Gly939,Thr941,Phe942
E566K	Drug1	Leu244,Tyr247,Lys285,Arg286,Ile289,Lys290,Ile293,Thr785,Lys826
E566K	Drug2	Gln132,Val133,Trp136,Cys137,Gly187,Ile190,Glu875,Leu879,Phe938,Thr941,Phe942
E566K	Drug3	Ile293,Phe777,Gly778,Gly781,Ala823,Gln824,Lys826,Gly827,Ile829,Gly830,Phe994,Ala995,Pro996
E566K	Drug4	Ser831,Ala834,Val835,Gln838,Gln990,Val991,Ser992,Ser993,Phe994,Ala995,Pro996,Asp997,Tyr998
Q1107P	Drug1	Tyr247,Glu282,Arg286,Lys290,Gly781,Glu782,Thr785,Arg789,Ala823,Gln824,Lys826
Q1107P	Drug2	Asp689,Glu690,Ser691,Ile692,Lys808,Asn809,Ala813,Leu814,Thr816,Arg817,His1007,Met1010,Ile1011
Q1107P	Drug3	Val133,Trp136,Cys137,Glu875,Leu879,Lys934,Phe938,Thr941,Phe942
Q1107P	Drug4	Ser831,Ala834,Val835,Gln838,Gln990,Val991,Ser992,Ser993,Phe994,Ala995,Pro996,Asp997,Tyr998
A999T	Drug1	Leu244,Tyr247,Glu282,Arg286,Ile289,Lys290,Ile293,Thr785,Arg789,Ala823,Lys826
A999T	Drug2	Ala259,Ala260,Ile261,Arg262,Thr630,Leu688,Phe804,Pro807,Lys808,Asn809,Thr810,Thr811,Leu814
A999T	Drug3	Ser831,Ala834,Val835,Gln838,Gln990,Val991,Ser993,Phe994,Ala995,Pro996,Asp997,Tyr998
A999T	Drug4	Ser831,Ala834,Val835,Gln838,Gln990,Val991,Ser992,Ser993,Phe994,Ala995,Pro996,Asp997,Tyr998

Table 7. Free energies of binding (ΔG_{bind}) of each docked receptor-drug pair. The predicted differential ligand affinity is given by $\Delta\Delta G_{mut} = \Delta G_{bind,mut} - \Delta G_{bind,wild}$. All values in kcal/mol.

Receptor	Valproate	Phenytoin	Carbamazepine	Phenobarbital
wildtype	-4.28	-5.48	-6.35	-5.24
G185V	-3.90	-6.38	-6.27	-5.62
R593C	-5.21	-5.52	-6.37	-5.74
E566K	-5.53	-6.17	-5.55	-6.15
Q1107P	-5.50	-5.47	-6.66	-5.52
A999T	-5.15	-5.65	-6.67	-6.48
$\Delta\Delta G_{G185V}$	0.38	-0.90	0.08	-0.38
$\Delta\Delta G_{R593C}$	-0.93	-0.04	-0.02	-0.5
$\Delta\Delta G_{E566K}$	-1.25	-0.69	0.80	-0.91
$\Delta\Delta G_{Q1107P}$	-1.22	0.01	-0.31	-0.28
$\Delta\Delta G_{A999T}$	-0.87	-0.17	-0.32	-1.24

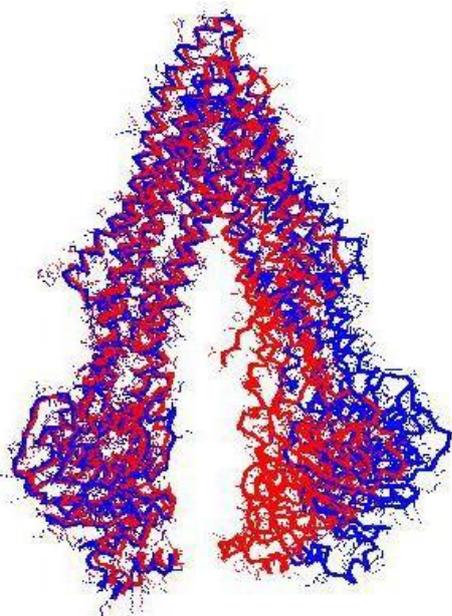


Fig. (2). Structural superposition of 3G5U (red) and 4Q9H (blue). Note the displacement of the C-terminal region of 4Q9H.

