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Several New Diverse Anticonvulsant Agents Discovered in a Virtual Screening Campaign Aimed at Novel Antiepileptic Drugs to Treat Refractory Epilepsy

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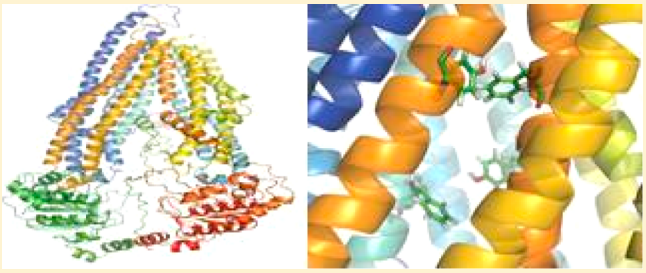
ABSTRACT: A virtual screening campaign was conducted in order to discover new anticonvulsant drug candidates for the treatment of refractory epilepsy. To this purpose, a topological discriminant function to identify antiMES drugs and a sequential filtering methodology to discriminate P-glycoprotein substrates and nonsubstrates were jointly applied to ZINC 5 and DrugBank databases. The virtual filters combine an ensemble of 2D classifiers and docking simulations. In the light of the results, 10 structurally diverse compounds were acquired and tested in animal models of seizure and the rotorod test. All 10 candidates showed some level of protection against MES test.



INTRODUCTION

Epilepsy is the most frequent chronic brain disorder, affecting around 50 million people worldwide.[1] Current antiepileptic drugs present two major limitations: on the one hand, even third generation antiepileptic agents present considerable and frequent adverse reactions;[2−4] on the other, current medications fail to control the symptoms in around one-third of the patients (condition known as refractory or intractable epilepsy).[1,5]

Experimental and clinical studies have suggested two possible pharmacological explanations to refractory epilepsy:[6] (a) a pharmacokinetic mechanism (the transporter hypothesis), i.e. limited bioavailability of the antiepileptic drug in the brain due to acquired or intrinsic overexpression or activation of efflux-transporters from the ABC protein superfamily at the blood−brain barrier and/or epileptic foci and (b) a pharmacodynamic mechanism (the target hypothesis), namely acquired or constitutive alterations to the structure and/or functionality of the molecular targets of antiepileptic drugs.

The transport hypothesis is supported by the fact that refractory epilepsy continues to be an issue even though approved antiepileptic agents act through a wide range of molecular mechanisms, which suggests a rather unspecific resistance mechanism. The main weakness of the transporter hypothesis is the uncertain interaction between antiepileptic drugs and multidrug transporters, since seemingly contradictory findings have been obtained when studying the transport of antiepileptic drugs in vitro.[7−12] It has been proposed that the transporter hypothesis might explain only a subset of nonresponsive patients.[13] On the other hand, transporters may assume greater importance at epileptic loci, where localized up-regulation of multidrug transporters has been demonstrated.[14,15] Remarkably, the studies that confirmed the interaction between multidrug transporters and some antiepileptic drugs have relied on modified model systems which either remove the diffusion component of drug transport of highly permeable drugs or consider the modified transport features of pathologic tissue.[7,8] It should also be considered that an association has been observed between disease severity and response to treatment[15] and that early pharmacologic treatment seems to influence disease progression in those patients with bad prognosis,[16,17] thus suggesting that achieving control of symptoms early (e.g., by choosing and adequate drug treatment) may be critical in those patients with high risk of recurrence.

P-glycoprotein (Pgp) is one of the ABC efflux transporters linked to refractory epilepsy, being up-regulated at the blood−brain barrier and epileptic loci of patients with intractable epilepsy.[18−21] Recently, we have reported a highly specific three-model ensemble of 2D classifiers capable of differentiating Pgp-substrates from nonsubstrates.[22] Here, we have applied this model ensemble in combination with a previously reported topological model capable of identifying anticonvulsants active in the Maximal Electroshock Seizure (MES) test[23−25] to ZINC 5 and DrugBank databases,[26,27] in order to find possible candidates for the treatment of Pgp-mediated refractory epilepsy. From the database compounds chosen as candidates, a subset of 380 molecules was selected for a structure-based virtual screening by docking. We docked this subset into the 3D structure of human Pgp, obtained from an homology model based on the crystal structure of mouse Pgp

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(pdb code: 3G60).[28] After this analysis, 10 compounds were selected for acquisition and subsequent pharmacological evaluation.

