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Intramolecular Distribution of Hydrophobicity Influences Pharmacological Activity of Propafenone-type MDR Modulators

Lipophilicity is one of the major determining physicochemical descriptors for P-glycoprotein (P-gp) inhibitory activity. Recently, Pajeva and Wiese showed that in case of P-gp interaction, lipophilicity may be regarded as space-directed property. In the present study, a series of propafenone-type P-gp inhibitors with systematically varying hydrophobicity distribution within the molecules were synthesised and pharmacologically tested. QSAR studies on the basis of multiple linear regression analysis showed that with increasing lipophilicity of the substituents on the amine moiety, the statistical significance of the indicator variables, denoting the substitution pattern on the central aromatic ring system, also increases. This indicates that the distribution of hydrophobicity within the molecules influences the mode of interaction with P-gp.

Keywords: Multidrug resistance; P-Glycoprotein, Propafenone; Hydrophobicity distribution

Received: December 12, 2003; Accepted: March 5, 2004 [FP862] DOI 10.1002/ardp.200300862

Introduction

The phenomenon of multidrug resistance (MDR) has become a major obstacle in the treatment of cancer with chemotherapeutic drugs. The development of broad specificity mechanisms of resistance to multiple classes of drugs is strongly associated with the overexpression of membrane-bound drug efflux pumps such as P-glycoprotein (P-gp) [1]. P-gp is a 170-kDa membrane protein that belongs to the family of ABC (ATP-Binding Cassette) transporters and functions as an ATP-dependent efflux protein for a large variety of structurally and functionally diverse drugs and natural products. These include anthracyclines, epipodophyllotoxines, actinomycin D, vinca alkaloids, colchicine and taxol [2]. All ABC transporters share a common architecture consisting of four domains. Two transmembrane domains form a pathway across the membrane through which solutes can move. They consist of multiple membrane-spanning segments and contain the substrate-binding site. Additionally, two highly conserved nucleotide-binding domains are located at the cytoplasmatic side of the membrane and couple ATP hydrolysis to substrate translocation [3]. Recently, three X-ray structures of full-length bacterial ABC transporters have been obtained, the MsbA lipid flippase from E. coli [4], the vitamin B12 transporter BtuCD from *E. coli* [5], and the lipid flippase MsbA from *V. cholerae* [6]. Additionally, the nucleotide-binding domains of HisP, MJ0796, MJ1267, Malk and TAP1 have been crystallised [7–11]. The low-resolution three-dimensional structure of P-gp in the presence and absence of ATP has recently been resolved at 10-Å resolution by electron cryomicroscopy of negatively stained crystals [12]. Very recently, two protein homology models of P-gp on the basis of the X-ray structure of MsbA were published [13, 14]. However, both the detailed mechanism of transport and the ligand/protein interaction remain unresolved up to now.

It has been shown that drugs with the ability to inhibit P-gp lead to resensitisation of resistant tumour cells. Among them are numerous structurally and functionally diverse drugs, such as verapamil, dihydropyridines, phenothiazines, thioxanthenes, amiodarone, and even flavonoids, steroids and detergents. In an attempt to systematically explore structure-activity relationships within the class of P-gp inhibitors, we used the class Ic anti-arrhythmic agent propafenone as template (Figure 1A).

Our results obtained so far show that pharmacophoric substructures such as H-bond acceptors and one or more aromatic rings seem to be important for the biological activity. Additionally, overall lipophilicity of the compounds represents a major determinant for propafenone-type MDR modulators (Figure 1B) [15]. However, results of a CoMFA study performed on our data set suggest that lipophilicity influences pharma-

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Figure 1. A. Chemical structure of propafenone. **B.** Summary of the results of structure-activity relationship studies on propafenone analogues.

cological activity in a space-directed manner rather than as a general physicochemical determinant [16]. To further investigate this hypothesis, we synthesised and tested a series of propafenone analogues designed on the basis of altered distribution of hydrophobicity within the respective molecules.

Methods

Chemistry

Compounds were prepared analogous to previously described procedures (Table 1) [17-23]. Briefly, an appropriate phenol (1) was O-alkylated with epichlorhydrine. Reaction of the resulting epoxides with the corresponding amines lead to the target compounds 1-32. Phenols were synthesised via aldol condensation of hydroxyacetophenone derivatives with the appropriate aldehyde and subsequent catalytic hydrogenation (Scheme 1). The chemical struc-

ture, physicochemical parameters and biological activity of compounds 1-32 are given in Table 1.

