0026-895X/96/061122-09$3.00/0

MOLECULAR PHARMACOLOGY, 49:1122–1130 (1996).

Structural Requirements for Activity of Propafenone-type Modulators in P-Glycoprotein-Mediated Multidrug Resistance

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Received August 15, 1995; Accepted March 7, 1996

SUMMARY

The sodium channel blocker propafenone and a series of analogs have been identified as effective modulators of P-glycoprotein-mediated multidrug resistance in human tumor cells. A series of closely related structural homologues showed a highly significant correlation between lipophilicity and pharmacological effect. Reduction of the carbonyl group as well as conversion to a methylether led to a remarkable decrease in activity, whereby lipophilicity lost its predictive character as the main determinant for modulator potency. Similarly, the relative positioning of the acyl- and propanolamine side chains also influences activity, so the distance between carbonyl group and nitrogen atom seems important.

Clinical resistance to drugs poses a major problem in cancer treatment as well as in antimicrobial therapy; development of resistance frequently is not restricted to individual drugs. The phenomenon that cells acquire cross-resistance to a panel of drugs when exposed to a single drug has been termed MDR (1). Drugs with the ability to induce this type of resistance are structurally and functionally diverse and include anthracyclines, epipodophyllotoxins, actinomycin D, vinca alkaloids, colchicine and taxol as well as antimicrobial agents (2, 3).

Classic MDR in tumor cells has been shown to be mediated by an energy-dependent efflux pump termed PGP, which is located in the plasma membrane (4). In humans, PGP is encoded by the *mdr1* gene (5). Substances have been identified that reestablish sensitivity of PGP-expressing tumor cells toward cytostatic drugs through inhibition of this efflux pump (6, 7); these include calcium channel blockers like the phenylalkylamine verapamil; the dihydropyridines nifedipine, nimodipine, and nicardipine; and the benzothiazepine diltiazem. In addition, steroids, cyclosporin A and its nonimmunosuppressive congeners, the antipsychotic phenothiazines as well as the structurally related thioxanthenes, rauwolfia alkaloids, and the antimalaria agent quinine have been identified as inhibitors of PGP (8). Specific chemosensitizers have been designed that inhibit PGP without demonstrating the inherent pharmacological effects of the parent drugs (e.g., cardiac effects, immunosuppression, and nephrotoxicity). These include (*R*)-verapamil, the dihydropyridine dexniguldipine, and nonimmunosuppressive cyclosporins, which have been tested in clinical phase I and II studies in cancer patients (9, 10). In addition to its effects on tumor cell resistance, PGP has been reported to play a role in host-mediated resistance toward tuberculostatic drugs (3).

Attempts have been made to characterize common structural features of PGP-blocking agents. In general, active compounds have high lipophilicity and, with the exception of cyclosporin and its derivatives, have aromatic ring systems in the molecule. Most substances also possess a tertiary nitrogen atom that is positively charged at physiological pH. A computer-based analysis has been performed of substructures of substances that have the property to interact with PGP (11, 12).

We recently reported that analogs of the class 1c antiarrhythmic drug propafenone, as well as the parent compound, are effective in restoring drug sensitivity in PGP-expressing human CCRF-CEM T lymphoblasts (13, 14).

Propafenone (**1a**) is in clinical use as an antiarrhythmic agent due to its ability to block cardiac sodium channels. The substance also has weak β-adrenoreceptor-blocking activity (15). Analogs with modification of the nitrogen substituents and structural modification of the phenylpropanone moiety were synthesized. These compounds were tested for their potential to reverse PGP-mediated MDR in an attempt to define structural requirements for an interaction with PGP.

Our data demonstrate that a wide range of structural modifications at the nitrogen atom influence activity, mainly by determining the lipophilicity of the molecule. Modification

This work was supported by OENB (Austrian National Bank) Grant 4728 and by the Ministry for Science, Research and Art (B.T.) and the University of Vienna (B.T.).

ABBREVIATIONS: PGP, P-glycoprotein; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

of the carbonyl group as well as the relative positioning of the two side chains lead to a marked change in activity, by which lipophilicity loses its importance as a factor for predicting activity.

