Interactions of human P-glycoprotein transport substrates and inhibitors at the drug binding domain: Functional and molecular docking analyses

Interactions of human P-glycoprotein transport substrates and inhibitors at the drug binding domain: Functional and molecular docking analyses

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ABSTRACT

Rhodamine 123 (R123) transport substrate sensitizes P-glycoprotein (P-gp) to inhibition by compound **2c** (*cis–cis*) *N*,*N*-bis(cyclohexanolamine)aryl ester isomer in a concentration-dependent manner in human *MDR1*-gene transfected mouse T-lymphoma L5178 cells as shown previously. By contrast, epirubicin (EPI) concentration changes left unaltered **2c** IC_{50} values of EPI efflux. To clarify this discrepancy, defined molecular docking (DMD) analyses of 12 *N*,*N*-bis(cyclohexanolamine)aryl esters, the highly flexible aryl ester analog **4**, and several P-gp substrate/non-substrate inhibitors were performed on human P-gp drug- or nucleotide-binding domains (DBD or NBD). DMD measurements yielded lowest binding energy (LBE, kcal/mol) values (mean ± SD) ranging from −11.8 ± 0.54 (valspodar) to −3.98 ± 0.01 (**4**). Lys234, Ser952 and Tyr953 residues formed H-bonds with most of the compounds. Only **2c** docked also at ATP binding site (LBE value of −6.9 ± 0.30 kcal/mol). Inhibition of P-gp-mediated R123 efflux by 12 *N*,*N*-bis(cyclohexanolamine)aryl esters and **4** significantly correlated with LBE values. DMD analysis of EPI, ^{3}H-1EPI, ^{3}H-2EPI, ^{14}C-1EPI, ^{14}C-2EPI, R123 and **2c** before and after previous docking of each of them indicated that pre-docking of either **2c** or EPI significantly reduced LBE of both EPI and R123, and that of both ^{3}H-2EPI and ^{14}C-2EPI, respectively. Since the clusters of DBD amino acid residues interacting with EPI were different, if EPI docked alone or after pre-docking of EPI or **2c**, the existence of alternative secondary binding site for EPI on P-gp is credible. In conclusion, **2c** may allocate the drug-binding pocket and reduce strong binding of EPI and R123 in agreement with P-gp inhibition experiments, where **2c** reduced efflux of EPI and R123.

1. Introduction

Fighting cancer still represents a challenge, since many patients die from the disease. One of the mainstays of cancer management is chemotherapy, often performed by administering various agents simultaneously. However, the success of chemotherapy is limited due to drug resistance of tumor cells and high treatment-related toxicity [1,2]. Multi-drug resistance (MDR) is a phenomenon of cancer cells characterized by cross-resistance to many structurally and mechanistically unrelated anticancer drugs [3–7]. P-glycoprotein encoded by the *ABCB1/MDR1* gene is an important mechanism of MDR and is upregulated in many clinically resistant and refractory tumors [7]. Overexpression of P-glycoprotein (P-gp) is causatively linked to accelerated efflux of chemotherapeutic agents. P-gp uses ATP as energy source to fuel drug efflux [8]. Pharmacological inhibition of P-gp and other MDR-conferring drug transporters is a promising strategy to revert MDR [9]. The previously published crystal structure of P-gp [10] provided the molecular basis to better understand poly-specific drug binding and how this protein can accommodate a drug in multiple conformations. Compounds mimicking P-gp substrates can allocate the drug-binding domain (DBD) and competitively inhibit its function. This may lead to decreased drug efflux and increased intracellular concentrations of anticancer agents. Exploratory chemistry aimed at identifying novel MDR reverters led to the synthesis of compounds inspired by pervilleine A and verapamil [11,12]. Structurally, these compounds are *N*,*N*-bis(cyclohexanol)amine aryl esters formed by

Abbreviations: CSA, cyclosporin A; DBD, drug-binding domain; DMD, defined molecular docking; EPI, epirubicin; LBE, lowest binding energy; MDA, molecular docking analysis; MDR, multidrug resistance; MFI, mean fluorescence intensity; P-gp, P-glycoprotein; R123, rhodamine 123; VMD, Visual Molecular Dynamics.

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a scaffold, where a basic linker tethers two aromatic moieties. In our previous study [11], P-glycoprotein inhibition by three sets of four isomers of *N,N*-bis(cyclohexanolamine)aryl esters was assessed on rhodamine 123 (R123) efflux in human *MDR1*-gene transfected mouse T-lymphoma L5178 cells and on ATPase activity of human P-gp-enriched intestinal *Spodoptera frugiperda* membranes (*Sf*9). The most active compounds inhibited P-gp with IC_{50} values much lower than those of cyclosporin A (CSA) or GF120918 (elacridar). It was concluded that the isomeric geometry and restriction of molecular flexibility of *N,N*-bis(cyclohexanolamine)aryl esters were crucial for their presentation to and P-gp inhibition as transport substrates, R123 and epirubicin seemingly cooperating with them to this inhibition [11].

In the present study, we report on P-gp inhibition by compound **2c**, which is a *N,N*-bis(cyclohexanol)amine aryl ester. Compound **2c** (*cis–cis*) was investigated for its inhibition of epirubicin and R123 efflux as a function of concentration of these two agents. Furthermore, the binding modes of *N,N*-bis(cyclohexanol)amine aryl esters on P-gp were compared with those of EPI and R123 to evaluate P-gp inhibition of those compounds *in silico*. To this end, we have introduced the co-docking approach for molecular docking analysis (MDA) of substrates/inhibitors to P-gp drug- or nucleotide-binding domains (DBD or NBD) for the first time. This is an extension to concepts of molecular pharmacological analysis employed to investigate drug/receptor interactions.

The present findings shed light on the molecular mechanism of P-gp inhibition by *N,N*-bis(cyclohexanolamine)aryl esters, especially **2c**.

2. Materials and methods

2.1. Chemicals

McCoy's 5A medium, heat-inactivated horse serum, L-glutamine, sodium orthovanadate (Na_3VO_4, V_i), colchicine, R123, dimethylsulfoxide (DMSO) and epirubicin hydrochloride (EPI) were purchased from Sigma Chemical Co. (Milan, Italy). Penicillin (10,000 UI/ml) and streptomycin (10 mg/ml) mixture was purchased from Lonza (Basel, Switzerland) and cyclosporin A (CSA) from Alexis Biochemicals (San Diego, CA, USA). Three sets of *N,N*-bis(cyclohexanol) amine aryl esters (each composed of four geometrical isomers) designated as **1a–d**, **2a–d**, and **3a–d** along with the highly flexible aryl ester analog **4** were investigated. The synthesis of these esters has already been described [12–14]. V_i solution (0.1 M) was prepared by dissolving the salt in hot water and adjusting the pH to 7.4 with addition of 0.1 M HCl or 0.1 M NaOH. The aryl esters along with EPI carrying radioactive ^3H or ^{14}C at selected positions employed in this study are depicted in Fig. 1.