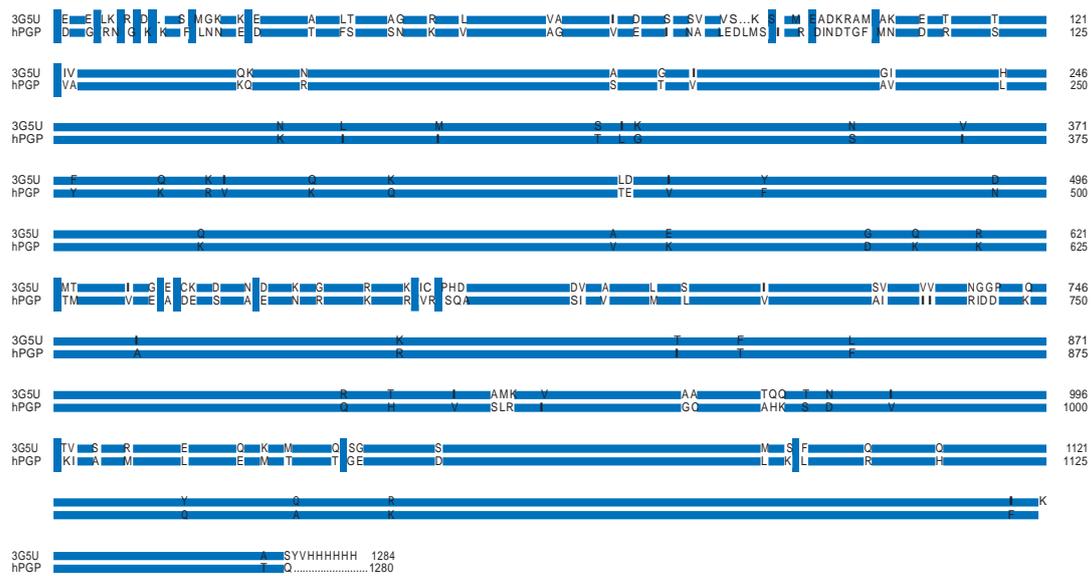


Fig. (3). Alignment of human PGP (target) and mouse 3G5U (template). Identical residues are highlighted and gaps are indicated by.

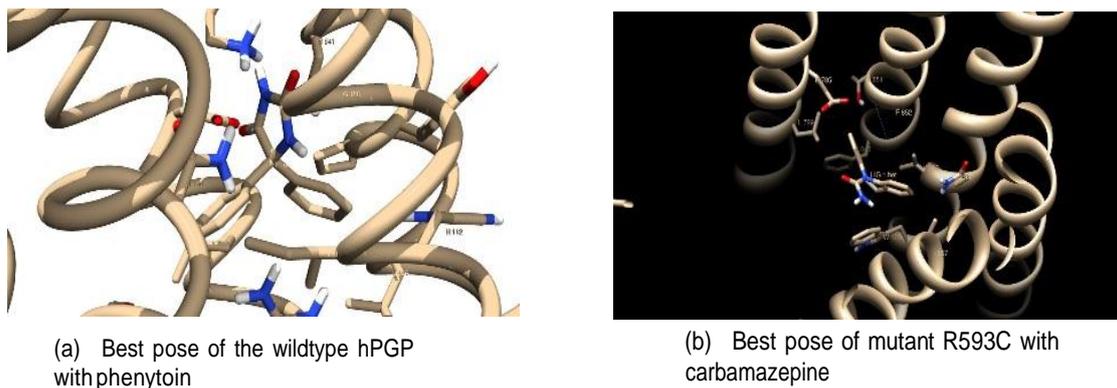


Fig. (4). Illustration of best pose comparisons.

4. DISCUSSION

The evolutionary conservation of the location of the prioritised SNPs was computed using the multiple alignments constructed from PGP homologous sequences non-redundant at 40% sequence identity. The quantile scores of each position with respect to hPGP sequence are given in the Supporting information. All five polymorphism locations ranked at least in the 50th quantile, and the E566K in particular ranked 97th. The polymorphisms were found in evolutionarily conserved positions. Experimental evidence is available on some of the polymorphisms [39 - 41].

