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UNDERSTANDING POLYSPECIFICITY WITHIN THE SUBSTRATE-BINDING CAVITY OF THE HUMAN MULTIDRUG RESISTANCE P-GLYCOPROTEIN

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Abstract

Human P-glycoprotein (P-gp) controls drugs bioavailability by pumping out of the cells many structurally-unrelated drugs. The x-ray structure of the mouse P-gp ortholog was solved with two SSS- and one RRR-enantiomers of the selenohexapeptide inhibitor QZ59, found within the putative drug-binding pocket of the membrane domain outer leaflet. This offered the first opportunity to localize the well-known H- and R- drug-substrate sites in light of QZ59 inhibition mechanisms that were characterized here *in cellulo* and modelled towards Hoechst 33342 and daunorubicin transport. We found that QZ59-SSS competes efficiently with both substrates, displaying $K_{I,app}$ values of 0.15 and 0.3 μ M, respectively 13 and 2 times lower than corresponding $K_{m,app}$. In contrast, QZ59-RRR non-competitively inhibited daunorubicin transport with moderate efficacy ($K_{I,app} = 1.9 \mu$ M) and displayed a mixed-type inhibition towards Hoechst 33342 transport, resulting from a mainly non-competitive ($K_{i2,app} = 1.6 \mu$ M) and a poor but significant competitive tendency ($K_{i1,app} = 5 \mu$ M). These results suppose a positional overlap of QZ59 – drug-transport sites, total for the SSS enantiomer and partial for the RRR one. Crystal structures analysis suggests that the H site overlaps both QZ59-SSS locations while the R-site overlaps the most embedded one.

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Keywords

P-glycoprotein; multidrug efflux ABC transporters; drug efflux mechanism; drug-binding sites; cancer; drug resistance

Introduction

The human P-glycoprotein (P-gp) is an integral membrane protein that actively pumps endo/exogenous compounds out of cells using the energy derived from ATP binding and hydrolysis [1]. P-gp is up-regulated in many cancer cells, where it reduces the intra-cellular concentrations of many chemotherapeutic drugs, thereby imparting multidrug resistance (MDR) [2]. P-gp is one of the best-known pumps in this context, in addition to Multidrug Resistance Protein 1 (MRP1/ABCC1) and Breast Cancer Resistance Protein (BCRP/ABCG2); all these proteins belong to the same ATP-binding cassette (ABC) transporter superfamily [3]. The implication of such pumps in chemoresistance necessitates the elucidation of the mechanisms of multidrug export and its selective inhibition. P-gp transports a broad spectrum of molecules sharing a marked hydrophobicity but structurally divergent [4, 5]. This polyspecificity is partly explained by the existence of at least three predicted drug-binding sites, termed H-, R-, and P-site. The H-site binds Hoechst 33342 and quercetin, the R-site preferentially binds rhodamine 123 and anthracyclins such as daunorubicin and doxorubicin, while the P-site binds the likes of prazosin and progesterone [6, 7]. A fourth site has also been suggested, which may bind non-transported modulators like GF120918 or nicardipine [8].

Among the different drug-binding sites of P-gp, the H- and R- sites have been more rigorously investigated. The use of thiol-reactive substrates in competition with drugs coupled to cysteine scanning mutagenesis has contributed to identify several amino acid residues from transmembrane domain close, or participating to the drug binding sites (reviewed in [9]). Notably, I340(TM6), A841(TM9), L975-V981-V982(TM12) were mapped in the R-site [10]. Location of the H-site is more controversial since fluorescence resonance energy transfer investigations using Hoechst 33342 suggested a location in the cytoplasmic/inner leaflet side of the transporter [11] while the use of a pharmacophore pattern for the same dye suggested a location within the outer leaflet [12].

The recent mouse P-gp (Abcb1a) x-ray structures revealed a large, internal drug-binding cavity enclosed between the two ‘halves’ of the ‘full’ transporter [13, 14]. For the first time the pump was co-crystallized with the RRR and SSS enantiomers of the cyclic selenohexapeptide, QZ59 [13]. One molecule of QZ59-RRR, and two of QZ59-SSS were identified in their respective co-crystal structures. As not uncommon for membrane proteins, these 3D-structures were obtained at a rather modest resolution of 3.8 – 4.35 Å but, thanks to the 3 Selenium atoms they included, QZ59s could be unambiguously located, within the outer leaflet interface of the drug-binding cavity, QZ59s were found to inhibit the mouse P-gp-mediated efflux of drugs such as colchicine and calcein, and the verapamil-stimulation component of mouse P-gp’s ATP hydrolysis activity [13]. Until now, no other compound, as either inhibitor or substrate drug, has ever been co-crystallized, making QZ59 enantiomers

unique tools for localizing more precisely the drug-binding sites, at least inside the co-crystallized pump conformation. A detailed characterization of their inhibition mechanisms, competitive, non-competitive, mixed..., would let us know if they share, none, one or even several drug transport sites, which ones, and to what extent. Such information is crucial to understand the transport mechanism mediated by ABC pumps; it is also critical to guide the design of compounds acting at the molecular level with the highest efficiency.