2.2. Cell lines and cultures

The L5178Y mouse T-lymphoma parent cell line and the same cell line transfected with a recombinant *MDR1A* retroviral vector (pHa MDR1/A) [15] were a generous gift from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD, USA). Human *MDR1*-expressing cells were selected by culturing the transfected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype [16]. The L5178 MDR1 cell line was grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 100 UI/ml penicillin and 0.01 mg/ml streptomycin. Cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C. If the cells reached confluence, they were harvested and plated for up to 20 subsequent passages and for drug treatment. Cultures were initiated at a density of 2×10^5 cells/ml and grown exponentially to about 2×10^6 cells/ml in 48 h. Cells were counted in a Burker cytometer (VWR International, Albertslund, Denmark) before use. Their viability was always greater than 95% as tested by Trypan Blue exclusion.

2.3. Cell loading with R123 or epirubicin

R123 and EPI influx at 37 °C occurred rapidly into L5178 MDR1 cells, where it reached a steady-state concentration in about 10 min. If cells were transferred to medium without either R123 or EPI, their fluorescence quite rapidly decayed (apparent $t½ < 3$ min)

Compound	Structure	Ar_1	Ar_2
1a (*cis/trans*)	II	A	B
1b (*trans/trans*)	II	A	B
1c (*cis/cis*)	II	A	B
1d (*trans/cis*)	II	A	B
2a (*cis/trans*)	II	A	C
2b (*trans/trans*)	II	A	C
2c (*cis/cis*)	II	A	C
2d (*trans/cis*)	II	A	C
3a (*cis/trans*)	II	A	D
3b (*trans/trans*)	II	A	D
3c (*cis/cis*)	II	A	D
3d (*trans/cis*)	II	A	D
4	I	A	C

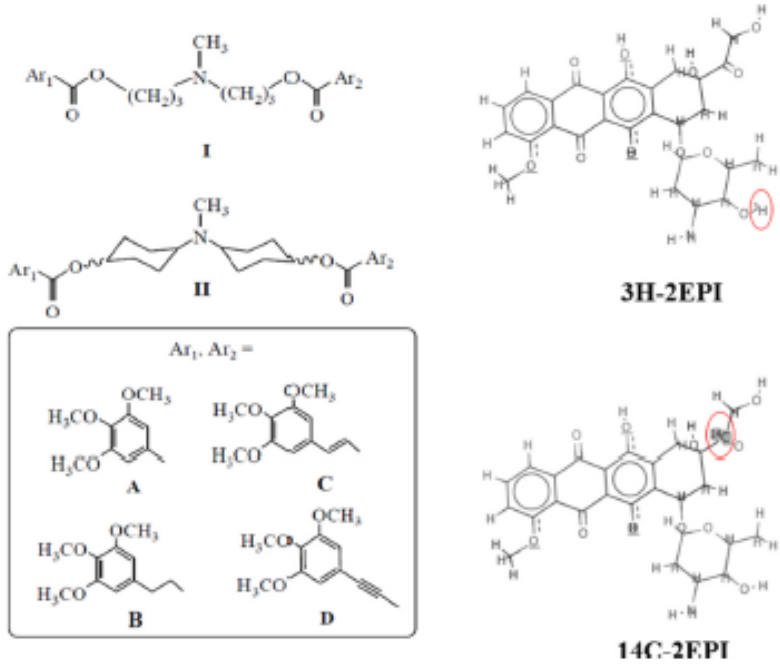


Fig. 1. Structures of some substrates/inhibitors of human P-glycoprotein investigated in the present study.

indicating fast substrate efflux. This efflux was mostly dependent on the capacity of P-gp, as the wild-type cells retained fluorescence for a long time. The same happened, if P-gp in L5178 MDR1 cells was inhibited by V_i in the case of R123, or CSA in the case of EPI. An incubation period of 20 min was chosen to attain a steady-state concentration of R123 or EPI, even in the presence of V_i or CSA, respectively. Under these conditions, the fluorescence values obtained were much higher and already maximal at an R123 concentration of 5×10^{-6} M and at an EPI concentration of 3×10^{-6} M, which were then selected as standard concentrations for all assays.

2.4. Cooperation of R123 and epirubicin with **2c** for P-gp inhibition

L5178 MDR1 cells (2×10^6/ml) were re-suspended in serum-free McCoy's 5A medium and 0.5 ml aliquots of the cell suspension were distributed into Eppendorf centrifuge tubes. **2c** was added at different concentrations and samples were incubated for 10 min at room temperature. R123 was added at different concentrations (5×10^{-6} M, 1×10^{-5} M, 2×10^{-5} M and 5×10^{-5} M) in the presence or absence of V_i. V_i was selected as positive control for a standard inhibitor, since already at 5×10^{-3} M it maximally inactivated P-gp efflux [17]. The IC_{50} for V_i in L5178 MDR1 cells was about 7×10^{-4} M in the present study. Cells were incubated for 20 min at 37 °C, washed twice and re-suspended in 0.5 ml PBS containing 1% heat-inactivated horse serum [11]. For EPI efflux experiments, different concentrations (3×10^{-6} M, 1×10^{-5} M, and 5×10^{-5} M) of EPI were applied comparable to those of the former experiment with R123. CSA was selected instead of V_i as positive control for a standard inhibitor, since it can maximally inactivate the P-gp efflux pump at concentrations as low as 50 μM. The IC_{50} for CSA determined on L5178 MDR1 cells was 6.7×10^{-7} M [11]. R123 or EPI retained by cells after washings were quantified by their respective fluorescence using a FACS Calibur flow cytometer (Becton–Dickinson, San José, CA, USA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 585/42 nm band-pass filters). FACS histograms were gated to include only individual, viable cells on the basis of forward and side-light scatter and was based on acquisition of 10,000 cells. Fluorescence signals were analyzed by the BDIS CellQuest software (Becton–Dickinson). The mean fluorescence intensity (MFI) was used for comparison among different conditions. This method is a slight modification of the previous protocol [18], which was chosen for the transfected/wild-type cells [19]. The original and the modified methods generated quite comparable results as assessed by assaying P-gp inhibition with GF120918 (elacridar).

