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ABCB1 Structural Models, Molecular Docking, and Synthesis of New Oxadiazolothiazin-3-one Inhibitors

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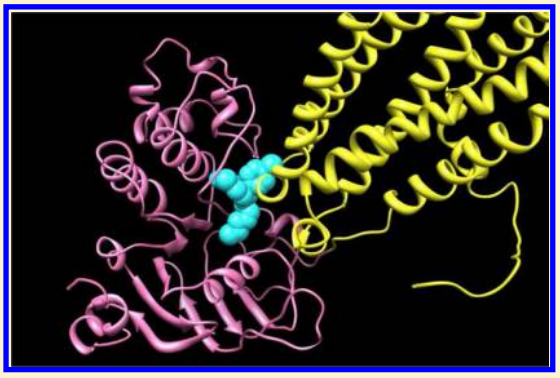
Supporting Information

ABSTRACT: Docking methods are powerful tools for in silico screening and drug lead generation and optimization. Here, we describe the synthesis of new inhibitors of ABCB1 whose design was based on construction and preliminary confirmation of a model for this membrane transporter of the ATP-binding cassette family. We chose the strategy to build our three-dimensional model of the ABCB1 transporter by homology. Atomic coordinates were then assayed for their reliability using the measured activity of some oxadiazolothiazin-3-one compounds. Once established their performance by docking analysis, we synthesized new compounds whose forecasted activity was tested by MTT and cytofluorimetric assays. Our docking model of MDR1, MONBD1, seems to reliably satisfy our need to design and forecast, on the basis of their LTCC blockers ability, the inhibitory activity of new molecules on the ABCB1 transporter.



KEYWORDS: *ABCB1 inhibitors, multidrug resistance, docking, drug design, LTCC blockers compounds, oxadiazolothiazin-3-ones*

The overexpression of the multidrug resistance-1 (MDR1 or ABCB1) gene has been associated with the onset of intrinsic or acquired drug resistance in many tumor histotypes.[1−3] This gene encodes for the membrane ATP-dependent transporter P-glycoprotein-170 (Pgp-170) able to pumping out of the cells a number of cationic or neutral hydrophobic xenobiotics and drugs[4,5] by an entropically driven flip-flop mechanism accounting for the broad range of substrates exported. It acts as bellows that widen the lower strand of the bilayer hunting for xenobiotics from the cytoplasmic interface or the lipid bilayer itself and then push these substrates out of the cell.

Protein structure consist of two protein halves each made by a cytoplasmatic nucleotide binding domain (NBD1 and NBD2), which acts synergistically on the substrate, and by a 6 transmembrane helices domain (TMD) forming a channel (Figure 1A). Both the NBDs share motifs typical of the domains involved in ATP binding and highly conserved within the transporter family. They contain the Walker A and B motifs as well as the ABC signature motif.[6]

Many compounds have been used to overcome MDR,[7,8] but today no one has shown a significant clinical efficacy because of a detrimental unbalance between pharmacodynamic, pharmacokinetic, and toxic activities.[9−13] In this framework, we have considered different LTCC (L-type calcium channels) blocker compounds (derivatives of [1,2,4]oxadiazolo[3,4-c][1,4]-thiazin-3-ones **1** and **2**[14]), which were already tested for their ability to compete for excretion with a typical drug excreted by Pgp-170, doxorubicin, causing its accumulation into multidrug-resistant A2780/DX3 cells (Figure 2).[14]

Here, we describe the construction of a 3D model of Pgp-170 that was used for drug modeling studies followed by the synthesis of the new compounds so designed (**2m−x**). In particular, to perform this study, we chose the strategy of modeling the Pgp-170 transporter by homology. This method may be useful to rationally design novel moieties that can act as inhibitor of this protein and that can be directed to the TMD and/or the NBD1 and NBD2 domains located within the cytoplasm. We tested a databank of small molecules by simulating docking procedures to these three main zones of Pgp-170.