EXPERIMENTAL SECTION

Drug Sources. 2,2-Dimethyl-3-(2-methylprop-1-enyl)-cyclopropane-1-carboxylate (I) was acquired from Princeton Biomolecular Research. 7,7-Dimethylbicyclo[2.2.1]heptane-1-carboxamide (II) and 1-hydroxycycloheptanecarboxylic acid (VIII)were provided by Sigma Aldrich. Thioctic acid (III) was a generous gift from Bagó Laboratories. Metformin (IV) was a kind gift from the Medications Production Unity (UPM) from the Faculty of Exact Sciences, National University of La Plata. Mannitol (V) was acquired from Anedra. Sorbitol (VI) was a kind gift from Prof. P. De Urraza (Microbiology course). *N*-(*tert*-Butoxycarbonyl)-L-isoleucine (VII) was acquired from Otava Ltd. *N*-(3,3-Dimethylbutan-2-yl)-2-methylfuran-3-carboxamide (IX) was provided by UkrOrgSyntez Ltd. EDTA (X) was a kind gift from the Analytical Chemistry lab, Department of Chemistry, Faculty of Exact Sciences, National University of La Plata. Thioctic acid was a kind gift from Bagó Laboratories.

Computational Classification Models. To prioritize the early identification of non substrates of efflux transporters, initially we applied a three-model ensemble of 2D classifiers capable of differentiating Pgp-substrates from nonsubstrates.[22] Briefly, the average output of the following three models is used to predict whether a given drug candidate is or is not a Pgp-substrate:

$$\text{class} = -0.40\text{nCl} - 1.48\text{nTB} + 2.70\text{MATS3p} + 0.067\text{GGI2} \quad (1)$$

$$\text{class} = -0.64Y\ \text{index} + 0.11\text{GGI2} - 0.75\text{S-108} - 1.28\text{nSO2N} - 0.45\text{Cl-088} + 0.54\text{N-069} + 0.56\text{C-033} + 0.37n\text{RSR} \quad (2)$$

$$\text{class} = 20.61 + 0.12\text{nHAcc} - 22.95\text{MATS2m} + 0.042\text{mlogP2} + 0.38\text{nCaH} + 0.22\text{nCO} \quad (3)$$

where, nCl represents the number of chlorine atoms; nTB represents the number of triple bonds; MATS3p is Moran autocorrelation of lag 3, weighted by atomic polarizabilities; GGI2 is Galvez' topological charge index of second order; *Y* index stands for the Balaban *Y* index; S-108 represents the number of R = S; Cl-089 represents number of Cl attached to sp2 carbons; N-069 represents a primary aromatic amine or a primary amine bonded to a halogen atom; C-033 symbolizes X—CH···X fragments (X being halogen); nRSR represents the number of sulfures; nHAcc corresponds to the number of H-bond acceptors and; mlogP2 is the square of Moriguchi's log of the octanol/water partition coefficient. *Class* stands for a binary variable which assumes a value of −1 for Pgp-nonsubstrates and a value of 1 for Pgp-substrates. All these molecular descriptors have been computed by Dragon software for molecular descriptor calculation v. 4.0 (Talete SRL, 2003). Figure 1 illustrates the classificatory ability of the combination of these models (using the average value of the three outputs as classification criterion). Note that the ability to classify the 125-compound training set and the 125-compound test set are practically identical, indicating balanced explanatory and predictive powers (absence of overfitting).

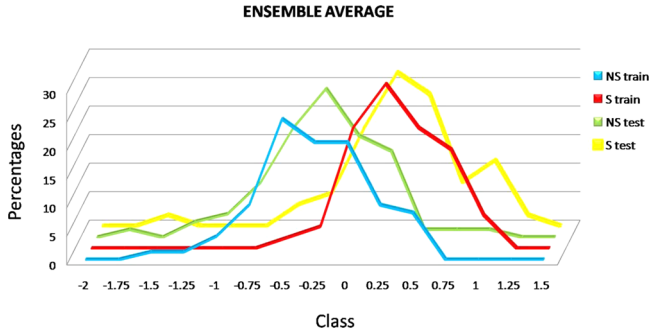

Figure 1. Distribution of the training set substrates (S train), training set nonsubstrates (NS train), test set substrates (S test), and test set nonsubstrates (NS test) over the average class predicted by each of the three models that compose the ensemble.