2.5. Data analysis and statistics of cell experiments

Data are reported as mean ± SEM of at least three independent experiments each of them run in triplicate. The fluorescence data are expressed as mean of arbitrary fluorescence units derived from histogram plots of 10,000 cells examined. The percent P-gp inhibition exerted by a single compound was calculated as described [11]. The relative fluorescence (*i.e.*, percent inhibition of P-gp) was calculated as mean fluorescence intensity (MFI) of a discrete sample divided by the MFI in the presence of 5×10^{-3} M V_i or 5×10^{-5} M CSA, times 100:

$$\text{Relative fluorescence} = [\text{MFI of sample}/(\text{MFI of sample} + V_i, \text{ or CSA})] \times 100.$$

The denominator represents MFI of the sample, if inactivation or complete preclusion of the function of P-gp active efflux is attained. The numerator is the resulting signal caused by test compound inhibiting the function of P-gp active efflux. The P-gp-blocking activity was described by α_{max}, which expresses the efficacy of the inhibitor and by IC_{50}, which measures its potency; α_{max} varies between 0 (in the absence of the inhibitor) and 1 (if the amount of R123 or EPI found in L5178 MDR1 cells was that determined in the presence of 5×10^{-3} M V_i or 50 μM CSA, respectively). The IC_{50} measures the potency of the inhibitor and represents the concentration that causes half-maximal increase ($\alpha = 0.5$) of intracellular R123 or EPI concentrations. IC_{50} values were obtained by best fitting the concentration/inhibition curves, according to the one-site or two-site models. In order to establish which equation was more appropriate, the two models were compared using the F test (GraphPad Prism version 5, GraphPad Inc., San Diego, CA, USA).

For the correlation between IC_{50} and LBE values, Pearson's correlation test was used as a relative measure of the linear dependency of these two variables (WinSTAT, Kalmia).

2.6. Molecular docking

The previously generated homology model of human P-gp was used for molecular docking studies [20]. AutoDock 4 [21] was used for defined molecular docking (DMD) calculations. As a first step, blind docking was performed to evaluate the docking poses. Then, defined docking was conducted on the drug binding pocket or ATP binding site. Drug binding amino acid residues of P-gp were identified as His61, Gly64, Leu65, Met69, Ser222, Leu304, Ile306, Tyr307, Phe336, Leu339, Ile340, Ala342, Phe343, Gln725, Phe728, Phe732, Leu762, Thr837, Ile868, Gly872, Phe942, Thr945, Tyr953, Leu975, Phe978, Ser979, Val982, Gly984, Ala985, Met986, Gly989, Gln990, and Ser993 [10]. ATP binding site involves Trp1044, Val1052, Gly1070, Ser1071, Ser1072, Gly1073, Gly1074, Gly1075, Lys1076, Ser1077, Thr1078, Gln1118, Glu1119, Tyr1167, Gln1175, Leu1176, Ser1177, Gly1178, Gly1179, Gln1180, Asp1200, Glu1201, Ala1205, Leu1206, Asp1207, His1232 [10,22,23]. A grid map was chosen to cover these residues. Three to six independent docking calculations were conducted, with each 2,500,000 evaluations and 250 runs being performed. Lamarckian Genetic Algorithm was chosen for the docking calculations. The corresponding lowest binding energies (LBE) and predicted inhibition constants (pK_i) were obtained from the docking log files (dlg). Mean ± SD of binding energies were calculated from the independent dockings. For visualization of docking results, AutoDock Tools and Visual Molecular Dynamics (VMD) were used (Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign). Surface representation images showing the binding pocket of human P-gp were generated using VMD software.

For co-docking calculations, our attention was focused mostly on the drug-binding pocket of P-gp, since all compounds tested here preferentially bound there. EPI, ^{3}H-2EPI, ^{14}C-2EPI, R123 and **2c** were selected to evaluate the effect of pre-docked compounds on binding energies and docking pose of the same and/or other compounds.

3. Results

3.1. Cooperation of R123 and EPI with **2c** for P-gp inhibition

2c inhibited P-gp function, as shown by decreased R123 or EPI efflux upon cell treatment. For a better comparison of how increasing R123 or EPI concentrations enhanced the potency of **2c** to inhibit P-gp, relative IC_{50} values and concentration-inhibition graphs are shown in Fig. 2. The concentration–inhibition curve shifted to the left with increasing R123 concentrations, while maximal

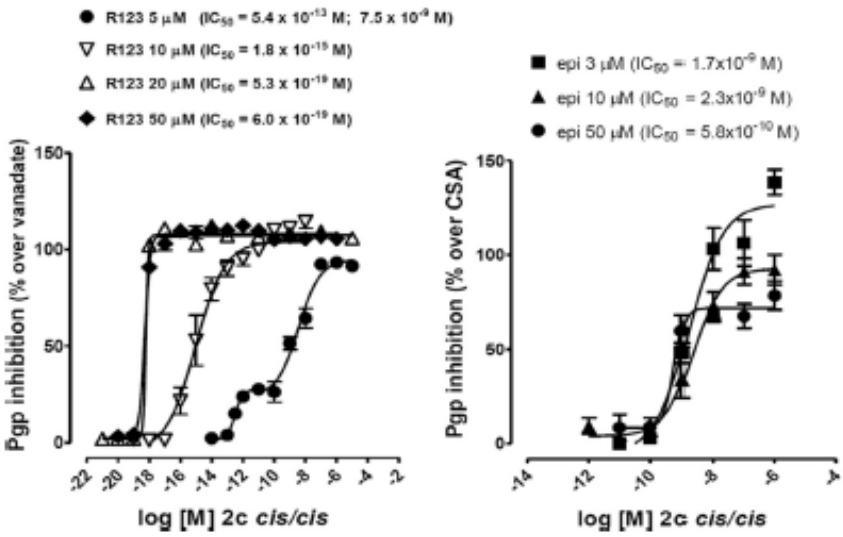


Fig. 2. P-glycoprotein inhibition by **2c** as shown by R123 and EPI retention in L5178 MDR1 cells. The panel on the left side is taken from [11].

inhibition values remained comparable at all R123 concentrations employed. Increasing EPI concentrations caused slightly lower maximal inhibition values of P-gp, suggesting competitive antagonism of EPI toward inhibition by **2c** regarding P-gp function. Notably, **2c** increased its potency from 10^6 up to 10^{10} fold, if the R123 concentrations were raised from 5 µM to 50 µM. By contrast, raising the EPI concentrations from 3 µM to 50 µM did not significantly affect **2c** IC_{50} values of cellular EPI efflux.