We addressed this question in the present study. Our results revealed distinct inhibitory mechanisms of the QZ59 enantiomers toward drug-substrates binding to the H and R sites. As expected, such mechanisms allowed us to localize these substrate-binding sites within the transporter's binding cavity.

Results

In order to test the effects of QZ59 inhibitors on the transport by P-gp of substrates binding to the H- and R-sites, as detailed in the experimental section, we used whole cells in order to provide a native environment for the pump [15] in terms of membrane lipid composition, substrate partitioning and bioavailability, protein trafficking, and membrane potential. Concentrations and kinetic parameters estimated under these conditions remain only apparent but, most importantly for the present study, fully comparable.

We measured the transport of daunorubicin and Hoechst 33342 mediated by P-gp (Fig. 1), from which we estimated with equation 2.1 (Table 1) maximal rates, V_m , of 41 ± 3 and 34 ± 3 pmol.s⁻¹.mg⁻¹ (of cell protein content) and Michaelis constants, K_m of 0.7 ± 0.2 and 2 ± 0.4 μM for daunorubicin and Hoechst 33342, respectively. These values are in the same range that those previously reported for daunorubicin [15] and Hoechst 33342 [16] showing that the system set up here allows to characterize the QZ59 inhibition towards these substrates. For the further experiments, data sets were subjected to a detailed enzymatic analysis (fully displayed in Table S1) using non-linear regressions, and modeled using the equations displayed in Table 1. We then evaluated the models by statistical tools such as Goodness-Of-Fit (GOF) and corrected Akaike Information Criterion (AIC_c). As classically carried out, the choice of the best model was guided either by looking for the largest drop in AIC_c or, equally, the largest AIC_c when using the basal Michaelis-Menten equation as reference (eq. 2.1).

Mechanism of drug efflux inhibition by QZ59-RRR

We first measured the QZ59-RRR-affected inhibition of Hoechst 33342 (used to probe the H-site) and daunorubicin (used to probe the R-site) efflux by human P-gp. Transport data in the absence or presence of increasing concentrations of the drug-substrate and QZ59-RRR were collected using the cell line NIH3T3-P-gp over-expressing human P-gp, compared to non-expressing NIH3T3 cells as control (Fig. 2A–F).

The mixed inhibition model (eq. 6.1) best fitted the data set for the inhibition of Hoechst 33342 export (Fig. 2A–C), leading to the largest AIC_c (174.8, Fig. 2B). A detailed analysis of model fitting and scoring is displayed in Figure S1A, legend and comments. This mixed inhibition has a strong non-competitive contribution as suggested by the inhibition constants

$K_{i1,app}$ (for P-gp alone) of $5 \pm 2 \mu\text{M}$ and $K_{i2,app}$ (for the P-gp-Hoechst 33342 complex) of $1.6 \pm 0.2 \mu\text{M}$ (panel C; Table S1). Indeed, the non-competitive inhibition model (eq. 4.1) confirmed this tendency, giving a comparably close $AICc$ (171.7, panel B). As drawn in panel C, this non-competitive mixed inhibition suggests that at the $K_{i2,app}$ concentration ($1.6 \mu\text{M}$) QZ59-RRR binds to an inhibitory site, distinct to the H- site, while at high concentrations, i.e. in the range of $K_{i1,app}$ ($> 5 \mu\text{M}$), QZ59-RRR tends to share the H- site.