We have applied this model ensemble in combination with a previously reported topological model capable of identifying anticonvulsants active in the Maximal Electroshock Seizure (MES) test.[23−25] The latter is also based on 2D molecular descriptors from Dragon:

$$\text{class} = 8.110 - 2.206\text{HVcpx} - 4.277\text{BIC2} + 0.443\text{GATS7e} + 1.089\text{GATS8p} \quad (4)$$

where class now indicates whether a given compound does or does not elicit protection in the Maximal Electroshock Seizure model (1 indicates an active drug; −1 an inactive one). HVcpx represents the graph vertex complexity index; BIC2 symbolizes the Bond Information Content (neighborhood symmetry of second order); GATS7e denotes Geary autocorrelation − lag 7, weighted by atomic Sanderson electronegativities, and GATS8p stands for Geary autocorrelation − lag 8, weighted by atomic polarizabilities. Further information on these descriptors and model interpretation can be found in ref 24. The combination of models 1, 2, and 3 has been jointly applied with model 4 in a virtual screening campaign on the ZINC 5 and DrugBank databases to select anticonvulsant candidates for the treatment of Pgp-mediated refractory epilepsy. The applicability domain of the four models has been estimated through the leverage (or extent of extrapolation) approach.[29] It consists in computing the leverage h_i for each compound of the database. The leverage is defined as $h_i = x_i^{\mathrm{T}}(\mathbf{X}^{\mathrm{T}}\mathbf{X})^{-1}x_i$, where x_i is the descriptor vector of the considered compound i and $\mathbf{X}$ is the model matrix derived from the training set descriptor values. The warning leverage is generally fixed at $3k/n$, k being the number of model parameters and n being the number of training set compounds. According to this screening, 380 compounds passed to the docking stage.

Docking. The structures were docked and scored by Autodock Vina docking program.[30] The starting protein was prepared from the crystal structure of the mouse Pgp (PDB code: 3G5U) by a homology modeling performed with I-TASSER.[31] The resulting initial geometry was then minimized with AMBER11 software.[28]

The position of the substrate RRR-QZ59 in the mouse experimental structure (pdb code: 3G60) defined the center of the "docking active site" with a 24 × 24 × 24 A^3 grid volume. We used the default Autodock Vina parameters for the docking variables and the 20 energetically most favorable binding poses were outputted for each molecule. Gasteiger charges were

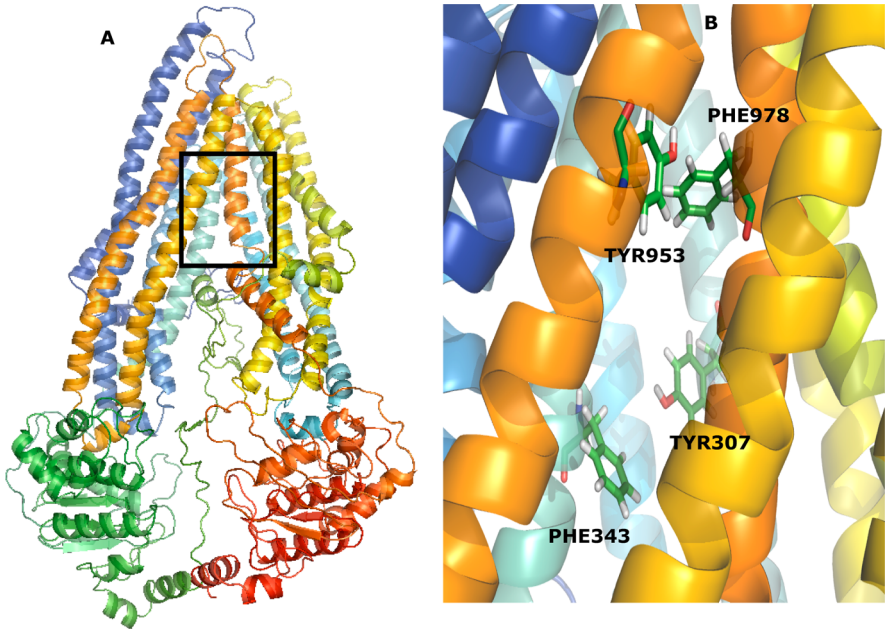

Figure 2. (A) Structure of P-gp obtained from homology modeling and used for docking calculations. The "docking active site" is highlighted. (B) Docking active site. The residues that were allowed to rotate in the docking campaign are highlighted as follows: carbon atoms are colored in green, oxygen atoms in red, nitrogen atoms in blue, and hydrogen atoms in white.

calculated for the ligands and the receptor, and the compounds were docked in its protonation state at pH 7.4.