P-gp inhibitory activity

The pharmacological activity of the compounds was measured in a zero trans efflux protocol using daunorubicine as the fluorochrome [21]. Briefly, multidrug resistant CCRF-CEM VCR1000 cells were incubated with daunorubicine, and the decrease in mean cellular fluorescence in dependence of time was measured in the presence of various concentrations of the modulator. EC_{50} values were calculated from the concentration response curve of efflux V_{max}/K_m *vs.* concentration of the modulator. Thus, the effect of different modulators on the transport rate is measured in a direct functional assay. Values are given in Table 1 and are the means of at least three independently performed experiments. Generally, interexperimental variation was below 20%.

Calculation of incremental logP values

The increment logP (logP_{incr}) of each acyl substituent was calculated as outlined in Figure 2 using Chem-Draw[®] [24], choosing the atom-based method from Broto [25]. This method was chosen because values derived by the Ghose-Crippen algorithm [26] gave inconsistent results. Thus, adding one methylene group increases logP from -0.69 (9–11) to -0.04 (12), whereas addition of two methylene groups increases logP only from 1.21 (13) to 1.57 (14–16).

Multiple linear regression analysis

Multiple linear regression analyses were performed using the Analysis Functions implemented in Excel 7.0.

Results and discussion

A set of 32 compounds with altered distribution of hydrophobicity within the molecules was synthesised and pharmacologically tested. A series of acylaryloxypropanolamines was prepared in order to evaluate the influence of intramolecular hydrophobicity distribution and substitution pattern on the central aromatic ring on their P-gp inhibitory activity. The compound design is based on the definition of two hydrophobic areas: one in the vicinity of the nitrogen atom and one on the central aromatic ring. Thus, the compounds synthesised vary in these two positions, with morpholine, piperidine, p-F-phenylpiperazine and o-tolylpiperazine Table 1. Chemical structure, physicochemical parameters and MDR-modulating activity of compounds 1-32.



General formula for compounds 1-32.

No	R1	R2 [†]	logP _{inkr}	EC50	Anal. [‡]
1	o-COCH₃	А	-0.55	67.32	[17]
2	m-CO-CH ₃	А	-0.55	128.427	[18]
3	p-COCH ₃	А	-0.55	302.05	[18]
4	o-COCH ₂ CH ₃	А	-0.09	207.203	[18]
5	o-CO(CH ₂) ₂ Ph	А	1.38	3.645	[19]
6	m-CO(CH ₂) ₂ Ph	А	1.38	3.475	C, H, N, CI
7	p-CO(CH ₂) ₂ Ph	А	1.38	6.875	[18]
8	o-CO(CH ₂) ₂ Napht.	А	2.50	0.73	[18]
9	o-COCH ₃	В	-0.55	31.96	[20]
10	m-COCH ₃	В	-0.55	9.073	[18]
11	p-COCH ₃	В	-0.55	48.973	[18]
12	o-COCH ₂ CH ₃	В	-0.09	14.3065	[21]
13	o-CO-Ph	В	0.74	1.2	[22]
14	o-CO(CH ₂) ₂ Ph	В	1.38	0.5988	[20]
15	m-CO(CH ₂) ₂ Ph	В	1.38	0.424	[21]
16	p-CO(CH ₂) ₂ Ph	В	1.38	0.972	[21]
17	o-CO(CH ₂) ₂ Napht.	В	2.50	0.172	[18]
18	o-COCH ₃	С	-0.55	2.087	[21]
19	m-COCH ₃	С	-0.55	10.072	[18]
20	p-COCH ₃	С	-0.55	11.885	[18]
21	o-COCH ₂ CH ₃	С	-0.09	0.836	C, H, N, CI
22	o-CO-Ph	С	0.74	0.147	[23]
23	o-CO(CH ₂) ₂ Ph	С	1.38	0.07	[20]
24	m-CO(CH ₂) ₂ Ph	С	1.38	1.1195	[21]
25	p-CO(CH ₂) ₂ Ph	С	1.38	2.535	[21]
26	o-COCH ₃	D	-0.55	0.249	C, H, N, CI
27	m-COCH ₃	D	-0.55	0.537	C, H, N, CI
28	p-COCH ₃	D	-0.55	1.55	C, H, N, CI
29	o-CO(CH ₂) ₂ Ph	D	1.38	0.0267	[20]
30	m-CO(CH ₂) ₂ Ph	D	1.38	0.074	C, H, N, CI
31	o-COCH ₂ CH ₃	D	-0.09	0.168	C, H, N, CI
32	p-COCH ₂ CH ₃	D	-0.09	0.863	C,H,N