The inhibition of P-gp-mediated daunorubicin efflux by QZ59-RRR is displayed in Fig. 2D-F and detailed in Fig. S1B. The initial kinetic analysis suggested a non-competitive model (Table S1). Additionally, we observed an inhibition mediated by daunorubicin itself upon its own efflux, which is clearly visible at high daunorubicin and QZ59-RRR concentrations in Fig. 2D. Regression analyses carried out on these data with equations 4.6 and 4.7 including these effects gave the same $AICc$ scores (191.1) for the competitive (eq. 4.6) and non-competitive (eq. 4.7) models (Fig. 2E). These models however did not correctly fit the data obtained at high daunorubicin and QZ59-RRR concentrations (Fig. 2E, S1B). Such data suggested that QZ59-RRR may enhance the daunorubicin-mediated self-inhibition. Indeed, introducing this effect into equations 4.8 and 4.9 led to the largest increase of $AICc$ values, 225.2 and 227.4 for models including competitive and non-competitive inhibition by daunorubicin (panel E). Although these models are fairly close in their fits (Fig. S1B), the latter model (eq. 4.9) with the significantly higher $AICc$ is more likely to be true. Thus, the QZ59-RRR-mediated inhibition of daunorubicin transport is non-competitive ($K_{I,app} = 1.9 \pm 0.4 \mu\text{M}$), added of a daunorubicin-mediated self-inhibition enhanced by QZ59-RRR ($K_{SI,app} = 7 \pm 3 \mu\text{M}$).

Taken together, our data suggest that QZ59-RRR inhibits drug efflux mainly through an inhibitory site, distinct to the R- and H- transport sites. At high concentrations however, QZ59-RRR partially shares or overlaps with the H-site, and also enhances the self-inhibitory action of R-site drug-substrates.

Mechanism of drug efflux inhibition by QZ59-SSS

The mouse P-gp-QZ59 co-crystal structures reveal distinct binding sites for the RRR and SSS enantiomers of QZ59, the latter being present in 2 different locations. We tested whether this distinction has a bearing on the mechanism of drug efflux inhibition.

The inhibition P-gp-mediated Hoechst 33342 efflux by QZ59-SSS is presented in Fig. 3A–C.

The data at different concentrations of QZ59-SSS revealed a sigmoidal distribution, suggesting a cooperative behavior of the pump triggered by QZ59-SSS binding. Moreover, data at higher inhibitor concentrations revealed an activation effect by QZ59-SSS on the efflux of Hoechst 33342. We carried out the simulations accordingly, exploring the effects of cooperativity, inhibition and activation independently or together using equations 2.1 to 3.5. A detailed analysis is displayed in Figure S2A, legend and comments. Among these models, competitive inhibition models best fitted the data set (eq. 3.1–3.5, panel B). Furthermore, the models that included activation and cooperativity together (eq. 3.4, 3.5) led to the largest $AICc$ (95.4 and 105.7), in contrast to the models that included them separately (eq. 3.2,

3.3: 47.9 and 79.9). The best model (eq. 3.5, GOF displayed in Fig. S2A) suggests that QZ59-SSS competes with Hoechst 33342 transport (panel C), doing this with a very good efficiency as evidenced by the low value of the inhibition constant, $K_{I,app}$ of 0.15 ± 0.04 μM , which is 13 times lower than the $K_{m,app}$ (2 μM). At high QZ59-SSS and Hoechst 33342 concentrations, QZ59-SSS tends to activate the Hoechst 33342 efflux, in a cooperative manner. The estimated activation constant $K_{A,app}$ of 4.5 μM suggests that this effect is mediated either through binding to additional low-affinity sites and/or with a poor efficacy.

The effects of QZ59-SSS on P-gp-mediated daunorubicin efflux are displayed in Fig. 3D–F. A detailed analysis is displayed in Figure S2B, legend and comments. The distribution of the values suggested an inhibition by daunorubicin at high substrate and QZ59-SSS concentrations as observed with QZ59-RRR, although to a lower extent. Competitive and mixed inhibition models (eq. 3.9 and 6.9, panel E) gave the largest $AICc$, 199 and 199.5, respectively. The mixed inhibition model had a marginally better fit, as displayed in panel D and confirmed by the corresponding GOFs (Fig. S2B). As drawn in panel F, the mixed inhibition model suggests that QZ59-SSS competes with daunorubicin at the R transport site. This effect occurs with an inhibition constant $K_{i1,app}$ of 0.35 ± 0.08 μM , which is ~ 2 times lower than the K_m of daunorubicin transport. According to this model, QZ59-SSS also binds to the P-gp-daunorubicin complex on an inhibitory site, with an inhibition constant $K_{i2,app}$ of 1.3 ± 0.9 μM . $K_{i2,app}$ is ~ 4 times higher than $K_{i1,app}$ indicating a marked competitive inhibition tendency. This is confirmed by the close $AICc$ obtained with the competitive model (eq. 3.9), leading to a $K_{I,app}$ of 0.28 ± 0.05 μM . At high concentrations, daunorubicin reaches its self-inhibitory site similar to previously described for QZ59-RRR on daunorubicin efflux. This effect is limited since the inhibition constant $K_{SI,app}$ is estimated to 16 μM (panel F).