Materials and Methods

Cell Lines

The CCRF-CEM T lymphoblast cell line and the resistant line were obtained as described previously (16). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum under standard culture conditions. The resistant CCRF vcr1000 cell line was maintained in medium containing 1000 ng/ml vincristine. The selecting agent was washed out at least 1 week before the experiments. The cell line used in our study was selected in the presence of increasing doses of vincristine without prior mutagenization (16). This cell line was chosen due to its distinct PGP expression, and it does not show the mutation at codon 185 (17). In addition, no significant contribution was observed of other factors to MDR.[1]

The mouse lymphoma cell line L5178Y was infected by a retroviral vector carrying the human *mdr1* gene. The vector was grown in and encapsidated by the packaging cell line PA12MDR1/$_{A\text{-}1}$. The viral RNA was from a replication-defective, amphotropic virus that was extruded by the cells into surrounding medium (18). Polybrene was added to a final concentration of 2 μg/ml in this filtered supernatant, which was then diluted 1:5 with growth medium. Again, polybrene was added to a final concentration of 2 μg/ml. The cells to be infected were plated in medium containing the virus and polybrene. After 2 days, this medium was replaced with McCoy's 5A medium containing 10% horse serum, to which 60 ng/ml colchicine was added to select resistant cells (19). These resistant cells were termed L5178Y VMDR C.06. The line was maintained in colchicine-containing culture medium.

Rhodamine 123 and Daunomycin Efflux Studies

Rhodamine efflux studies were performed with modifications of reported methods (20–22). Cells were pelleted, the supernatant was removed through aspiration, and the cells were resuspended at a density of 1×10^6/ml in RPMI 1640 medium containing rhodamine 123 (Sigma Chemical Co., St. Louis, MO) at a final concentration of 0.2 μg/ml (0.53 μmol/liter). Cell suspensions were incubated at 37° for 15 min. Tubes were chilled on ice and pelleted at 500 × *g* in an Eppendorf 5403 centrifuge (Eppendorf, Hamburg, Germany). Supernatants were removed, and the cell pellet was resuspended in medium that was prewarmed to 37° and contained either no modulator or chemosensitizer at various concentrations ranging from 3 nM to 500 μM, depending on solubility and the expected potency of the modifier. Eight concentrations (serial dilution 1:2.5) were tested for each modulator. After 30, 60, 90, and 120 sec, aliquots of the incubation mixture were transferred to tubes containing an equal volume of ice-cold stop solution (RPMI 1640 medium containing verapamil at a final concentration of 10 μg/ml). Zero time points were determined by immediately pipetting rhodamine 123- preloaded cells into ice-cold stop solution. Non-PGP-expressing parental CCRF-CEM cells were used as controls for simple plasma membrane diffusion, in which initial rhodamine 123 fluorescence levels were adjusted to be equal to initial levels observed in resistant cells. Samples drawn at the respective time points were kept in an ice water bath and measured at ≤1 hr with a Becton Dickinson FACSCALIBUR flow cytometer (Becton Dickinson, Vienna, Austria). Viable cells were gated on the basis of forward and side scatter. The excitation and emission wavelengths were 488 nm and 534 nm, respectively. Five thousand gated events were accumulated for the determination of mean fluorescence values. The time-dependent decrease in mean fluorescence values was linear over time for ≥2 min and was expressed as a percentage of zero time points to allow comparison of independent experiments.

In daunomycin efflux experiments we used an analogous experimental protocol in which the daunomycin concentration was 3.2 μmol/liter and the preloading time was 30 min. Time points were 60, 120, 180, and 240 sec. Efflux was linear with time for ≥4 min. Otherwise, conditions were identical to those outlined for the rhodamine 123 efflux experiments.

Cytotoxicity Assays

The assay is based on the cellular reduction of MTT (Sigma Chemical Co.) in mitochondria of viable cells to water-insoluble formazane. The assay was performed as described previously (14).

Mathematical Model for the Determination of Modifier ED_{50} Values

Initial efflux rates were calculated from the time-dependent linear decrease in mean fluorescence through the use of linear regression analysis. ED_{50} values were determined from dose-response curves of modifier concentration versus initial efflux rate or versus fractional survival for MTT assays. Data points of at least two independently performed experiments were fitted according to the equation given below, using the method of nonlinear least squares.

$$y = y_i - \frac{ME \cdot c}{ED_{50} + c}$$

where y is the initial rate of efflux as a function of modifier concentration, c is the modifier concentration, y_i is the initial rate of efflux observed in the resistant cell line in the absence of modulator, and ME is the modulator efficacy. The initial efflux rates observed in resistant cells were corrected for simple diffusion by subtracting the efflux rates observed in the parental line.