We consider the ligands as flexible and the docking active site as rigid for all the residues but TYR307 PHE343 TYR953 PHE978 (Figure 2). We based the selection of the mobile ligands (as well the other docking conditions cited before) in a previous investigation.[28] Initially we detect the aminoacids that interact with the inhibitors in the experimental complexes (pdb codes: 3G60 and 3G61) and defined them as flexible in an initial docking of a set of known substrates and nonsubstrates (docking data set). The results showed that some of the amino acids (PHE343, PHE978) presented different conformations depending on the ligand, whereas others adopted practically the same conformation in all the ligands from the docking data set. Therefore we choose another set of flexible residues that includes PHE343, PHE978, and two more residues that interact with the structures according to the initial docking (TYR307 and TYR953). These mobile residues presented a better capacity to discriminate known Pgp-substrates from non-substrates of the docking data set.[28]

Finally we performed a docking simulation in the same condition with the 380 compounds that passed the 2D binary classifier virtual screening stage, and we used the values of the docking scores to predict the ability of the compounds to bind the Pgp. We considered as substrates the structures that showed a docking score lower than −7.4 kcal/mol. Conversely, we predict as nonsubstrates those compounds that presented weaker binding energy. This optimal threshold criterion was supported by the inspection of the "receiver operating characteristic" (ROC) curve calculated for de docking data set.[28]

Anticonvulsant Activity. Pharmacological testing was performed according to standard procedures provided by the Antiepileptic Drug Development (ADD) Program of the National Institute of Neurological and Communicative Disorders and Stroke.[32] Swiss mice provided by the Faculty of Veterinary, National University of La Plata, weighing between 18 and 23 g at the time of testing were used as experimental animals. Mice are housed in colony cages on a 12 h light/dark cycle and provided with food and water ad libitum. The tested drugs were dissolved in either saline or PEG 30%

To assess protection in the MES test, a maximal volume of 10 mL/kg of the freshly made solutions was injected intraperitoneally (i.p.) to groups of three mice, at doses of 30 and 100 mg/kg, the two lowest doses suggested by the ADD. Maximal electroshock seizures were elicited in mice by delivering a 60 Hz/50 mA electrical-stimulus for 0.2 s via ear clip electrodes, at 0.5 and 4 h after the drug injection, using an UGO Basile equipment. A drop of saline solution was applied on each ear before placing the electrodes to ensure adequate electrical contact. In these conditions, maximal seizures are produced in virtually all normal mice. The maximal seizure typically consists of a short period of tonic flexion followed by a longer period of tonic extension of the hind limbs and a final clonic episode. Blockade of the hind limbs tonic extension due to the drug treatment is taken as the end point. The tonic component is considered abolished if the hindleg tonic extension does not exceed a 90 degree angle with the trunk. *N*-(3,3-Dimethylbutan-2-yl)-2-methylfuran-3-carboxamide was also tested at doses of 20 and 300 mg/kg in order to confirm activity.

To assess protection in the subcutaneous pentilentetrazol (scPTZ) model, 1−2 mice were administered doses of 30 and 100 mg/kg of the tested candidate i.p. At 0.5 and 4 h after administration of the candidate, the freshly made solution of PTZ (1.7% in 0.9% saline solution) was administered subcutaneously (s.c.) into a loose fold of skin in the midline of the neck in a volume of 5 mL/kg body weight. This produces clonic seizures in 97% of animals tested. Immediately after PTZ injection, animals were located in individual cages during 30 min for behavioral evaluation. Occurrence and duration of clonus seizures were determined. Absence or less than 5 s duration of clonic convulsions indicate protection against this model.