3.2. Molecular docking on P-gp

3.2.1. Blind molecular docking calculations

All compounds investigated, 13 aryl esters as well as CSA, EPI, elacridar, R123 and valspodar were subjected to blind molecular docking calculations. They all docked with high affinity to the DBD with the exception of compound **4**, whose affinity was relatively low. Remarkably, at this phase of the study **2c** was the only compound binding with high affinity to the nucleotide binding domain (NBD) of P-gp among all the *N,N*-bis(cyclohexanol)amine aryl esters investigated. Amino acids in NBD interacting with **2c** were Lys1061, Gly1224, Glu1223, Arg1225, Lys1220 and Asp 1219. The amino acids of DBD interacting with various compounds were Ser222, Phe303 and Phe343 (Fig. 3, right side). These results appeared promising and encouraged us to perform defined molecular docking calculations at the DBD for all compounds, and at NBD for **2c**. Since R123 proved to dock at NBD, its behavior underwent more detailed analyses in co-docking simulation at the NBD with the P-gp substrate/inhibitor **2c**.

3.2.2. Defined molecular docking: comparison of lowest binding energies with experimental IC_{50} values

Defined molecular docking (DMD) at the drug binding pocket pointed out that all aryl esters, except **4**, showed high LBE values that were comparable to those of the control compounds. As reported in Table 1, low binding energy (LBE) values ranged from −11.80 ± 0.54 (valspodar) to −3.98 ± 0.01 (**4**). The value of the EPI binding site at DBD was −8.97 ± 0.18 kcal/mol. This seems to be a preferential binding site of P-gp for EPI. The amino acids of DBD involved in H-bond formation with most of the aryl esters are shown in Fig. 3 and Table 1. They were Lys234, Ser952 and Tyr953. All these residues contribute to the cavity formation of the drug binding pocket of P-gp.

In order to assess whether any correlation exists, between LBE and IC_{50} values of R123 efflux from L5178 MDR1 cells of 12 *N-N*-bis(cyclohexanol) amine aryl esters and compound **4**, the relative data were plotted (Fig. 4). The IC_{50} values of cellular R123 efflux retention by L5178 MDR1 cells and LBE values of the aryl esters were significantly correlated (*R*-value = 0.69, *p*-value = 0.009), indicating good matching of the *in vitro* and *in silico* data. This represents a good indicator for the reliability of our computational protocol to estimate binding affinities of substrates and inhibitors of P-gp.

3.2.3. Co-docking calculations at the DBD and NBD of P-gp

In order to assess, whether a P-gp substrate/inhibitor could interfere at the DBD with another substrate/inhibitor, docking of transport substrates (EPI, R123 and **2c**) to P-gp alone or to complexes of P-gp pre-docked with each of the transport substrates were performed. Docking of EPI to P-gp already complexed with **2c**, R123 or EPI revealed that only the **2c**–P-gp complex had a significantly reduced LBE value (Table 2). Remarkably, if EPI docked to EPI–P-gp complex, the LBE value was not statistically different from the LBE value of EPI docking to P-gp alone. This suggests that EPI docked at a second binding site characterized by a binding affinity, which is comparable to that of the primary site of docking (EPI alone). The amino acid residues participating in docking of EPI under both conditions are shown in Table 3. Once the EPI structure underwent slight changes, *i.e.*, by substitution of one H atom at position 2 with ^3H or of one C atom at the same position with ^{14}C, the resulting two isotopically labeled EPIs bound to P-gp with LBE values very similar to that of EPI. Notably, however, if both ^3H-2EPI or ^{14}C-2EPI docked to the EPI–P-gp complex, the resulting LBE values significantly decreased. This finding suggests that the two radiolabeled EPI structures docked to a binding site, which was different from the primary binding site of EPI to P-gp (third binding site of EPI to P-gp).

R123 docking to P-gp complexed with **2c** yielded a significantly lower LBE value, as compared to that of R123 single docking. Remarkably, R123 docking to P-gp complexed with EPI gave a slightly lower but significant LBE value.

2c docking at the P-gp complex with both, EPI or R123, gave LBE values which were not significantly different from the LBE value obtained by **2c** single docking. The lack of effect of EPI pre-docked at DBD, on **2c** docking may be explained by the pose shown

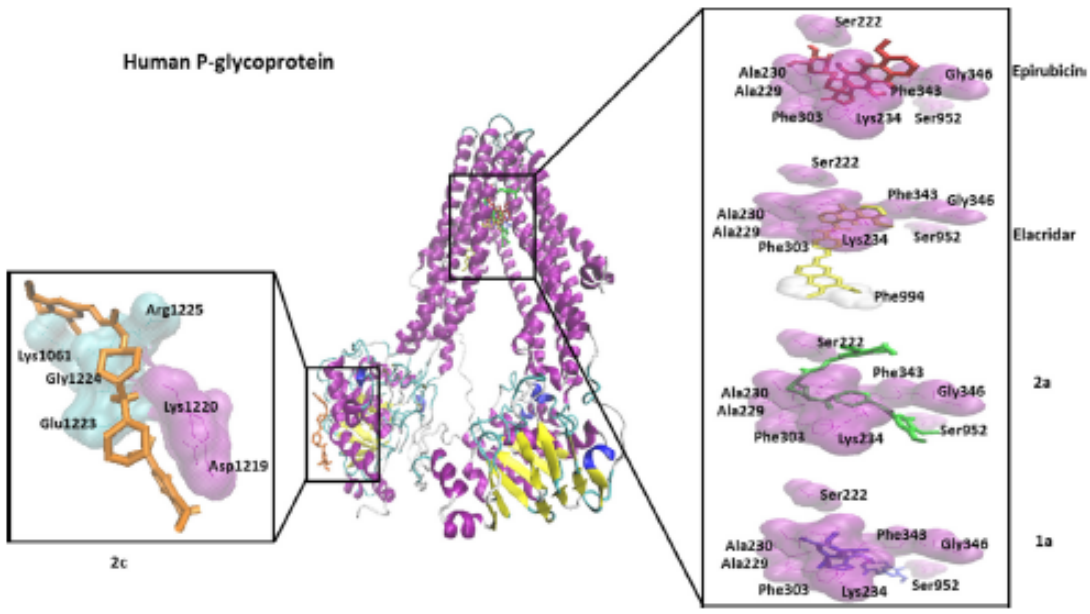


Fig. 3. Docking poses of **1a** (purple bond representation), **2a** (green bond representation), **2c** (orange bond representation), elacridar (yellow bond representation), epirubicin (red bond representation), R123 (blue bond representation) on human P-glycoprotein (ribbon and surface representation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Defined molecular docking of 12 *N,N*-bis(cyclohexanol)amine aryl esters, compound **4** and other P-glycoprotein substrates/inhibitors at the DBD.