Synthesis of Compounds

o-Acylphenoxypropanolamines **1a–l** were synthesized in analogy to the previously described procedure (14). Synthesis of **6a–9b** was achieved as follows (Fig. 1): condensation of commercially available *meta*- or *para*-hydroxyacetophenone (**2c** and **2d**) with benzaldehyde gave the hydroxychalcones **3c** and **3d**, which were converted to the corresponding hydroxyphenones **4c** and **4d** through subsequent reduction of the double bond via catalytic hydrogenation (23). Phenol **4a** was prepared via reduction of commercially available *o*-hydroxyphenylpropiophenone **2a** (24). The corresponding methoxy derivative **4b** was synthesized via acidic methanolysis of **4a**. Phenols (**4a–4d**) were reacted with epichlorohydrine to give the epoxides **5a–5d**, which were refluxed in piperidine to yield the desired amines **6a–9b**.

Melting points were determined with a Kofler melting point apparatus and are uncorrected. Infrared spectra were recorded as thin films on salt disks with a Perkin Elmer 298 spectrophotometer. Mass spectrometry was performed with a Shimadzu QP 1000 spectrometer by G. Reznicek (Institut für Pharmakognosie, University of Vienna, Vienna, Austria). Gas chromatography/mass spectrometry was performed by L. Jirovetz (Institut für Pharmazeutische Chemie, University of Vienna, Vienna, Austria) with a Hewlett Packard 5890A gas chromatograph equipped with a Hewlett Packard-5970 mass spectrometry detector and a 59970 ChemStation data system. NMR spectra were recorded with a Bruker AC 80 and a Varian Unity plus 300 system with tetramethylsilane as internal standard. Microanalyses were done by J. Theiner (Institut für Physikalische Chemie, University of Vienna, Vienna, Austria). Satisfactory carbon, hydrogen, nitrogen, and chloride analyses (±0.4%) were obtained for all hydrochlorides.

[1] V. Gekeler, unpublished data.

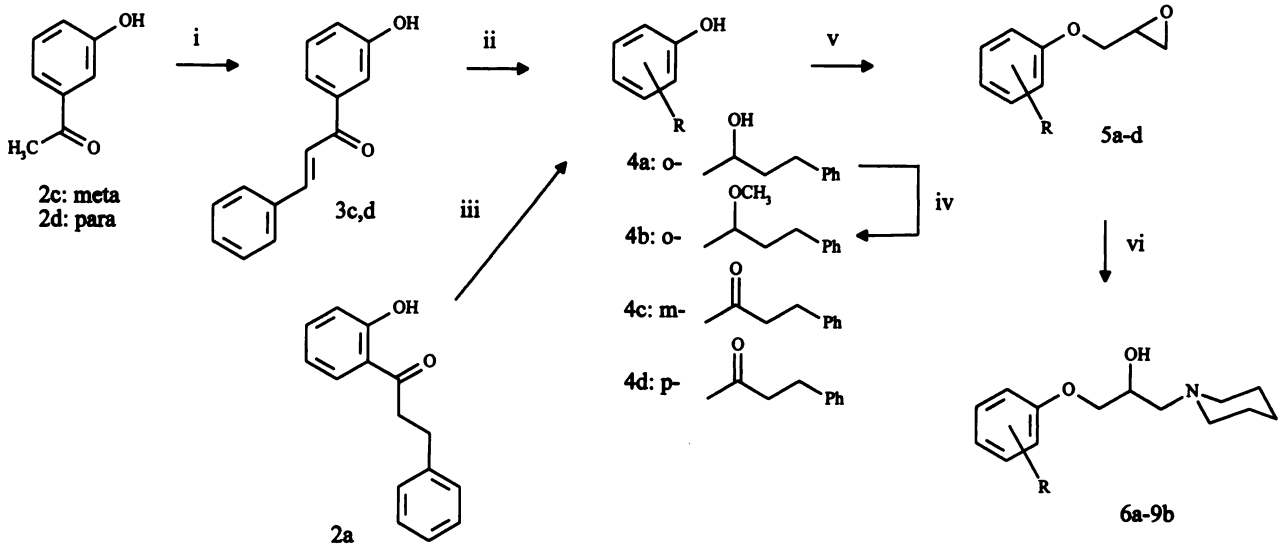


Fig. 1. Synthesis of **6a–9b**. *i*, NaH, benzaldehyde; *ii*, H_2, Pd/C; *iii*, $NaBH_4$, methanol; *iv*, H^+, methanol; *v*, NaOH, epichlorohydrine; *vi*, amine, reflux.