The hPGP residues binding the ligand in each hPGP-drug pair represent the drug-specific binding pocket (Table 6). These residues contributed to stabilizing the docked complex by forming hydrogen bonds and Van der Waals interactions with the substrates. It is observed that the binding site of wildtype hPGP is identical for phenytoin and carbamazepine. This is a 14-residue binding pocket in the internal cavity lined by four hydrophobic residues (Ile144, Val179, Leu890, Leu924), three charged residues (Arg148, Asp886, Lys934) and four polar residues (Ser180, Asn183, Tyr928, Ser931). In contrast, the variant proteins bound the drugs in alternative variable regions, notably a binding pocket involving Gln132, Val133, Trp136, Ile190, Glu875, Leu879, Phe938, Thr941, and Phe942 that bound all drugs except valproate. It was remarkable that for a given variant hPGP, the binding pocket interactions differed for each drug. The R593C hPGP variant bound phenytoin very close to the mutation site, suggesting evidence for a local conformational change in binding the drug.

Further clarity on these observations could be obtained on an examination of the estimated binding free energies of

the wildtype and variant hPGPs with the different drugs of interest and the corresponding predicted differential ligand affinity given by $\Delta\Delta G_{\text{mut}} = \Delta G_{\text{bind,mut}} - \Delta G_{\text{bind,wt}}$ (Table 7). It was observed that all but three of the differential ligand affinities were negative. This implied that the variant hPGP bound each drug with a stronger affinity than the wildtype hPGP. The maximum range of differential response was observed with variant E566K ($-1.25 \text{ kcal/mol} < \Delta\Delta G_{\text{mut}} < 0.80 \text{ kcal/mol}$).

Two features indicated the neutrality of wildtype hPGP with respect to binding anti-epileptic drugs. First, the binding pocket appeared constant for both phenytoin and carbamazepine. Second, the binding energy with the drug was higher relative to the variants and hence less tight. On the other hand, there were two features that indicated that variant hPGPs would assist in the development of drug resistance.

First, variant hPGPs bound each drug in a different location in the internal cavity. Variability in location affords a better search of the optimal binding modes of the drug. Second, consistently lower binding energies were observed, implying stable drug-PGP complexes for possible energetic extrusion of the drug. The *in silico* analysis showed that polymorphisms could have played a role in relocating the optimal drug-binding cavity for a higher affinity, relative to the wild-type hPGP.

An elevated affinity between a variant hPGP and the drug could suggest a potential differential adverse response to therapy. From Table 7, it is observed that this is the case for 17 out of the 20 drug-protein combinations studied. Experimental studies are necessary to validate these results and determine whether the magnitude of any differential adverse responses could translate to the threshold for the development of pharmacoresistance. By contributing to drug resistance, PGP polymorphisms could conceivably play an adverse role in the treatment of multiple epilepsy subtypes. One example is electrical status epilepticus during slow sleep ('ESES'), a condition which could primarily affect children [42] and should best be treated early. The response to use of valproate and diazepam treatment regimens for ESES is quite heterogeneous [43 - 45], indicating inter-individual variability and a possible role for polymorphisms of pharmacogenomic importance, including those of PGP analyzed in our study.

CONCLUSION

Though hPGP is well-documented as a modifier of drug bioavailability in many conditions, its role in antiepileptic drug resistance has been controversial. At least two alternative mechanisms could explain the hPGP-mediated epileptogenic phenotype. Our work suggests that polymorphisms are a viable mechanism of PGP action that could lead to drug resistance acquisition independent of other mechanisms. It is interesting that all the polymorphisms appeared to result in gain-of-function. Coupled with the observation that somatic mutations could have a similar effect to identical inherited polymorphisms, this would suggest that PGP is a potential oncogene in the context of cancer drug resistance.

Developing a drug resistance strategy to combat drug resistance is a top priority. Our work has highlighted that *MDR1* polymorphisms could potentially lower the threshold for development of pharmacoresistance. This gain-of-function process in hPGP offers a novel candidate target in the fight against antiepileptic drug resistance. Experimental validation of our work is necessary to apply our findings towards achieving pharmacosensitive response in epilepsy treatment. Our methodology is extendable to studies investigating the effect of genetic polymorphisms on phenotypes in other diseases and conditions.

AUTHORS' CONTRIBUTION

Dr. Ashok Palaniappan conceived and designed the experiments. Experiment was performed and data was analyzed by Ms. Sindhu Varghese and Dr. Ashok Palaniappan. Dr. Ashok Palaniappan prepared the figures and/or tables.

Dr. Ashok Palaniappan drafted the work and revised it critically for important content.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Website along with the published article.

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