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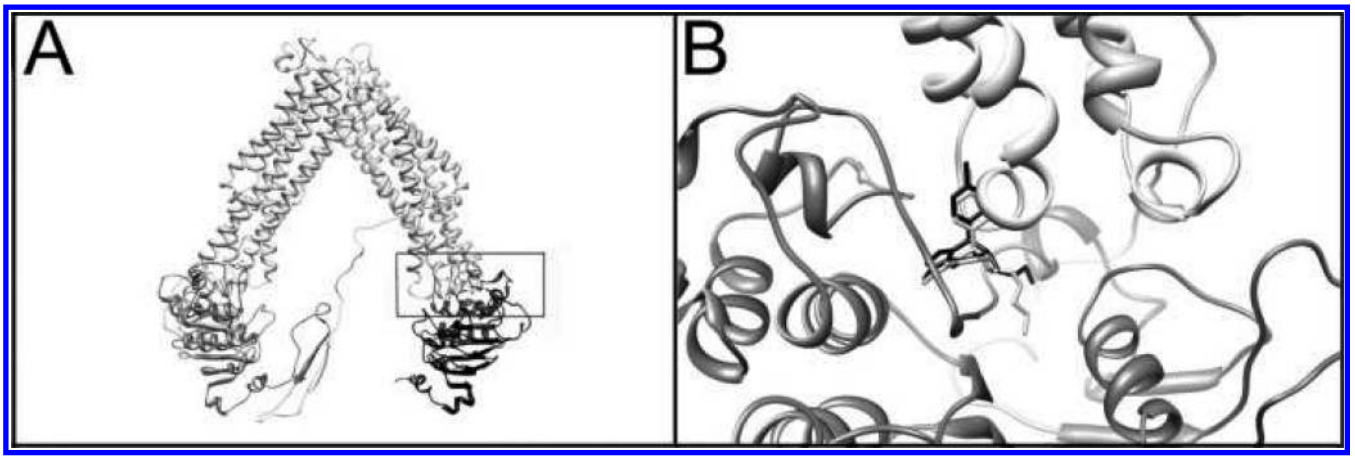

Figure 1. (A) Ribbon representation of the three-dimensional structure of the MDR1 in open conformation. NBD1 is on the left of the figure; NBD2 stands on the right, in a darker gray. The transmembrane helices are drawn in a lighter gray. The small molecules binding site area is represented enclosed by a rectangle. (B) Superposition of **2k** (lighter gray sticks) to **2l** (darker gray sticks) inside the ligand binding pocket. NBD2 is drawn as a dark gray ribbon. The relative position of the transmembrane helices TM4 and TM5 of MDR1 in a closed conformation (light gray) is reported to highlight the sterical clashes with the ligands (which are preventing the pump closure).

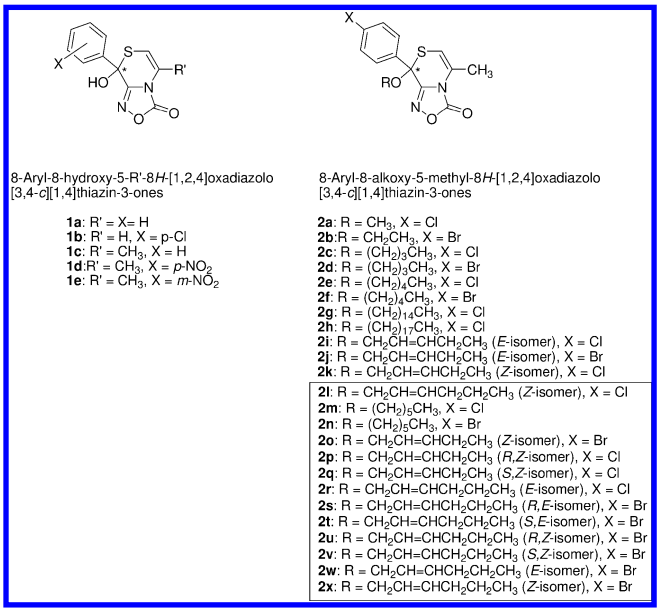

Figure 2. Oxadiazolothiazin-3-one derivative structure. In the box, the molecules **2l**−**2x** synthesized and/or selected following MONBD1 docking indications.

The availability of the crystal structures of the MDR1 from mouse,[15] a protein sharing 80.9% amino acids identity with the human Pgp-170, allowed us to build a first three-dimensional model to be used as a target (MOMDR1). The simulations using MOMDR1 as a target were not supported by in vitro analysis. In fact, out of the LTCC blockers previously tested,[14] the most promising candidates would be **2b**, **2d**, **2e**, and **2f** each bound to NBD2. Acceptable binding modes would also be found for **2j** and **2k** but with lower calculated binding affinity compared to a second model built based on the homology with the human NBD1 domain (MONBD1).

MONBD1 was built using as a template the three-dimensional coordinates of human MDR1 Nucleotide Binding Domain 1, this domain sharing 52.4% sequence identity with human NBD2, as previously described.[16]

Eventually, **1c** would be bound to NBD1, while no evidence of activity was found in in vitro analysis.