[†] A: morpholine, B: piperidine, C: p-F-phenylpiperazine, D: o-tolylpiperazine.

[‡] Satisfactory C, H, N, and CI elemental analyses (± 0.4%) were obtained.

on the amine side and various acyl groups on the central aromatic core (Table 1). Each compound group (according to the substituent on the nitrogen atom) was separately submitted to a multiple linear regression analysis using logP_{incr} and two indicator variables (substituent in meta position: $I_m = 1$, else: $I_m = 0$; substituent in para position: $I_p = 1$, else: $I_p = 0$) as independent variables and log(1/EC₅₀) values as



Scheme 1. General scheme for synthesis of compounds 1-32; i: NaOH, epichlorohydrine; ii: MeOH, amine; reflux.



Figure 2. Calculation of incremental logP values (logP_{incr}).

dependent variable. The corresponding equations retrieved are given in Table 2. See also Figure 3. Additionally, the complete data set was analysed via multiple linear regression analysis.

All four subsets of compounds gave statistically significant equations. In the group of the morpholine-substituted compounds (lowest hydrophobicity on the amine side) both indicator variables $(I_m \text{ and } I_p)$ did not significantly contribute to the variance in the data set (P =0.58 and 0.63, respectively). The activity of the compounds solely depends on their lipophilicity values (P =0.002). Increasing lipophilicity on the amine side gives rise to increasing significance of the coefficients of the two indicator variables I_m and I_p . Although in case of the piperidines a certain influence of the substituent's position on activity is present (I_m : P = 0.10; I_p : P =0.32), still no significance on the 95% confidence level was obtained. In case of p-F-phenylpiperazine and otolylpiperazine derivatives, the influence of both indicator variables is statistically significant, with meta and para substitution showing a negative influence on activity (P < 0.01). This indicates that with increasing lipophilicity on the amino terminus the influence of the substituent's position on the central aromatic ring gains increasing relevance.

The interaction of ligands with P-gp occurs within the lipid bilayer. It has been shown that both the lipid environment and the ligand/lipid interaction influences pharmacological activity of the compounds. Recent studies on AcrB, a multidrug exporter from *E. coli*,

showed that interaction of ligands with the transport protein is mainly based on hydrophobic interactions [27]. Our results indicate, that the hydrophobicity distribution within the molecules may also be regarded as a determinant for P-gp inhibitory potency. The substructure with the higher partial lipophilicity acts as an anchor for the compounds and thus influences the orientation of the molecule in the binding region. In case of the morpholines, the central aromatic moiety acts as hydrophobic anchor, and in case of p-F-phenylpiperazines and o-tolylpiperazines, the highly lipophilic amine acts as anchor. Only in this case, the position of the acyl substituent plays a role, possibly due to steric interaction. This hypothesis is further supported by the results of Wiese and Pajeva, who denoted lipophilicity as a space-directed property rather than a simple physicochemical measure [16]. This space-directedness might be indicative of different orientations within the binding region, which might then influence transport efficiency.

However, all equations obtained are misbalanced with regard to the number of compounds used per descriptor. To further strengthen our hypothesis, additional sets of compounds will be synthesised and tested.

Acknowledgments

The present work was generously supported by the Austrian Science Fund (project 17014-B11). K. Pleban thanks the University Vienna for financial support.