Taken together, these data suggest that QZ59-SSS competes with drug substrates, which are historically known to bind the H- and R- sites, with a positional substrate overlap much more marked for the H-site than for the R-site. Additionally, at higher concentrations this inhibitor tends to activate the efflux of drugs that bind the H-site.

Discussion

Owing to its significant role in imparting multidrug resistance properties to cancer cells, P-gp is a validated therapeutic target. The inhibition of its drug export function is critical in order to improve the efficacy of many clinically important chemotherapeutic drugs. Thus, understanding the inhibitory mechanisms pertinent to this pump helps for developing more potent and selective inhibitors. The system set up here allowed to estimate the apparent transport and inhibition constants; we found them from 0.1 to 25 μM , values which correlate well with K_D recently estimated for compounds interacting with P-gp [17, 18].

QZ59-RRR and -SSS enantiomers are two inhibitors that were co-crystallized with mouse P-gp [13], each in one of the 6 inward-facing conformations resolved to date [19]. The mouse P-gp is 87% identical in protein sequence to human P-gp making that the 3D structural information obtained with the mouse P-gp can be reasonably correlated to the inhibitory mechanism of QZ59 compounds towards the human P-gp. We found that in contrast to RRR,

the SSS enantiomer competes with drugs binding to canonical H- and R-sites. This result is intriguing because QZ59-RRR and -SSS are distributed in a common groove in the outer leaflet of the membrane domain (Fig. 4A) while R- and H-drugs-binding sites are distinct, as previously reported [7].

This contradiction is only apparent. Indeed, the $K_{I,app}$ for QZ59-SSS competitive inhibition toward Hoechst 33342 and daunorubicin, 0.15 and 0.3 μM respectively, let us suppose an overlap between drugs and QZ59-SSS, as the corresponding $K_{m,app}$ are 13 and 2 times higher. In addition, QZ59-RRR displays a moderate competitive tendency toward Hoechst 33342 while it acts strictly non-competitively toward daunorubicin. This suggests that the H site broadly overlaps the QZ59-binding groove, contrarily to the R site which shares it partially. A docking simulation carried out with Hoechst 33342 in the mouse QZ59-SSS-P-gp x-ray structure indeed gave the 3 best locations along the groove (Fig. S3A). Notably, as defined the H site is bordered by residues F299, Y303 and Y306 (Fig. 4A) that have been proposed to be part of it [12]. Another docking simulation done with daunorubicin suggested that the 4 best locations overlap the most embedded QZ59-SSS (Fig. S3B). We got exactly the same result by docking the thiol-reactive analog of rhodamine, methanethiosulfonate (MTS)-rhodamine (Fig. S3C) used previously by Loo and Clarke for mapping cysteine-mutagenized residues protected from its modification by rhodamine B [10]. Interestingly, docking showed that while the rhodamine moiety binds to the same R-site, the MTS moiety occupies a much larger space indeed surrounded by the cysteine-mutagenized residues protected by rhodamine B. As positioned, the R site overlaps the most embedded QZ59-SSS location and almost not that of QZ59-RRR (Fig. 4A), an observation fully consistent with the QZ59 enantiomers inhibition patterns. When looking at residues forming each H- and R-pocket (Fig. 4B–C), about 2/3 are distinct and 1/3 common, coherent with the competition between each drug above 2 μM early reported [7]. Note that Pajeva *et al.* in a recent study in which they tested elacridar and tariquidar-based inhibitors of P-gp indirectly reached to the same conclusions [20].

The location overlap between Hoechst 33342 and QZ59-RRR suggested in Fig. 4A reflects rather well the mixed non-competitive nature of the inhibition pattern resulting from a poor competitive component ($K_{i1,app}$ of 5 μM) and a non-competitive one ($K_{i2,app}$ of 1.6 μM), close to the $K_{m,app}$ (2 μM). Such a modest range of inhibition constants suggests that QZ59-RRR may shift in the presence of Hoechst 33342, similarly to the situation observed with proflavin and ethidium bound to the multidrug-binding transcription repressor QacR [21]. Putting together inward-facing conformations resolved to date [13, 14] (video S1) shows that the R and H drug-binding site locations that we propose here (Fig. 4) only exist at a given time, a situation which seems reasonable in a context of drug translocation. Experimentally determined structures of P-gp with bound drug substrates and inhibitors, along with computational simulations, will help in getting a better understanding of this fascinating membrane protein pump.