Being the result of this simulation not compatible with the results obtained by in vitro screening, we repeated the docking procedures using as a target the atomic model MONDB1. Structural superposition of the two NBD2 models (MOMDR1 and MONBD1) shows a substantial identity among the two structures. The overall root-mean-square deviation (rmsd) calculated on the position of 256 Cα atoms, in fact, was 2.21 Å. Nevertheless an important difference occurs in the proximity of the supposed binding site where the helix Asn1105-Leu1113 stands closer to loop Tyr1133−Ser1141 in MOMDR1 model with respect to NBD1 derived model. This difference seems to be sufficient to inhibit the binding capacity of the docked molecule to this site rendering MOMDR1 not suitable for our in silico test. Moreover, the supposed ligand binding pocket of NBD2 is placed in correspondence to a cleft that in the open conformation of MDR1 is occupied by the loop connecting TM helixes TM4 and TM5. The docking procedures adopted consider the protein as a rigid object, and thus it would be not possible for a molecule to bind within the considered cavity, due to steric hindrance. This reason made us think to target our molecules to the MONBD1. All the data about in vitro screening were obtained from our previous paper.[14]

On the basis of the models built, we docked 32 molecules (16 moieties, Figure 2: **1a**−**e** and **2a**−**2k** in *R* and *S* configurations; in **1** this approach is significant for interaction study, really enantiomer pairs spontaneously transform one into the other)[17] using as target the homology model of NBD2 domain. These simulations allowed us to screen our library and to determine some ligands to be further compared with in vitro analysis (see Figure S4, Supporting Information, as an example of the different docking of molecules with different forecasted inhibitory activities).

The *S*-isomers appear more effective than the *R*-ones. This different biological activity is well in line with expectations. In fact, while for most chemical reactions (without interactions with chiral reagents) the two enantiomers are indistinguishable; in contrast, they can show different biological effects because of the presence of chiral centers in living organisms. Because the best fitting in our docking model always corresponded to the *S*-configuration, we compared the Pgp-170 inhibiting activity of our *S* compounds (calculated as the percent reduction of IC_{50}s

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of doxorubicin obtained with compound concentrations giving per se 5% reduction of cell proliferation)[14] separated in three groups of high, modest, or absent fitting, as suggested by docking analysis. Our statistical analysis showed a correlation with $p = 0.007$, while, in contrast, the MOMDR1 model did not correlate with the in vitro activity of our molecules (Figure 3)

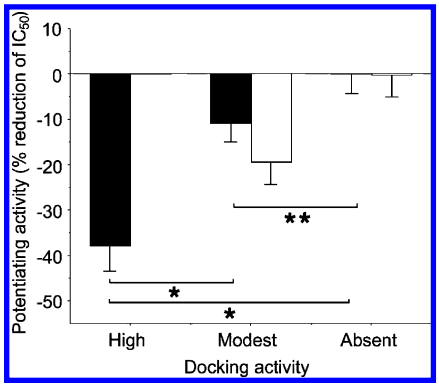

Figure 3. Comparison between data about the relative activity of our molecules separated in three grouping variables of high [$n = 4$ and 0, for MONBD1 (black bars) and MOMDR1 (white bars) docking models], modest ($n = 6$ and 11), and absent activity ($n = 6$ and 5), as classified by our docking analysis. The Kruskall–Wallis test for nonparametric data was used for the correlation between groups ($p = 0.007$ for MONBD1 model), while the nonparametric Mann–Whitney test was used to compare the groups in pairs (*$p = 0.011$; **$p = 0.109$). Data shown as mean ± SE.

confirming that our docking model may be considered reliable as reference for the molecular design of new compounds with the same mechanism of MDR1 inhibiting activity. The results of the simulations were in good agreement with the experiments carried in vitro using the racemic mixtures of all compounds and allowed to identify molecules with a potential activity on Pgp-170. The use of data of racemic mixtures could seem ambiguous, but their use seemed reasonable on the basis of our docking analysis and helped us to avoid wasting time to separate all compounds in their *S*- and *R*-isomers. However, our choice was also confirmed and rewarded by the results obtained with the enantiomers of **2k**, **2p**, and **2q** (see later). Moreover, the not extremely low theoretical values for K_i constants evaluated by docking analysis, from 10^{-6} to 10^{-8} M, may justify the fact that when using for comparison the data obtained from concentrations giving per se 0% reduction of cell proliferation we did not observe a significant correlation.