Compound	Lowest binding energy (kcal/mol)	pK_i (μM)	Amino acids involved in H-bonds
Valspodar	−11.80 ± 0.54	0.04 ± 0.03	–
Cyclosporin A	−10.40 ± 0.82	0.003 ± 0.003	–
Elacridar	−10.90 ± 0.68	0.02 ± 0.02	Lys234, Phe994
2a	−9.50 ± 0.51	0.14 ± 0.09	Lys234
1a	−9.00 ± 0.14	0.26 ± 0.06	Ser952
Epirubicin	−8.97 ± 0.18	0.27 ± 0.07	Lys234, Ile306
2b	−8.90 ± 0.06	0.32 ± 0.03	Ser952
2d	−8.70 ± 0.79	0.65 ± 0.57	–
R123	−8.61 ± 0.01	0.49 ± 0.01	–
3a	−8.60 ± 0.10	0.47 ± 0.08	–
1c	−8.60 ± 0.87	1.01 ± 1.29	Tyr953
3b	−8.60 ± 0.31	0.57 ± 0.25	Gln944, Tyr953
2c	−8.38 ± 0.17	0.73 ± 0.19	–
3c	−8.40 ± 0.46	0.88 ± 0.63	Glu875
3d	−8.30 ± 0.12	0.85 ± 0.15	–
1b	−8.30 ± 0.57	1.11 ± 1.05	Lys234
1d	−7.80 ± 0.25	1.97 ± 0.79	–
4	−3.98 ± 0.01	1238 ± 2.00	–

For each compound, lowest binding energy, predicted inhibition constant (pK_i) and amino acids (AA) involved in hydrogen bonding are given. Docking simulation underwent three independently repeats with 2,500,000 calculations and 250 runs for every repeat. Figures represent the mean ± SD of LBE and pK_i from three independent docking calculations.

in Fig. 5, where the docking of both compounds to DBD is shown. It is evident that the two compounds docked at two quite separated sites of DBD with only Phe343 overlapping at both sites (see also Table 3).

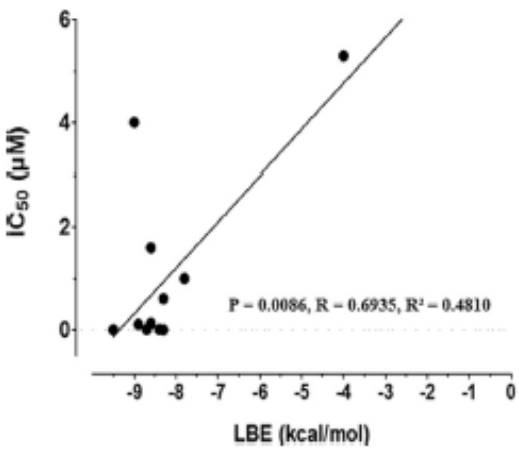
The amino acid residues of the DBD interacting with **2c**, epirubicin, ^{3}H-2EPI and ^{14}C-2EPI in docking and co-docking simulations are shown in Table 3. As mentioned before, **2c** and EPI docked at DBD with Phe343 as the only overlapping residue. Remarkably, co-docking measurements of EPI and its radiolabeled isomers revealed two additional binding sites for EPI characterized by Gln132 and Asp188 as H-bond forming amino acids. This is comparable to the second binding site for EPI which was also observed for the EPI docking to EPI–P-gp complex.

A third binding site for the radio-labeled EPI isomers was evident, if the H-bond forming amino acids Asp241, Ser993 and Phe994 were considered. The H-bond forming amino acids of the docking site of EPI at the **2c**–P-gp complex revealed a hypothetical fourth EPI binding site at the DBD. In fact, these amino acids were Thr1208, Glu1211 and Arg1279, which were different from those found at the other EPI binding sites.

Remarkably, **2c** docked at the NBD with relatively high affinity (LBE value of −6.90 ± 0.30 kcal/mol). As shown in Table 4, R123 also docked at the NBD with an affinity comparable to that of **2c** (−6.92 ± 0.05 kcal/mol). Co-docking of **2c** to the R123–P-gp complex resulted in an LBE value of −7.04 ± 0.30 kcal/mol, which, however, did not significantly differ from the value obtained, if **2c** docked alone (−6.79 ± 0.33 kcal/mol). The cluster of amino acid residues interacting with **2c**, if co-docked at the R123–P-gp complex, was different from that observed, if it docked alone. This indicates a second binding site of **2c** in the NBD, characterized by an affinity comparable to that of the first binding site.

4. Discussion

Murine P-gp shares 87% sequence identity to human P-gp in a drug-binding competent state and its crystallographic structure has been defined, although at medium to low resolution. Its 6000 Å^3 cavity accommodates up to two identical or different low molecular weight substrates/inhibitors at the same time [10,24]. A previously created human P-gp homology model based on murine P-gp as template was used to perform *in silico* molecular docking calculations [20]. This homology model allowed to evaluate the binding energies and docking poses of selected *N,N*-bis(cyclohexanol)amine aryl esters on human P-gp in the present study. We hypothesized that this approach may explain, why these P-gp substrates/inhibitors that interact with P-gp in cellular systems, exhibit some unexpected, paradoxical features, which are not amenable to the classical paradigm of substrate/inhibitor–enzyme interaction.



Compound	Lowest binding energy (kcal/mol)	IC_{50} (μM)
2a	-9.4867	0.0023
1a	-9.0000	4.0000
2b	-8.8567	0.1100
2d	-8.7000	0.0060
3a	-8.6400	0.1200
1c	-8.5800	1.6000
3b	-8.5667	0.1300
2c	-8.3833	0.0075
3c	-8.3733	0.0064
3d	-8.3000	0.0015
1b	-8.3000	0.6100
1d	-7.8167	1.0000
4	-3.9700	5.3000

Fig. 4. Correlation of IC_{50} values of R123 efflux by L5178 MDR1 cells and lowest binding energy to the drug-binding domain of P-glycoprotein of 12 *N,N*-bis(cyclohexanol amine)aryl esters and compound **4**. IC_{50} values are those already published in [11]. The calculation has been performed using Pearson's correlation test.