Materials and Methods

Reagents

Daunorubicin and Hoechst 33342 were from Sigma Aldrich. QZ59-RRR and QZ59-SSS cyclic hexapeptides were synthesized according to Tao *et al.* [22]. Compounds were dissolved in 100 % DMSO at 20 mM stock concentration and stored at – 20° C.

Cell Culture

The NIH3T3 parental cell line and NIH3T3-P-gp drug-resistant cell line transfected with human MDR1/A-G185 [23], were from ATCC and used as previously described [24]. Cells were grown in a cell growth medium (CGM) containing Dulbecco's modified Eagle's medium (PAA laboratories), 10% fetal bovine serum (PAA laboratories) and 1% penicillin/streptomycin (PAA Laboratories). The NIH3T3-P-gp growth medium was also supplemented with 60 ng/mL colchicine. Cells were incubated at 37° C in humidified 5% CO₂.

Drug transport set up

We used settings of drug transport described by Spoelstra *et al.* [15], who fully characterized the cell P-gp-mediated daunorubicin efflux. Steady state conditions were used as described in this study, close to those described by Litman *et al.* [25], by incubating control cells and P-gp-expressing cells with up to 6 µM daunorubicin (instead of the 5 µM described in the reference study) for one hour in the culture medium. The same settings were used for Hoechst 33342: 5.10⁴ NIH3T3 or NIH3T3-P-gp cells were incubated as above for 24 h in CGM and then 1 h in CGM with or without 0–6 µM daunorubicin or Hoechst 33342 and with or without 0–4 µM QZ59s. After incubation, cells were washed with Phosphate Buffered Saline (PBS, PAA laboratories), trypsinised, and stored on ice for approximately 30 min before quantification of their intracellular drug amount by flow cytometry. We checked by flow cytometry that virtually no cell lysis occurred during the overall treatment since cell fragments and dead cells corresponded to less than 0.1%. Flow cytometry was carried out with a FACS Calibur cytometer or FACS LSR II from BD Biosciences. Daunorubicin and Hoechst 33342 were excited with 488 and 355 nm lasers; the corresponding emissions were recorded with a 530/30 band pass filter and 450/50 band pass filter, respectively. All experiments were standardized with mid-range FL1 fluorescence beads (BD Biosciences). Data was collected with CellQuest Pro 4.0 or FACSDiva 6.1.2 softwares and then exported to FlowJo (TreeStar) for analysis. To facilitate the kinetic analysis, arbitrary fluorescence values were converted to apparent intracellular drug concentrations (nmoles drug•mg⁻¹) by quantifying the intracellular drug amounts spectrophotometrically (see details in Fig. S4). This led to reasonable estimations, close to those reported earlier [15]. Under these conditions, drug range concentrations were then optimized, showing that a maximal accumulation was reached close to 6 µM drug, with or without QZ59 inhibitors (see Fig. S5) [15]. Although these drug/dye partly are well known to accumulate into the nucleus, this effect had a limited impact since we only considered the difference in accumulation between control NIH3T3 and NIH3T3-P-gp cells incubated in comparable conditions [25]. Intracellular drug accumulation was fitted using equations 1.1 or 1.2,

$$y=a * (1-e^{-bx}) \quad (1.1)$$

$$y=a/(1+e^{-(x-x_0)/b}) \quad (1.2)$$

Regression analyses

Equations 2.1 to 7.1 used or built to fit P-gp-mediated drug efflux are detailed in Table 1. Regressions were performed using *nls* function in the statistical software R (2.14.1 version). Scripts are available upon request. Model validation and selection was achieved firstly by evaluating each model by Goodness-Of-Fit (*GOF*) diagnostics (*Lowess* function in R) comparing predictions to observations, and secondly by calculating the Akaike's Information Criterion (*AIC*) score (equation 8, *AIC* function in R)),

$$AIC=-2 * \log \text{likelihood}+k * npar \quad (8)$$

In which, *npar* represents the number of parameters in the fitted model and *k* the penalty per parameter to be used (default *k* = 2). The log likelihood term is a measure of the fit between predicted and observed values (the lower the deviance value, the better the model); the *k*npar* term is a penalty for over-fitting by increasing the number of parameters [26]. Since in this study the data points/parameters ratio was within 14–40, we used the corrected *AIC* score, *AICc* (equation 9), which gives a more accurate score by taking into account the number of points and parameters:

$$AICc=AIC+2K * (K+1)/(N-K-1) \quad (9)$$

Where N is the number of data points and K is the number of parameters +1. The simplest model with the smallest loss of information due to the prediction gives the smallest *AICc* score [27]. Models were compared by calculating the difference of *AICc* between a reference model and the tested one (m), using as reference the Michaelis and Menten equation 2.1, without inhibition (Table 1). The choice of the best model was guided by the largest drop in *AICc*.