Out of docked molecules, **2g** and **2h**, indicated as good by simulations, were not considered as ligands due to the long hydrophobic tail, which was found entangled to the protein surface and not interfering with the protein activity. Compound **1a** showed a favorable clusterization with good calculated affinity constant not validated in vitro. Interestingly, **2j** and **2k** (*S*-isomers in *E*- or *Z*-configuration) were found to bind to NBD2 in a surface cleft deputy to host the loop connecting helices TM4 and TM5, **2j** (bromine substituted) being a larger favorite due to a better clusterization of the results (perhaps for the larger atomic radius of bromine in comparison to chlorine). The identification of this site as a potential binding site for our molecules justify that **1a** was not found as active by in vitro experiments, being its putative binding site was differently located.

Visual inspection of the three-dimensional structure of the protein model MOMDR1 showed that the ligand binding pocket is a mainly polar pocket in which six hydrophilic residues, E1084, R1085, R1110, S1117, R1188, and R1192, contribute to stabilize the ligand through hydrogen bonds with their polar groups, while residues F1086, L1109, L1113, I1115, I1121, and A1189 provide hydrophobic contacts. This site is about 11 Å apart from the ATP binding site as determined by similarity with the NBD1 domain, evidencing how our molecules are neither ATP competitors (see Figure S5, Supporting Information, for details) nor occupy the Pgp-170 drug binding site, which is hosted in a large hydrophobic cavity in the TMD domain (Figure 1B). Furthermore, the identified binding site is coincident with a cleft hosting the loop connecting TM4 and TM5 (residues A255-Q266) in the open conformation (Figure 1B); the presence of a molecule bound in this cavity could lead Pgp-170 to freeze in a closed conformation blocking its unpalatable export of anticancer drugs from the cell.

According to docking simulations, it appears evident how the alkyl chain of **2k** is pointing toward a deep hydrophobic hole made by residues V1080, L1083, L1113, I1115, I1196, L1198, and I1228.

To improve the ligand binding to this protein site, we modified the structure of **2k** into **2l** by lengthening acetal chain by a further CH_2. Interestingly, the comparison between dockings of **2k** and **2i** suggests a better influence of the *Z*-isomers on pharmacological activity, although when we considered the structures with 6 atom chains (**2l** vs **2r**), the *Z*-isomer did not seem to confer significant advantages in terms of inhibitory activity (**2r**, the *E*-isomer was considered a good ligand although displaying a less favorable clusterization of the docking simulation results). In this case, the best binding affinity was 8.7×10^{-8} M, similar to that of **2l**, suggesting a higher influence on activity for the alkyl chain length.

Thus, we considered two others molecules containing a saturated alkyl chain of six carbon atoms (**2m** and **2n**) with chlorine or bromine substituted moieties. As described, these modifications should lead to higher affinities or to understanding the influence of some structural modifications on pharmacological activity and should represent a first step for identification of more efficient MDR1 inhibitors.

The first of these modifications, which generated the synthesis of **2l** (see above), was suggested by docking analysis in order to increase the binding affinity (theoretical $K_i = 3.28 \times 10^{-8}$ M vs 1.11×10^{-7} M) and allowed the synthesis of one new *Z*-isomer that caused an increase of the potency, calculated as a decrease of IC_{50}, of about 71%, i.e., with a 24% increase of activity compared to **2k** (relative activity 44%, Table 1). As for **2m** and **2n**, they differentiate each other for chlorine or bromine in para position on the benzene ring. The measured in vitro activity of these molecules reflected the predictions of MONBD1. In fact, in vitro assays showed that only **2n** displayed a significant activity of 63 ± 10% of IC_{50} reduction (34% increase of activity compared to **2k**), underlining the importance of bromine and of length of the alkyl chain in determining the MDR1 inhibitory activity. The synthesis of **2o** similar to **2k** but with a bromine substituting chlorine confirmed the importance of halogen, realizing a 72% increase of activity compared to **2k** (44 vs 81%, Table 1).