General procedures for the synthesis of the epoxides 5a-5d and the amines 1c, 1l, and 6a–9b. The substances were prepared as described previously (14). Selected physicochemical data and ^1H NMR spectra are shown in Table 1.

1-(2-Hydroxyphenyl)-3-phenyl-1-propanole (**4a**): yield 89%; ^1H NMR (chloroform-*d*) 2.06–2.22 (m, 2 H, —C(O)CH_2—), 2.64–2.74 (m, 2 H, PhCH_2—), 3.04 (br, 1 H, —OH), 4.74 (t, 1 H, J = 6.6 Hz, ArCH(O)—), 6.77–7.29 (m, 9 H, arom H), and 8.03 (br, 1 H, phenol H); 2-(1-Methoxy-3-phenyl-propyl)phenol (**4b**)/2-(1-hydroxy-3-phenyl-propyl)phenol (**4a**) (1.81 g, 7.9 mmol) was dissolved in 20 ml of methanol, and 0.15 ml of concentrated sulfuric acid was added. The reaction mixture was refluxed for 1.5 hr, diluted with water, neutralized with $NaHCO_3$, and extracted twice with diethyl ether. The combined organic layers were dried over Na_2SO_4 and evaporated to dryness. Purification of the oily residue (1.71 g) via column chromatography (silica gel, petroleum ether/diethyl ether) gave 1.48 g (87%) **4b** as colorless oil that solidified slowly. [^1H NMR (chloroform-*d*) 1.88–2.38 (m, 2 H, —C(O)CH_2—), 2.60–2.88 (m, 2 H, PhCH_2—), 3.38 (s, 3 H, —OCH_3), 4.23 (dd, 1 H, J = 6.1, 8.0 Hz, —CH(O)—), 6.78–7.42 (m, 9 H, arom H), 7.93 (s, 1 H, —OH); ^{13}C NMR (chloroform-*d*) 31.84, 37.47 (PhCH_2CH_2—), 57.11 (—OCH_3), 84.83 (—CH(O)—), 116.91, 119.68, 124.87, 125.89, 128.32, 128.37, 128.41, 129.03, 141.39, 155.53 (arom C); IR (cm^{-1}) 3700–2900 (OH); MS (70 eV) 242 (M^+, 17.9), 210 (43.6), 137 (54.1), and 91 (100).

Synthesis of phenoles 4c and 4d. Commercially available acetylphenole (**2c** and **2d**) (14.7 mmol) was dissolved in 40 ml of ethanol, and 3.56 g of benzaldehyde (33.56 mmol) and 7.12 g of NaOH (50% w/w) were added. After being stirred for 3 days, the reaction mixture was acidified with 5 M HCl and extracted twice with diethyl ether. The combined organic layers were washed with water, dried over Na_2SO_4, and evaporated to dryness. The oily residue was purified via crystallization to give hydroxychalcones **3c** and **3d**.

Hydroxychalcone (**3**) (8 mmol) was dissolved in 30 ml of methanol and Pd on charcoal (10 g%); and ammonium formate (40 mmol) was added under argon atmosphere, and the reaction mixture was refluxed for 1.5 hr. The catalyst was filtered out, and the solvent was removed under reduced pressure. The oily residue was dissolved in ethylacetate and washed with water. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The oily residue was purified through column chromatography (silica gel, petroleum ether/diethyl ether). 1-(3-Hydroxyphenyl)-3-phenyl-propenone (**3c**): yield 63.8%; ^1H NMR (chloroform-*d*) 7.12–7.84 (m, 12 H, arom H, —OH); 1-(4-Hydroxyphenyl)-3-phenyl-propenone (**3d**): yield 41.0%; ^1H NMR (chloroform-*d*) 5.93 (s, 1 H, OH), 6.93–8.13 (m, 11 H, arom H); 1-(3-Hydroxyphenyl)-3-phenyl-1-propanone (**4c**): yield 44.7%; ^1H NMR (chloroform-*d*) 3.04 (t, 2 H, J = 7.5 Hz, PhCH_2—), 3.27 (t, 2 H, J = 7.5 Hz, —CH_2CO), 5.81 (br, 1 H, —OH), and 7.07–7.55 (m, 9 H, arom H); 1-(4-Hydroxyphenyl)-3-phenyl-1-propanone (**4d**): yield 60.0%; ^1H NMR (chloroform-*d*) 3.03–3.28 (m, 4 H, —CH_2CH_2—), 3.95–5.00 (br, 1 H, OH), 6.85 (d, 2 H, J = 11.7 Hz, arom H), 7.12–7.38 (m, 5 H, arom H), and 8.09 (d, 2 H, J = 11.7 Hz, arom H).