Table 2
Lowest binding energy (LBE) values of transport substrates (EPI, ^{3}H-2EPI, ^{14}C-2EPI, R123, **2c**) docked to P-gp alone or at complexes of P-gp pre-docked with each of the transport substrates.

Compound	Single docking LBE (kcal/mol)	Drug–P-gp complex	Double docking LBE (kcal/mol)	rLBE	Significance
EPI	−8.97 ± 0.18	**2c_Pgp**	−7.46 ± 0.15	1.51	$p < 0.01$
		R123_Pgp	−8.78 ± 0.08	0.19	–
		EPI_Pgp	−8.82 ± 0.04	0.15	–
^{3}H2-EPI	−9.37 ± 0.19	**EPI_Pgp**	−7.92 ± 0.22	1.45	$p < 0.05$
^{14}C2-EPI	−9.02 ± 0.48	**EPI_Pgp**	−7.95 ± 0.31	1.07	$p < 0.05$
R123	−8.61 ± 0.01	**2c_Pgp**	−6.89 ± 0.07	1.72	$p < 0.01$
		EPI_Pgp	−8.22 ± 0.02	0.39	$p < 0.01$
2c	−8.38 ± 0.17	**EPI_Pgp**	−8.66 ± 0.60	−0.36	$p = 0.22$
		R123_Pgp	−8.78 ± 0.62	−0.48	$p = -0.08$

Each docking experiment has been independently repeated three times with 2,500,000 calculations and 250 runs. Mean values ± SD of binding energies were calculated from three independent dockings.

Table 3
Amino acid residues interacting with **2c**, R123, EPI, ^{3}H-2EPI and ^{14}C-2EPI at the drug-binding domain of human P-glycoprotein.

Compound	Interacting residues	H-bonds
2c	Gly203, Ser222, Pro223, Gly226, Ala229, Phe303, Leu304, Tyr307, Val338, Gly341, Ala342, Phe343, Ala985	–
R123	Ser222, Leu225, Phe303, Leu304, Ile306, Tyr307, Tyr310, Leu339, Ala342, Phe343	–
EPI	Ser222, Leu225, Gly226, Ala229, Ala230, Lys234, Phe303, Ile306, Tyr307, Tyr310, Leu339, Phe343, Val345, Gly346	Lys234, Ile306
^{3}H-2EPI	Gly226, Lys234, Phe303, Leu304, Ile306, Tyr310, Leu339, Ala342, Phe343, Val345, Gly346	Lys234, Leu304
^{14}C-2EPI	Ser222, lys234, Phe303, Ile306, Tyr307, Tyr310, Leu339, Phe343, Val345, Gly346	Ser222, Lys234
Co-dockings		
EPI + **2c**_Pgp	Thr1208, Glu1211, lys1212, Gln1274, Thr1277, Lys1278, Arg1279	Thr1208, Glu1211, Arg1279
EPI + EPI_Pgp	Gln132, Val133, Trp136, Cys137, Asp188, Phe194, Gln195, Ala883, Phe938, Phe942	Gln132, Asp188
^{3}H-2EPI + EPI_Pgp	Thr240, Asp241, lys290, Lys826, Asn296, Ser992, Ser993, Phe994, Ala995, Pro996	Asp241, Asn296, Ser992, Ser993, Phe994
^{14}C-2EPI + EPI_Pgp	Asp241, Ile289, Lys290, Ile293, Asn296, Lys826, Ser993, Phe994, Ala995, Pro996	Asp241, Lys826, Ser993, Phe994
2c + EPI_Pgp	Met69, Phe72, Phe336, Ile340, Phe343, Ile864, Val865, Tyr953, Cys956, Phe957, Phe978, Val981, Val982, Ala985	–

Each docking simulation underwent three or six (**2c**) independent repeats with 2,500,000 calculations and 250 runs for every repeat.

We wanted to assess, firstly, the reliability of our computational protocol for estimating binding affinities to human P-gp of some substrates/inhibitors. The calculated LBE values proved to be significantly correlated to the IC_{50} values found in previous cell experiments [11], thus witnessing for the validity of our computational approach. To assess, whether the method of calculating binding affinities was reliable in general, other authors proceeded in a different manner. They compared the calculated binding affinities to COX-1 of some COX-1 inhibitors with the experimentally found binding free energies (ΔG^{bind}_{exp}) [25]. The latter ones were derived from the IC_{50} values with use of the Cheng–Prusoff equation [26]. The authors also found good concordance of *in silico* and experimental data, which also speaks for the validity of the computational approach.

The present investigation aimed to define the nature and extent of inhibition by *N,N*-bis(cyclohexanol)amine aryl esters of

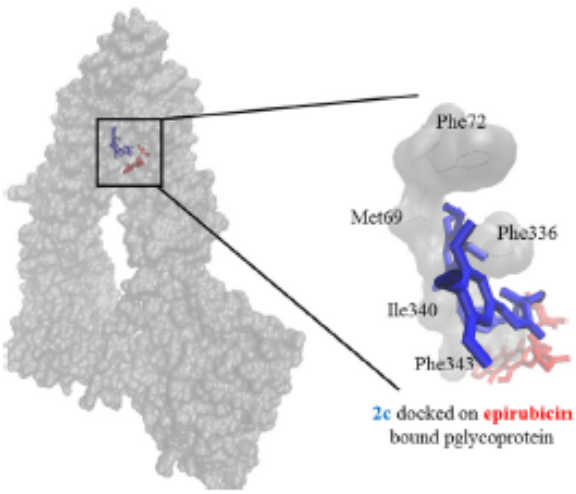


Fig. 5. Docking pose of **2c** (blue bond representation) on epirubicin (red bond representation). Bound human P-gp (gray surface representation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