Amine	logP _{incr}	I _m	I _p	Intercept	n	r ²	F
А	0.74 (0.31)	0.18 (0.82)	-0.15 (0.83)	-1.81 (0.53)	8	0.92	16.13
В	0.77 (0.17)	0.35 (0.45)	-0.19 (0.46)	-0.97 (0.28)	9	0.97	47.14
С	0.55 (0.27)	-0.99 (0.57)	-1.20 (0.56)	0.23 (0.34)	8	0.95	25.35
D	0.48 (0.08)	-0.36 (0.14)	-0.77 (0.15)	0.86 (0.10)	7	0.997	322.9
All	0.51 (0.33)	-0.13 (0.39)	-0.52 (0.82)	-0.42 (0.51)	32	0.32	4.32

Table 2. Coefficients (\pm SD) and statistical data for equations 1–5; the general equation is as follows: $log(1/EC_{50}) = a(logP_{incr}) + b(I_m) + c(I_p) + d$.



Figure 3. A. Plot of observed *vs.* calculated $\log(1/EC_{50})$ values for models A-D (Table 2); \blacksquare model A (morpholines), \bullet model B (piperidines), \blacktriangle model C (p-fluorphenylpiperazines), \checkmark model D (o-tolylpiperazines). **B.** Plot of observed *vs.* calculated $\log(1/EC_{50})$ values for the overall model (Table 2).

Experimental

Chemistry

Melting points were determined on a Reichert-Kofler hotstage microscope and are uncorrected. Elemental analyses were performed by J. Theiner (Mikroanalytisches Laboratorium, Institute of Physical Chemistry, University of Vienna). NMR spectra were recorded on a Bruker Avance 200 spectrometer (200 MHz for ¹H, 50 MHz for ¹³C) using CDCl₃ solutions at 28 °C. The centre of the solvent signal was used as an internal standard which was related to TMS with δ 7.26 ppm (¹H) and δ 77.0 ppm (¹³C). Column chromatographic purifications were performed on Merck silica gel 60 (70–230 mesh). Yields given below are not optimised and refer to analytically pure material.

General procedure for preparation of amines

To a solution of 5.0 mmol of the corresponding epoxide (see Refs. [15–21]) in 20 mL methanol, 5.1 mmol of the desired amine was added. The reaction mixture was heated at 50 °C (refluxed for **32**) till the reaction was completed (tlc control). The solvent was evaporated and the resulting oil purified via column chromatography (silica gel, CH_2Cl_2 /methanol/ NH_3 conc 200:10:1 – 400:10:1).

General procedures for formation of hydrochlorides

1.0 mmol of the amine was dissolved in ethyl acetate, and 1.2 mL of a 1 M solution of HCl in diethyl ether was added. The resulting precipitate was filtered off and recrystallised from ethyl acetate.

1-[3-(2-Hydroxy-3-morpholin-4-yl-propoxy)-phenyl]-3-phenylpropan-1-one (6)

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1-(2-{3-[4-(4-Fluoro-phenyl)-piperazin-1-yl]-2-hydroxy-propoxy}-phenyl)-propan-1-one (21)

Yield = 80.6%, yellowish-coloured oil; ¹H-NMR (CDCl₃): δ_{ppm} 1.23 (t, 3H, J = 7.25 Hz, -CH₃); 2.66–2.73 (m, 4H, -N-(CH₂)₂-); 2.84–2.94 (m, 2H, -CH₂-N-); 3.02–3.13 (dd, 2H, J = 7.26/14.53 Hz, CO-CH₂-); 3.17–3.22 (m, 4H, -(CH₂)₂-N-); 4.13–4.26 (m, 3H, O-CH₂-CH-); 6.89–7.10 (m, 6H, arom. H); 7.44–7.52 (m, 1H, arom. H), 7.70–7.74 (dd, 1H, J = 1.71/7.71 Hz, arom. H); ¹³C-NMR (CDCl₃): δ_{ppm} 8.50 (-CH₃); 36.77 (CO-CH₂-); 50.19 (-N-(CH₂)₂-); 53.4 (-N-(CH₂)₂-); 60.92 (-N-CH₂-); 65.31 (-CH-OH); 71.65 (O-CH₂-); 112.23, 115.02, 115.65, 117.14, 117.64, 120.83 (arom. CH); 128.87, (arom. C); 130.14 (arom. CH); 133.93 (arom. CH); 147.41 (arom. C); 157.82 (d, J = 238.5 Hz, arom. C–F); 157.7 (arom. C); 203,32 (CO).

21 hydrochloride: yield 96.3%; colourless crystals; mp 113-115 °C (ethyl acetate).