Calculation of Distribution Coefficient

The logarithm P values were calculated according to the method of Ghose *et al.* (25) with the software package MOLGEN (CHERS, Bratislava, Slowak Republic). To ensure that for nonhomologous molecules (**6a–9b**) the calculated values are in agreement with those estimated experimentally, a high performance liquid chromatography method was used for determination of the distribution coefficients (26). Compounds were eluted on an RP-18 column (10 μm, LiChrospher 100; Merck Vienna/Austria) with four different mobile phase compositions (buffer/methanol = 30:70, 35:65, 40:60, and 45:55 v/v; buffer: 16.7 mmol/liter Sörensen pH, 7.4). The k' values of each substance were estimated and, on basis of a linear dependence of logk' on the methanol content, extrapolated to an eluent with 0% organic modifier. An excellent correlation between the logarithm of these K_w values and the calculated logP was found (r = 0.99, 19 experiments).

Molecular Modeling Studies

Compounds **1g**, **8a**, and **9a** were generated with the molecular modeling system package SYBYL 6.1 (Tripos GmbH, München, Germany) and minimized using a Powel algorithm (1000 iterations, gradient 0.05). Distances between relevant substructures were estimated with the distance function.

Results

The rhodamine 123 assay has been widely documented as a direct and reproducible assay for measuring PGP-dependent efflux (20–22). ED_{50} values for all substances were determined, and data points were fitted to a mathematical model described in Materials and Methods. A representative dose-response curve for propafenone (**1a**) is shown in Fig. 2. A sigmoidal curve with an r_2 value of 0.988 was obtained. For all substances tested, r_2 values were >0.95. Table 2 depicts the chemical structure of the compounds tested and gives the

TABLE 1
Selected physicochemical data and NMR spectra analysis of newly synthesized compounds