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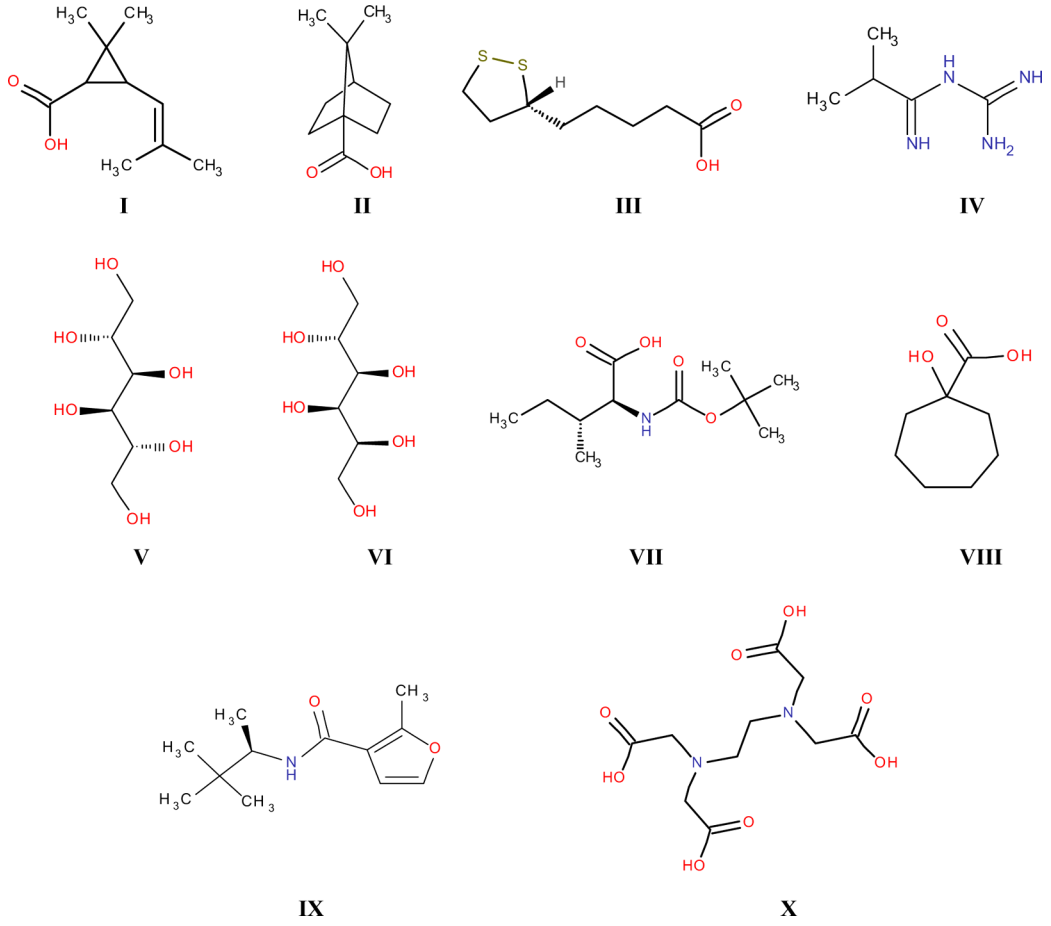

Figure 3. Candidates selected in the virtual screening campaign for acquisition and pharmacological testing.

Table 1. Number of Mice Protected by the Administered Drug in the MES and scPTZ Tests

		MES test[a]		PTZ test[a]		neurotoxicity[b]	
compound	doses (mg/kg)	0.5 h	4 h	0.5 h	4 h	0.5 h	4 h
I chrisantemic acid	30	0/3	1/3	0/2	0/2	0/5	0/5
	100	1/3	1/3	1/1	0/2	0/4	0/5
II 7,7-dimethyl-1-norbonane carboxamide	30	1/3	0/3	*c*	*c*	0/3	0/3
	100	3/3	0/3	*c*	*c*	0/3	0/3
III thioctic acid	30	0/3	1/3	*c*	*c*	0/3	0/3
	100	0/3	0/3	*c*	*c*	0/3	0/3
IV metformin	30	0/3	0/3	*c*	*c*	0/3	0/3
	100	0/3	1/3	*c*	*c*	0/3	0/3
V mannitol	30	0/3	1/3	*c*	*c*	0/3	0/3
	100	1/3	1/3	*c*	*c*	0/3	0/3
VI – Sorbitol	30	0/3	2/3	*c*	*c*	0/3	0/3
	100	0/3	0/3	*c*	*c*	0/3	0/3
VII *N*-(*tert*-butoxycarbonyl)-L-isoleucine	30	0/3	2/3	*c*	*c*	0/3	0/3
	100	0/3	2/3	*c*	*c*	0/3	0/3
VIII 1-hydroxycycloheptanecarboxylic acid	30	1/3	1/3	0/2	0/2	0/5	0/5
	100	0/3	2/3	0/2	0/2	0/5	0/5
IX *N*-(3,3-dimethylbutane-2-yl)-2-methylfuran-3-carboxamide	20	1/3	1/3	*c*	*c*	0/3	0/3
	30	1/3	0/3	*c*	*c*	0/3	0/3
	100	0/3	0/3	*c*	*c*	0/3	0/3
	300	0/3	1/3	*c*	*c*	0/3	0/3
X EDTA	30	2/3	0/3	*c*	*c*	0/3	0/3
	100	0/3	1/3	*c*	*c*	0/3	0/3