P-gp-mediated efflux of R123 and EPI in L5178 *MDR1*-transfected cells. The fluorescent compound R123 is a well-known substrate of P-gp [27] and its concentration inside the cell positively correlates to the degree of inhibition of P-gp. Moreover, the inhibition of P-gp-mediated R123 efflux by **2c** as well as the other *N,N*-bis(cyclohexanol)amine aryl esters persisted after wash-out of the inhibitor in an intermediate manner compared to CSA (a known substrate of P-gp) and GF120918 (*i.e.*, elacridar, known to be not a substrate of P-gp). This finding suggests that these esters behave as transport substrates of P-gp [27] and may compete for P-gp with R123, if acting on P-gp at the same recognition site as R123, [28]. If this assumption holds true, a reduced potency of the inhibitors upon increase R123 concentrations should occur, that would cause a rightward shift of the concentration-inhibition curve. The concentration–inhibition curves of P-gp-mediated R123 efflux by **2c** (*cis*/*cis*), however, yielded IC_{50} values in the range of 10^{-9} M to 10^{-19} M, indicating that the inhibition potency was clearly correlated to the R123 concentration. This paradoxical and unexpected mode of action is shared by the other *N,N*-bis(cyclohexanol) amine aryl esters, with the only exception of **3d** [11]. It is likely that R123 sensitizes P-gp to the inhibition by the *N,N*-bis(cyclohexanol)amine aryl esters. The positive interaction of R123 with some *N,N*-bis(cyclohexanol)amine aryl esters suggested the existence of more than a single binding site within the multifaceted drug-binding pocket of P-gp. Accordingly, the site pertaining to R123 – once it is bound to R123 – being capable of switching P-gp binding site of *N,N*-bis(cyclohexanol)amine aryl esters from a low to a high affinity conformation. This is consistent with data from several groups supporting the view that there are at least two, if not more, substrate-binding sites in the drug-binding pocket of P-gp [29–32]. The importance of synergistic effects of different substrates in inhibiting P-gp – including some powerful anthracyclines – previously raised our attention [11]. Differently from the previous study, however, the concentration–inhibition curves of P-gp-mediated EPI efflux by **2c** (*cis*/*cis*) yielded IC_{50} values in the range of 10^{-9}–10^{-10} M in the present study. This clearly shows that **2c** inhibition of P-gp was almost constant irrespective of EPI concentration changes. To clarify this discrepancy, DMD analyses of 12 *N,N*-bis(cyclohexanolamine)aryl esters, the highly flexible aryl ester analog **4**, and several P-gp substrate/non-substrate inhibitors were performed on NBD and DBD of human P-gp.

Molecular docking studies yielded support to functional findings, since the aryl esters proved to bind to the drug-binding pocket with comparable binding energies, similarly to some known substrates/inhibitors of P-gp. In fact, the *N,N*-bis(cyclohexanol) amine aryl esters bound to P-gp with comparable binding energies as R123, EPI, elacridar, CSA and valspodar. Additionally, **2c** and R123 proved to strongly dock at the ATP-binding site. This implies that **2c** may inhibit P-gp function in two ways: (1) inhibition of ATP binding at NBD, thus stopping energy supply of the pump, and (2) inhibition of drug efflux by competitive binding at the drug-binding pocket. Remarkably, **2c**, and **3d** as well, were the most powerful among the compounds tested to inhibit EPI-stimulated ATPase activity of membranes prepared from recombinant, baculovirus-infected, human P-gp enriched insect cells (*Sf9*) [11].

In an endeavor to simulate molecular docking in a more realistic way to the functional experiments in cells, we introduced a co-docking approach for the first time by docking simulations at P-gp nucleoside- and drug-binding domains of substrates/inhibitors. This computational co-docking approach consists of two distinct phases of the interaction of a substrate/inhibitor with P-gp. The first phase is the calculation of docking, if only one compound interacts with P-gp. The second phase takes place only after P-gp interacted with another compound that is supposed to interfere with the first one.

The premise upon which the interpretation of findings on co-docking experiment was based, consists of two intuitive elements, logically interconnected and complementary to each other. Firstly, if DMD simulations of compound **A** are performed at P-gp binding domains (NBD or DBD), after the same or another compound (both called **X**) had already docked at P-gp, the outcome depends on two options offered to compound **A**. The first option is that compound **A** cannot bind to the binding site, since this binding site has already been occupied by compound **X**. In this case, the LBE value of compound **A** should be less negative than the LBE value when it is docked alone. The second option takes place, if the LBE value found after the application of compound **A** to complex P-gp + compound

Table 4
Interacting amino acid residues for docking and co-docking of **2c** and R123 to human P-glycoprotein at the nucleotide binding domain.

Compound	Interacting residues	H-bonds	LBE (kcal/mol)
2c	Leu1031, Lys1061, Gln1193, His1195, Asp1219, Lys1220, Arg1222, Glu1223, Gly1224, Arg1225	lys1061, Arg1225	−6.79 ± 0.33
R123	Leu1234, Ser1235, Thr1236, Gln1238, Asn1239,His1257, Val1271, Gln1274, Thr1277, Lys1278, Arg1279	Asn1239, Gln1274	−6.92 ± 0.05
Co-docking			
2c + R123_Pgp	Lys1061, Gly1063, Gln1064, Thr1065, Leu1122,Ile1154, Phe1157, Ser1166, Thr1167, Lys1168, Val1169 Asn1239, Ala1240, Asp1241, Arg1279	–	−7.04 ± 0.30

Each docking simulation underwent three or six (**2c**) independent repeats with 2,500,000 calculations and 250 runs for every repeat. Figures represent the mean values ± SD of binding energy from three to six independent docking calculations.

X is different (increased or decreased) from the LBE value found after the application of compound **A** alone. This indicates that compound **A** docked at another drug-binding site characterized by a different LBE value. This represents the second intuitive element. In this case, the cluster of amino acid residues interacting with compound **A** should be different from that interacting with compound **A**, if it docked at P-gp alone. The novel LBE value indicative of a secondary binding site of compound **A**, should be smaller than or comparable to one of the primary binding sites of compound **A**, obtained if the same compound is applied alone. In the present study, we tried out many combinations, where compound **A** was interacting with the complex of P-gp plus compound **X**. In fact, we can conclude that the first option never occurred, *i.e.*, the LBE value never moved toward positive values. Perhaps, this happened because compound **A** could always find another binding site in proximity to the site occupied by compound **X**, at which it docked. This hypothesis is supported by the observation that the clusters of amino acid residues interacting with compound **A** were different in either cases, whether compound **A** docked at P-gp alone or at the P-gp plus compound **X** complex.