$$\Delta AICc_{(ref-m)}=AICc_{ref}-AICc_m \quad (10)$$

Akaike's weights were calculated for models with close *AICc*, typically giving a difference of ~3 and lower using equation 11,

$$\text{probability} = 100 * e^{-0.5\Delta AICc(m-\text{ref})} / (1 + e^{-0.5\Delta AICc(m-\text{ref})}) \quad (11)$$

A $AICc(m-\text{ref})$ of 0 (reference compared to itself) will give a probability of 0.5, thus 100% of chance to be correct; a $AICc(m-\text{ref})$ of 2, which corresponds to the default value of k in eq. 8, will give a probability of 0.27, thus 73% of chance that the reference model is correct and $73/(100-73)=2.7$ times more probable than the model m ; in the same way, a

$AICc(m-\text{ref})$ of 6 will give a probability of 0.05, thus $95/(100-95)=20$ times more probable than the model m , etc... [28].

Docking

Simulations were carried out with autodock vina 1.1.2 [29], preparing with autodocktools 4 [30] the Apo-mouse P-gp from the x-ray structure in which two QZ59-SSS molecules were resolved (PDB code 3G61) and ligands (QZR59-SSS, QZ59-RRR, Hoechst 33342, daunorubicin, MTS rhodamine). The docking box was designed to cover all the membrane region and using the following parameters: points in x-, y- and z-dimensions (50, 66, 60), spacing of 1 Å and a center grid box x, y and z of 20, 50 and 0. Exhaustivity was set up to the default value (8, testing 15 and 50 gave same results). These settings were tested by docking QZ59-SSS and QZ59-RRR on their corresponding P-gp 3D-structures, 3G61 and 3G60 (Fig. S6A–B). Scripts and 3D data are available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	ATP-binding cassette
P-gp	Pleiotropic glycoprotein
TM	Transmembrane
CGM	cell growth medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum + 1% penicillin/streptomycin)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffer Saline
AIC	AKAIKE Index Criterion

AICc	corrected AIC
GOF	Goodness-Of-Fit
MTS-rhodamine	methanethiosulfonate-rhodamine

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Figure 1. Intracellular accumulations and P-gp-mediated transports of daunorubicin and Hoechst 33342 in control and P-gp expressing NIH3T3 cells

Daunorubicin (A) and Hoechst 33342 (B) were added to NIH3T3 (triangles) and NIH3T3-P-gp (circles) cells and quantified as described in Methods. The net drug or dye accumulations corresponding to the difference in accumulation between both cell types are shown by squares. Data in triplicates were fitted as detailed in Methods with equations 1.1 (triangles and circles) and 2.1 (squares).

Figure 2. QZ59-RRR effect on P-gp-mediated drugs transport

A, D. Plots of Hoechst 33342 (A) and daunorubicin (D) transport rates as a function of drug and QZ59-RRR concentrations. The experiments were done in triplicate generating 84 (A) and 90 (D) measures respectively. Traces correspond to the best fit obtained with eq. 6.1 (A) or eq. 4.9 (D), Table 1. **B, E.** $AICc$ scores of the tested models in respect of the reference model (eq. 2.1). **C, F.** Reaction schemes of substrates transport and QZ59-RRR effects with the estimated constants. H33342 = Hoechst 33342; Dauno = daunorubicin; R, H = R- or H-transport sites; I= inhibition site. K_{i1} , K_{i2} , K_I , K_{SI} and K_m correspond to the inhibition and Michaelis constants of the reactions, as detailed in the text.