Anal. calcd. for $C_{22}H_{27}$ FN₂O₃ HCI: C, H, N, CI.

1-{2-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}ethanone (**26**)

Yield = 62%, yellowish-coloured oil; ¹H-NMR (CDCl₃): δ_{ppm} 2.22 (s, 3H, -CH₃); 2.56 (s, 3H, CO-CH₃); 2.45–2.60 (m, 4H, -N(CH₂)₂-); 2.68–2.92 (m, 6H, -CH₂-N-(CH₂)₂-); 4.00–4.16 (m, 3H, O-CH₂-CH-); 5.21 (s, 1H, -OH); 6.80–7.70 (m, 8H, arom. H); ¹³C-NMR (CDCl₃): δ_{ppm} 18.21 (-CH₃); 32.33 (-CH₃); 52.12 (-CH₂-N-(CH₂)₂-); 54.24 (-N-CH₂-); 61.13 (-N-CH₂-); 71.52 (CH₂-O), 65.82 (-CH-OH); 113.16, 119.31, 121.37, 123.74, 127.0, 130.81, 131.57, 134.13 (arom. CH), 128.84 (C); 133.0 (C); 151.67 (C); 158.48 (arom. C); 200.13 (CO). **26** *hydrochloride*: yield 99,8%; colourless crystals; mp

154–159°C (ethyl acetate).

Anal. calcd. for $C_{22}H_{28}N_2O_3$ ·HCI: C, H, N, CI.

1-{3-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}ethanone (27)

 $\begin{array}{l} \mbox{Yield} = 62.5\,\%, \mbox{ yellowish-coloured oil; 1H-NMR (CDCl_3): $^{5}_{ppm}$ 2.31 (s, 3H, -CH_3); 2.60 (s, 3H, CO-CH_3); 2.60-2.67 (m, 4H, -N-(CH_2)_2-); 2.84-2.97 (m, 6H, -CH_2-N-, -(CH_2)_2-N-Ph); $ 4.05-4.21 (m, 3H, O-CH_2-CH-); $6.99-7.58 (m, 8H, arom. H); 1C-NMR (CDCl_3): $^{5}_{ppm}$ 18.23 (-CH_3); $27.11 (-CH_3); $52.08, $54.18 (-CH_2); $60.81 (-CH_2); $65.83 (-CH); $70.88 (-CH_2); $113.58, $119.35, $120.33, $121.77, $123.63, $126.96, $129.99, $131.45 (arom. CH); $132.92 (arom. C); $138.82 (arom. C); $151.61 (arom. C); $159.35 (arom. C); $198.31 (CO). \end{array}$

27 *hydrochloride*: yield 82.5%; colourless crystals; mp 178-184°C (ethyl acetate).

Anal. calcd. for $C_{22}H_{28}N_2O_3$ HCI: C, H, N, CI.

1-{4-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}ethanone (28)

28 *hydrochloride*: yield 91%; colourless crystals; mp 187-194 °C (ethyl acetate).

Anal. calcd. for C₂₂H₂₈N₂O₃·HCI: C, H, N, CI.

1-{3-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}-3-phenyl-propan-1-one (**30**)

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30 *hydrochloride*: yield 89.9%; colourless crystals; mp 146-160°C (ethyl acetate).

Anal. calcd. for C₂₉H₃₄N₂O₃ HCI: C, H, N, Cl.

1-{2-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}-propan-1-one (**31**)

Yield = 90.52%, colourless oil; ¹H-NMR (CDCl₃): δ_{ppm} 1.16–1.23 (t, 3H, J = 7.33 Hz, -CH₃); 2.31 (s, 3H, -CH₃); 2.63–3.09 (m, 12H, -CH₂-N-(CH₂)₂-, -N-(CH₂)₂-, -CH₂-); 4.10–4.28 (m, 3H, O-CH₂-CH-); 6.96–7.05 (m, 4H, arom. H); 7.15–7.21 (m, 2H, arom. H); 7.40–7.48 (ddd, 1H, J = 1.77/ 6.44/7.70 Hz, arom. H); 7.66–7.70 (dd, 1H, J = 1.77/7.71 Hz, arom. H); ¹³C-NMR (CDCl₃): δ_{ppm} 8.58 (-CH₃); 17.85 (-CH₃); 36.90 (-CH₂-); 51.76, 53.87 (-N-(CH₂)₂-(CH₂)₂-N-); 60.75 (-N-CH₂-); 65.52 (-CH-OH); 71.13 (O-CH₂-); 112.80, 118.96, 121.00, 123.26, 126.57, 126.90 (arom. CH); 128.71 (arom. C); 130.19, 131.08 (arom. C); 132.59 (arom. C); 133.13 (arom. C); 151.26 (arom. C); 157.57 (arom. C); 203.39 (CO). *31 hydrochloride*: yield 85.8%; colourless crystals; mp 160–163°C (ethyl acetate).