Within this logical frame, we can match LBE values with the amino acid residues forming H-bonds and/or with the clusters of amino acid residues interacting with low molecular weight compounds in the various co-docking combinations. EPI docked on four different binding sites, R123 docked on three different binding sites and **2c** turned out to dock at three different binding sites of DBD of human P-gp, respectively. This supports the theory proposed by several authors [10,11,24,29–32] that the cavity of the drug-binding pocket of P-gp accommodates at once more than one molecule of low molecular weight substrate/inhibitor at the same time. Thus, it is legitimate to hypothesize that the pump effluxes more than one molecule of substrate at each stroke. Though suggestive, this hypothesis is negated by the stoichiometry of pump function, *i.e.*, the ratio of the number of ATP molecules consumed to the number of low molecular weight pump substrate molecules effluxed is higher than 1 [33]. This is in accordance to findings that the hydrolysis of at least two molecules of ATP is required for the transport of every substrate molecule [34–36]. The hydrolysis of one ATP molecule may be required to move the drug from the high-affinity "on" site to the low-affinity "off" site. The second ATP may then serve either to move the drug from the "off" site to the extracellular medium or to reset the conformation of P-gp to initiate the next cycle [37]. More recently, experimental evidence consistent with the stoichiometry measurements supported the role for ATP hydrolysis at two distinct steps during the catalytic cycle [38]. The suggestion arose that the additional ATP hydrolysis required to reset the conformation during a single turnover is common among ATP-binding cassette transporters.

The same phenomenon, *i.e.*, the accommodation of more than one molecule of low molecular weight substrate/inhibitor at the same time happened also at the NBD of P-gp. Therein, in fact, R123 and **2c** docked at two different sites, as shown by the clusters of amino acid residues interacting with them in the course of single docking simulations. Similarly, **2c** docked at another secondary binding site of the NBD in co-docking simulation, if R123 had already engaged its proper binding site.

Now, we shall try to find out how small changes in LBE have significant effects on K_{eq}. Inspecting the data on docking of three transport substrates to P-gp alone or to the complexes of P-gp pre-docked with the same transport substrates (Table 2), let us consider the case of R123 for simplicity. The LBE value of R123, if it docked alone was quite higher than that found, if R123 docked to P-gp already engaged by **2c**, ΔΔLBE amounting to 1.72. According to the Gibbs equation:

$$\Delta G = -RT \ln K_{eq}$$

K_{eq} (predicted equilibrium constant) for R123 resulted 2.15×10^6, if it docked alone to P-gp and 1.8×10^5 when it docked in the presence of **2c**. This means that in the presence of 10^{-6} M R123 concentration, the $Target_{free}/Target_{total}$ ratio for R123 were 0.31 and 0.89, respectively. In the first case, the amount of the R123-target complex corresponded to 69%, and 11% in the second case. Conversely, negative ΔΔLBE values indicating LBE increases and thus greater [R123-Target] fractions provide – although unexpectedly – the key to understand the mechanism of the paradoxical interaction of R123 and **2c**, observed in cell experiments. However, the small negative ΔΔLBE value reported in Table 2 was not statistically significant. This may encourage the performance of this kind of DMD on flexible docking systems.

An explanation of cell sensitization by R123 to **2c**-mediated P-gp inhibition may be found in R123 inhibition of function of isolated rat liver mitochondria [39]. Here, in fact, R123 concentrations above 1 μM inhibit ADP-stimulated (State 3) respiration of mitochondria (K_i = 12 μM) and ATPase activity of inverted inner membrane vesicles and partially purified F_1-ATPase (K_i = 126 μM and 177 μM, respectively). The smaller K_i for coupled mitochondria is accounted for by energy-dependent R123 uptake into the matrix. Above ~20 μM, R123 causes also rapid swelling of energized mitochondria. If the same happens in mitochondria of L 5178 MDR1 cells, the synergy of **2c** and R123 may represent a further example of the "collateral sensitivity" phenomenon of cancer cells [40].

Considering that hundreds of P-gp inhibitors have been published in the past four decades, the question may arise why further novel P-gp inhibitors are needed. In fact, although effective MDR modulators exist, they are still not approved for clinical use. This may at least in part be due to the fact that many P-gp inhibitors have not been specifically developed for this purpose. In general, they rather represent drugs for the treatment of other diseases and by chance they were found also to inhibit P-gp. The best example is the well-known P-gp substrate and inhibitor, verapamil, a calcium channel blocker employed for the treatment of cardiovascular diseases. Although verapamil's P-gp-binding properties are well established, its binding energy to P-gp is less (−4.34 kcal/mol; [41]) than that of **2c** reported in the present investigation (−8.38 kcal/mol). Another difference between these two agents is that **2c** binds to both DBD and NBD, whereas verapamil binds only to DBD [41]. This example illustrates that DBD's and NBD's chemical spaces are not yet fully explored and that improved P-gp inhibitors can still be found. Another considerable difference between *N,N*-bis(cyclohexanol)amine aryl esters and other P-gp inhibitors is that this class of compounds has been specifically developed on the basis of the existing knowledge of substrate recognition sites of P-gp, MRP1 and sister proteins [12].

Although not yet tested in detail, we speculated that compounds from this class might also be active against other ABC transporters [14]. This may turn out as an advantage, if it comes to the MDR modulation in the clinical setting, since tumors in patients may express several other ABC transporters in addition to P-gp. Hence, modulators inhibiting several efflux transporters at the same time could be expected to overcome drug resistance more efficiently than agents affecting only one drug transporter.

Another issue worth of consideration is that clinical trials with P-gp inhibitors largely failed so far [42–44]. Instead of giving up and abandoning this investigational field, the search for better P-gp inhibitors and better study designs should go on with even accelerated efforts. Numerous animal experiments indicate that P-gp inhibition and MDR modulation take place not only in cell cultures, but also in living organisms [45]. Despite the fact that several clinical trials with P-gp inhibitors have failed, it is sometimes overseen that an improvement of progression-free survival has been observed in a subset of breast cancer patients treated with standard chemotherapy (doxorubicin and cyclophosphamide)

plus dofequidar as P-gp inhibitor [46]. This indicates that P-gp inhibition and MDR modulation can be achieved *in vivo* and in patients. Hence, there is reason to believe that clinical modulation of MDR is possible if better study protocols will be applied. This may justify the quest for novel MDR modifying substances with improved features. For this purpose, molecular docking techniques such as that presented here are fundamental for clarifying the interactions between P-gp and inhibitors.

In conclusion, co-docking experiments showed that **2c** still bound to the drug-binding pocket, even if EPI or R123 allocated this site. Some *N,N*-bis(cyclohexanol)amine aryl esters could well have two or more distinct binding sites in the drug-binding pocket of P-gp. However, EPI and R123 yielded significantly weaker interaction, if **2c** allocated the site. This implies that **2c** may allocate the drug-binding pocket and prevent strong binding of EPI and R123. This is in concordance with the P-gp inhibition experiments, where **2c** inhibited P-gp and thus reduced efflux of EPI and R123.

Conflict of interest

The authors declare that there is no conflict of interest.

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