Figure 3. QZ59-SSS effects on P-gp-mediated drugs transport

A, D. Plots of Hoechst 33342 (A) and daunorubicin (D) transport rates as a function of drug and QZ59-SSS concentrations. The experiments were done in triplicate generating 72 data in both cases. Traces correspond to the best fits obtained with eq. 3.5 (A) or eq. 6.9 (D), Table 1. For clarity, traces in panel a have been drawn in solid lines for [QZ59-SSS] = 0 and 2 μM , and in dashed and dotted lines for [QZ59-SSS] = 0.5 and 1 μM . **B, E.** $AICc$ scores of the tested models in respect of the reference one (eq. 2.1). **C, F.** Reaction schemes of substrates transport and QZ59-SSS effects with the estimated constants. H33342 = Hoechst 33342; Dauno = daunorubicin; QZ59S = QZ59-SSS; R, H = R- or H-transport sites; I= inhibition sites; A= activation site. K_{i1} , K_{i2} , K_i , K_{Si} , K_A and K_m correspond to the inhibition, activation and Michaelis constants of the reactions, as detailed in the text.

Figure 4. Putative H and R drug-binding site locations in the (mouse) P-gp in respect to QZ59 inhibitors

A. QZ59 binding region of the mouse P-gp x-ray structure (PDB code 3G61) as shown in the lower-left inset. QZ59-SSS₁ and QZ59-SSS₂ as resolved in 3G61 are in green. QZ59-RRR as resolved in the mouse P-gp x-ray structure (PDB code 3G60) is included in this view in light blue. Hoechst 33342 (blue) and daunorubicin (red) correspond each to the best rated molecule location from independent docking simulations as detailed in Fig. S3A-B (-9.1 ± 0.3 and -10.4 ± 0.3 kcal/mol). Orange residues correspond to those reported to interact with Hoechst 33342 [12]. Yellow residues are those which, when replaced by a cysteine, are protected by rhodamine from thiol modification by MTS-rhodamine [10]. Areas surrounding the H- and R-sites and those being common to both are in blue, red and purple respectively. **B, C.** Residues forming the putative H- (B) and R- (C) drug-binding sites in the mouse P-gp inward-facing conformation in which QZ59-SSS were co-crystallized. Drawn with the same color code as in panel A. Drawn with Pymol 1.6.

Table 1
Enzyme inhibition models used in this study

Equations were used as detailed in Methods. S = substrate concentration, μM ; I = QZ59 concentration, μM ; V_m = maximal substrate efflux rate, in fmol of transported drug $\cdot \text{cell}^{-1} \cdot \text{h}^{-1}$; K_m = Michaelis constant, μM ; K_I = inhibition constant, μM ; K_{II} = inhibition constant for the $E+I \rightarrow E \cdot I$ partial reaction, μM ; K_{I2} = inhibition constant for the $E \cdot S+I \rightarrow E \cdot S \cdot I$ partial reaction, μM ; h = Hill number, K_A = activation constant, μM ; K_{SI} = substrate inhibition constant, μM .