Anal. calcd. for $C_{23}H_{30}N_2O_3$ HCI: C, H, N, CI.

1-{4-[2-Hydroxy-3-(4-o-tolyl-piperazin-1-yl)-propoxy]-phenyl}-propan-1-one (32)

Yield = 80,33 %, colourless crystals; mp = 97–105 °C (methanol); ¹H-NMR (CDCl₃): δ_{ppm} 1.21 (t, 3H, J = 7.26, -CH₃); 2.30 (s, 3H, -CH₃); 2.62–3.01 (m, 12H, -CH₂-N-(CH₂)₂-, -N-(CH₂)₂, -CH₂-); 4.07–4.21 (m, 3H, -CH₂-CH-); 6.95–7.21 (m, 6H, arom. H); 7.93–7.97 (m, 2H, arom. H); ¹³C-NMR (CDCl₃): δ_{ppm} 8.37 (-CH₃); 17.80 (-CH₃); 31.37 (-CH₂-); 51.71 (-N-(CH₂)₂-); 53.76 (-CH₂-N-); 60.37 (-(CH₂)₂-N-); 65.31 (-CH); 70.36 (O-CH₂-); 114.18, 118.92, 123.22, 126.54, 130.15, 131.04 (arom. CH); 130.21, 131.04, 132.51, 151.20, 162.40 (arom. C); 199.43 (CO).

32 hydrochloride: yield 98%; colourless crystals; mp 103-105 °C (ethyl acetate).

Anal. calcd. for C₂₃H₃₀N₂O₃·1.9 HCI: C, H, N, CI.

Cell lines

The human T-lymphoblast cell line CCRF-CEM and the multidrug-resistant line CCRF-CEM VCR1000 were provided by V. Gekeler (Byk Gulden, Konstanz, Germany). The resistant line was obtained by stepwise selection in vincristine- or daunorubicine-containing medium [28]. Cells were kept under standard culture conditions (RPMI 1640 medium supplemented with 10% fetal calf serum). The P-gp-expressing resistant cell line was cultured in the presence of 1000 ng/ mL vincristine. Prior to the experiments (1 wk), cells were transferred into medium without selective agents or antibiotics. 334 Ecker et al.

Efflux essay

Daunorubicine efflux studies were performed as described [19]. Briefly, cells were pelleted, the supernatant was removed by aspiration, and cells were resuspended at a density of 1 \times 10⁶/mL in PRMI 1640 medium containing 3 μ mol/L daunorubicine. Cell suspensions were incubated at 37 °C for 30 min. After this time, a steady state of daunorubicine accumulation was reached. Tubes were chilled on ice and cells were pelleted at 500 \times g. Cells were washed once in RPMI 1640 medium to remove extracellular daunorubicine. Subsequently, cells were resuspended in medium prewarmed to 37 °C, containing either no modulator or chemosensitisers at various concentrations ranging from 3 nM to 500 μ M, depending on solubility and expected potency of these modifiers. Generally, eight serial dilutions were tested for each modulator. After 1, 2, 3 and 4 min, aliguots of the incubation mixture were drawn and pipetted into four volumes of ice-cold stop solution (RPMI 1640 medium containing verapamil at a final concentration of 100 µM). Parental CCRF-CEM cells were used to correct for simple membrane diffusion, which was less than 3% of the efflux rate observed in resistant cells. Samples drawn at the respective time points were kept in an ice water bath and measured within an hour on a Becton Dickinson FACSCalibur (Becton Dickinson, Heidelberg, Germany) as described. Dose response curves were fitted to the data points using non-linear least squares, and EC₅₀ values were calculated.

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