The in vitro inhibitory activity was also tested for the single enantiomers of **2k**: **2p** and **2q**. Looking at their physical properties (see ECD, α_D in Supporting Information) and

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Table 1. Comparison between the Parent Compound 2k and the New ABCB1 Inhibitors Designed by the Docking Model MONBD1

	compd	MDR1 inhibitory activity[a]	gain/loss in inhibitory activity compared to **2k**	gain/loss in doxorubicin accumulation compared to **2k**[b]
presence of Cl or Br	**2k**	−44 ± 6%	100%	100%
	2o	−81 ± 8%	184%	139%
	2m	−23 ± 10%	52%	18%
	2n	−63 ± 10%	143%	72%
absolute configuration	**2p**	−16 ± 13%	36%	48%
	2q	−64 ± 10%	145%	136%
alkyl chain length	**2k**	−44 ± 6%	100%	100%
	2l	−71 ± 7%	161%	131%
Z- or *E*-isomers	**2k**	−44 ± 6%	100%	100%
	2i	−16 ± 7%	36%	35%
	2l	−71 ± 7%	161%	131%
	2r	−78 ± 9%	177%	109%

[a]The activity was evaluated as percent reduction of doxorubicin IC_{50}. Data shown as mean ± SD. [b]The gain/loss in doxorubicin accumulation was calculated compared to that of lead compound **2k** (100%).

considering some our previous results,[18−20] we suggest to consider the levogire **2p** as the *R*-enantiomer and the dextrogire **2q** as the *S*-one, respectively. From in vitro inhibitory activity results (Table 1) only **2q** (the *S*-enantiomer) showed a highly significant pharmacological activity (64% of IC_{50} reduction, with a 36% increase of activity compared to the racemic mixture **2k**, while **2p** was virtually inactive: −16 ± 13%). This result once again confirms the predictive reliability of our MONBD1 model emphasizing the importance of configuration of inhibitors and the lower suggested activity of *R*-enantiomers.

To confirm all these results, we also assayed cytofluorimetrically the ability of newly synthesized compounds to increase the accumulation of doxorubicin in our A2780/DX3 cell model in comparison with the original **2k**. From results in Table 1, it is evident that the compounds showing activity in antiproliferative assay were also more prone to block the efflux of doxorubicin via the ABCB1 transporter.

Considering our docking results, indicating that the presence of the *S*-configuration, the unsaturated 6C chain, and the bromine in benzene ring could increase the activity of our ABCB1 transporter inhibitors, we finally designed four molecules (**2s**−**2v**) all containing the bromine and all possible combinations of *R*/*S* and *Z*/*E* conformations. They were obtained from racemic **2w** and **2x** by chiral separation. We then activated the docking analysis to study their possible activity as MDR1 inhibitors.

Once we performed the docking analysis, surprisingly, we obtained for these molecules and, especially for those in *S* configuration, a score contradicting previous hypotheses. In fact, the sum of the above active chemical configurations was supposed to improve the score for the inhibiting activity, if compared to the single or coupled structural combinations. Instead, the activity predictable by docking analysis was not much better or even simply better than reference compound **2k**. In particular, (*S*,*Z*) **2v** had a score similar to that of **2k** (high activity), while the other stereoisomers had a lower score (modest activity).

All these data were also confirmed in vitro. In fact, the reduction of doxorubicin IC_{50} on resistant A2780/DX3 cells by the IC_{50} of molecules with the configurations *R*,*E* (**2s**), *S*,*E* (**2t**), *R*,*Z* (**2u**), and *S*,*Z* (**2v**) was 30 ± 9%, 27 ± 12%, 37 ± 15%, and 47 ± 15%, respectively, remembering that the reduction of the IC_{50} due to the activity of the reference compound **2k** was on average 44%.

Our results suggest, on one hand, that, in the case of our inhibitors, the simultaneous presence of single structural modifications, able per se or even when coupled to improve the inhibition of the MDR1 transporter, may not be able to cause an additive or synergistic effect in our test conditions. On the other hand, these results confirm the validity and reliability of our hybrid structural model for predicting the inhibitory activity of our ABCB1 inhibitors prompting us for a further development of new ABCB1 inhibitors.

Therefore, we are designing, synthesizing, and testing new derivatives of our more active compounds **2o** and **2r** to still increase the inhibitory activity of these compounds.

In conclusion, our docking model of MDR1, MONBD1, in spite of the relatively low number of structures used to confirm its validity, seems to reliably satisfy our need to forecast, considering their chemical structure, the inhibitory activity of our compounds on the Pgp-170 transporter and to design new molecules with an inhibitory mechanism.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and instruments used, synthesis and spectroscopic details of compounds, enantiomer separation, description of docking simulation, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ABCB1, ATP-binding cassette subfamily B member 1; LTCC, L-type calcium channels; MDR1, multidrug resistance 1; NBD, nucleotide binding domain; Pgp-170, P-glycoprotein-170; TMD, transmembrane domain

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