Eq. ^a	Models	Velocity equations
2.1	No inhibition (NI) ^b	$V_m^* S / (K_m + S)$
3.1	QZ59 competitive inhibition (CI) ^c	$V_m^* S / (K_m^* (1 + I / K_I) + S)$
4.1	QZ59 non-competitive inhibition (NCI) ^d	$V_m / (1 + I / K_I) * S / (K_m + S)$
5.1	QZ59 uncompetitive inhibition (UI) ^e	$V_m / (1 + I / K_I) * S / (K_m / (1 + I / K_I) + S)$
6.1	QZ59 mixed inhibition (MI) ^f	$V_m / (1 + I / K_{I2}) * S / (K_m^* (1 + I / K_{II}) / (1 + I / K_{I2}) + S)$
2.2	Cooperativity (coop. ^g) + NI	$V_m^* S^h / (K_m^h + S^h)$
3.2	Coop. + QZ59 CI	$V_m^* S^h / (K_m^h * (1 + I / K_I) + S^h)$
4.2	Coop. + QZ59 NCI	$V_m / (1 + I / K_I) * S^h / (K_m^h + S^h)$
5.2	Coop. + QZ59 UI	$V_m / (1 + I / K_I) * S^h / (K_m^h / (1 + I / K_I) + S^h)$
6.2	Coop. + QZ59 MI	$V_m / (1 + I / K_{I2}) * S^h / (K_m^h * (1 + I / K_{II}) / (1 + I / K_{I2}) + S^h)$
3.3	QZ59 CI and activation (act. ^h)	$V_m^* (1 + I / K_A) * S / (K_m^* (1 + I / K_I) / (1 + I / K_A) + S)$
4.3	QZ59 NCI and act.	$V_m^* (1 + I / K_A) / (1 + I / K_I) * S / (K_m / (1 + I / K_A) + S)$
3.4	Coop. + QZ59 CI and act.	$V_m^* (1 + I / K_A) * S^h / (K_m^h * (1 + I / K_I) / (1 + I / K_A) + S^h)$
3.5	Coop. + QZ59 CI and act. + QZ59 effect on coop.	$V_m^* (1 + I / K_A) * S^{h*} / (K_m^{h*} * (1 + I / K_A)^{h*} * (1 + I / K_I) / (1 + I / K_A) + S^{h*} / K_A)$
2.6	substrate (sub. ⁱ) CI	$V_m^* S / (K_m + S^* (1 + S / K_{SI}))$
3.6	Sub. CI + QZ59 CI	$V_m^* S / (K_m^* (1 + I / K_I) + S^* (1 + S / K_{SI}))$
4.6	Sub. CI + QZ59 NCI	$V_m / (1 + I / K_I) * S / (K_m + S^* (1 + S / K_{SI}))$
5.6	Sub. CI + QZ59 UI	$V_m / (1 + I / K_I) * S / (K_m / (1 + I / K_I) + S^* (1 + S / K_{SI}))$
6.6	Sub. CI + QZ59 MI	$V_m / (1 + I / K_{I2}) * S / (K_m^* (1 + I / K_{II}) / (1 + I / K_{I2}) + S^* (1 + S / K_{SI}))$
2.7	Sub. NCI	$V_m / (1 + S / K_{SI}) * S / (K_m + S)$
3.7	Sub. NCI + QZ59 CI	$V_m / (1 + S / K_{SI}) * S / (K_m^* (1 + I / K_I) + S)$
4.7	Sub. NCI + QZ59 NCI	$V_m / (1 + S / K_{SI}) / (1 + I / K_I) * S / (K_m + S)$
5.7	Sub. NCI + QZ59 UI	$V_m / (1 + S / K_{SI}) / (1 + I / K_I) * S / (K_m / (1 + I / K_I) + S)$
6.7	Sub. NCI + QZ59 MI	$V_m / (1 + S / K_{SI}) / (1 + I / K_{I2}) * S / (K_m^* (1 + I / K_{II}) / (1 + I / K_{I2}) + S)$
2.8	Sub. CI + QZ59 effect on sub. CI	$V_m^* S / (K_m + S^* (1 + S / K_{SI}^* I / K_I))$
3.8	Sub. CI + QZ59 CI + QZ59 effect on sub. CI	$V_m^* S / (K_m^* (1 + I / K_I) + S^* (1 + S / K_{SI}^* I / K_I))$
4.8	Sub. CI + QZ59 NCI + QZ59 effect on sub. CI	$V_m / (1 + I / K_I) * S / (K_m + S^* (1 + S / K_{SI}^* I / K_I))$
5.8	Sub. CI + QZ59 UI + QZ59 effect on sub. CI	$V_m / (1 + I / K_I) * S / (K_m / (1 + I / K_I) + S^* (1 + S / K_{SI}^* I / K_I))$
6.8	Sub. CI + QZ59 MI + QZ59 effect on sub. CI	$V_m / (1 + I / K_{I2}) * S / (K_m^* (1 + I / K_{II}) / (1 + I / K_{I2}) + S^* (1 + S / K_{SI}^* I / K_{I2}))$
2.9	Sub. NCI + QZ59 effect on sub. NCI	$V_m / (1 + S / K_{SI}^* I / K_I) * S / (K_m + S)$
3.9	Sub. NCI + QZ59 CI + QZ59 effect on sub. NCI	$V_m / (1 + S / K_{SI}^* I / K_I) * S / (K_m^* (1 + I / K_I) + S)$
4.9	Sub. NCI + QZ59 NCI + QZ59 effect on sub. NCI	$V_m / (1 + S / K_{SI}^* I / K_I) / (1 + I / K_I) * S / (K_m + S)$
5.9	Sub. NCI + QZ59 UI + QZ59 effect on sub. NCI	$V_m / (1 + S / K_{SI}^* I / K_I) / (1 + I / K_I) * S / (K_m / (1 + I / K_I) + S)$
6.9	Sub. NCI + QZ59 MI + QZ59 effect on sub. NCI	$V_m / (1 + S / K_{SI}^* I / K_{I2}) / (1 + I / K_{I2}) * S / (K_m^* (1 + I / K_{II}) / (1 + I / K_{I2}) + S)$

^aEq. = equation;

^bNI = no inhibition;

^cCI = competitive inhibition;

^dNCI = non-competitive inhibition;

^eUI = uncompetitive inhibition;

^fMI = mixed inhibition;

^gcoop. = cooperativity;

^hact. = activation;

i_{sub} = substrate.

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