[a]Number of protected animals/number of tested animals. [b]Number of animals displaying motor impairment/number of tested animals. [c]Compound was not tested.

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The Rotorod test was used to determine possible neurotoxic effects of the test drugs. Control animals are manipulated as described above except that they receive vehicle (saline) instead of the drug solution.

RESULTS

From the sequential screening described before, 380 structures were classified as nonsubstrates of Pgp by the four 2D classifiers, and they belong to their applicability domains. 275 of them passed the docking screening as nonsubstrates. From them, 10 diverse compounds (average intermolecular Tanimoto similarity using atom pairs as fingerprint system: 0.329) were selected for acquisition and subsequent pharmacological evaluation on the basis of their structural diversity, accessibility, price, and previous use as either drugs or food additives (see Figure 3).

Results obtained from the anticonvulsant assays are presented in Table 1. All candidates showed anticonvulsant activity in the MES test. No sign of ataxia was observed at the Rotorod test. None of the animals in the control groups were protected against seizures. The anticonvulsant activity of compounds similar to I has already been described by Bialer.[33]

It is worth mentioning that we have recently reported the anticonvulsant activities of other non-nutritive sweeteners than sorbitol and mannitol.[34] Their anticonvulsant activities reported here provide new evidence to what we have called the sweetener hypothesis: a structural link between the receptor that unchains sweet response in mouth and some of the molecular targets of antiepileptic medications (presumably, metabotropic glutamate receptors). Both are permitted as food additives in both America and Europe.[35,36] EDTA is a widely used additive in cosmetics and food, which has undergone extensive safety studies.[37] Thioctic acid (also known as α-lipoic acid) is a dietary supplement usually used at doses of 100−200 mg/day that has also undergone long-term safety studies.[38] Metformin is an oral diabetic medication currently used in clinical practice. These few examples illustrate the potential of using Drugbank for virtual screening purposes which can be encompassed in knowledge-based drug repurposing or reprofiling: the possibility of finding second uses to known, well-characterized drugs through rational approaches, leading to drugs with well-characterized safety and pharmacokinetic profiles.[39−41]

The high level of consensus between the 2D classifiers of Pgp-substrates and nonsubstrates and the structure-based approach should also be highlighted. More than 70% of the candidates predicted as nonsubstrates by the 2D ligand-based approach were also signaled as nonsubstrates by docking.

CONCLUSIONS

Experimental results confirmed the predictions of the MES classificatory model. 275 new potential anticonvulsants for the treatment of refractory epilepsy have been identified through application of 2D classificatory models and docking in virtual screening campaigns. What is more, these 10 hits have been predicted as non-Pgp substrates, suggesting they might be applied for the treatment of Pgp-mediated refractory epilepsy.

Early recognition of substrates of efflux transporters is a key step in drug development projects in order to avoid transporter-mediated multidrug resistance issues, especially when the drug under development is aimed at the treatment of health conditions with high prevalence of drug resistance (e.g., epilepsy).

The ligand- and structure-based approaches to distinguish Pgp-substrates and nonsubstrates presented a high level of consensus. The results presented here should be complemented by experimental determination of the interaction between the candidates and Pgp (for instance, through adequate transport experiments), to experimentally validate the predictions of the three-model ensemble.

The application of virtual screening to knowledge-based drug repurposing is interesting, since it provides a rational basis in the search of second medical uses, which had been traditionally based in serendipitous findings or clever exploitation of drug secondary effects (e.g., sildenafil, aspirin, minoxidil, and many others). It provides a cost-efficient way to develop innovative medications in relatively short time, considering that the toxiciology and pharmacokinetics of repurposed drugs have already been studied.

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Notes

The authors declare no competing financial interest.

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