Compound	Formula	m.p.	Recrystallization solvent	^{1}H NMR (in case of the hydrochlorides **1c**, **1l**, and **6a** through **9b**, NMR of the free base is presented); chloroform-*d*, δ (ppm)
		°		
1c	$C_{20}H_{25}NO_3 \cdot HCl$	144–147	i-PrOH	2.20 [s, 6 H, —N(CH_3)$_2$], 2.37–2.58 (m, 2 H, —CH_2N—), 2.88–3.48 (m, 4 H, PhCH_2CH_2—), 3.40–4.10 (br, 1 H, —OH), 3.85–4.13 [m, 3 H, ArOCH_2CH(O)—], 6.85–7.75 (m, 9 H, arom H)
1l	$C_{17}H_{25}NO_3 \cdot HCl$	148–151	i-PrOH	1.17 (t, 3 H, *J* = 7.4 Hz, —CH_3), 1.40–1.66 (m, 6 H, —$CH_2CH_2CH_2$—), 2.28–2.67 [m, 6 H, —CH_2N(CH_2)$_2$—], 3.04 (qu, 2 H, *J* = 7.4 Hz, COCH_2—), 3.87 (br, 1 H, —OH), 4.03–4.14 [m, 3 H, ArOCH_2CH(O)—], 6.96 (d, 1 H, *J* = 8.0 Hz, arom H-3), 6.99 (t, 1 H, *J* = 8.0 Hz, arom H-5), 7.44 (dt, 1 H, *J* = 2.0, 8.0 Hz, arom H-4), 7.67 (dd, 1 H, *J* = 2.0, 8.0 Hz, arom H-6)
4b	$C_{16}H_{18}O_2$	Oil		1.88–2.38 [m, 2 H, —C(O)CH_2—], 2.60–2.88 (m, 2 H, PhCH_2—), 3.38 (s, 3 H, —OCH_3), 4.23 [dd, 1 H, *J* = 6.1, 8.0 Hz, —CH(O)—], 6.78–7.42 (m, 9 H, arom H), 7.93 (s, 1 H, —OH)
5a	$C_{18}H_{20}O_3$	Oil		Mixture of diastereomeres; 1.95–2.31 [m, 2 H, —C(O)CH_2—], 2.52–2.98 (m, 5 H, epoxide CH_2, PhCH_2—, —OH), 3.13–3.42 (m, 1 H, —CH—), 3.83–4.40 (m, 2 H, ArOCH_2—), 4.78–5.09 [m, 1 H, ArCH(O)—], 6.74–7.47 (m, 9 H, arom H)
5b	$C_{19}H_{22}O_3$	Oil		Mixture of distereomeres; 1.94–2.02 (m, 2 H, epoxide CH_2), 2.63–2.86 (m, 4 H, PhCH_2CH_2—), 3.21–3.27 (m, 1 H, epoxide CH), 3.25, 3.26 (2s, 3 H, —OCH_3), 3.88–3.97 (m, 1 H, ArOCH_a—), 4.13–4.20 (m, 1 H, ArOCH_b—), 4.59–4.67 (m, 1 H, —CH(O)—), 6.80–7.41 (m, 9 H, arom H)
5c	$C_{18}H_{18}O_3$	Oil		2.78 (dd, 1 H, *J* = 2.7, 4.8 Hz, epoxide (CH_a), 2.92 (t, 1 H, *J* = 4.8 Hz, epoxide CH_b), 3.04–3.09 (m, 2 H, PhCH_2—), 3.26–3.31 (m, 2 H, —CH_2CO), 3.36–3.39 (m, 1 H, epoxide CH), 3.97 (dd, 1 H, *J* = 6.0, 11.1 Hz, ArO-CH_a—), 4.30 (dd, 1 H, *J* = 3.0, 11.1 Hz, ArOCH_b—), 7.12–7.57 (m, 9 H, arom H)
5d	$C_{18}H_{18}O_3$	Oil		2.76–2.95 (m, 2 H, epoxide CH_2), 3.03–3.28 (m, 4 H, —CH_2CH_2—), 3.36–3.38 (m, 1 H, —CH—), 4.01 (dd, 1 H, *J* = 5.4, 12.9 Hz, ArOCH_a—), 4.33 (dd, 1 H, *J* = 2.7, 12.9 Hz, ArOCH_b—), 6.95 (d, 2 H, *J* = 11.7 Hz, arom H), 7.12–7.38 (m, 5 H, arom H), 7.95 (d, 2 H, *J* = 11.75 Hz, arom H)
6a	$C_{23}H_{31}NO_3 \cdot HCl$	129–132	EtAc	1.22–1.73 (m, 6 H, —$CH_2CH_2CH_2$—), 1.97–2.98 [m, 11 H, PhCH_2CH_2—, —CH_2N(CH_2)$_2$—, —OH], 2.80–3.60 (br, 1 H, —OH), 3.86–4.18 [m, 3 H, ArOCH_2CH(O)—], 4.83 [t, 1 H, *J* = 6.7 Hz, ArCH(O)—], 6.78–7.40 (m, 9 H, arom H)
6b	$C_{28}H_{33}N_2O_3F \cdot 2HCl$	88–90	EtAc	Mixture of distereomeres; 2.12–2.24 (m, 2 H, PhCH_2CH_2—), 2.50–2.85 [m, 8 H, PhCH_2—,—CH_2N(CH_2)$_2$—], 3.09–3.12 [m, 4 H, —(CH_2)$_2$NAr], 3.30 (br, 1 H, OH), 3.60 (br, 1 H, OH), 3.98–4.12 [m, 3 H, PhCH_2CH(O)—], 4.85 [m, 1 H, PhCH(O)—], 6.86–7.30 (m, 13 H, arom H)
7a	$C_{24}H_{33}NO_3 \cdot HCl$	115–119	EtAc	1.35–1.82 (m, 6 H, —$CH_2CH_2CH_2$—), 1.82–3.08 [m, 11 H, —CH_2N(CH_2)$_2$—, PhCH_2CH_2—, —OH], 3.23 (s, 3 H, —OCH_3), 3.80–4.17 [m, 3 H, ArOCH_2CH(O)—], 4.58, 4.60 [2t, 1 H, *J* = 5.9 Hz, ArCH(O)—], 6.77–7.45 (m, 9 H, arom H)
7b	$C_{29}H_{35}N_2O_3F \cdot 2HCl$	79–82	EtAc	Mixture of distereomeres; 2.00–2.06 (m, 2 H, PhCH_2CH_2—), 2.54–2.81 [m, 8 H, PhCH_2—, —CH_2N(CH_2)$_2$—], 3.08–3.18 [m, 4 H, —(CH_2)$_2$NAr], 3.25, 3.26 (2s, 3 H, —OCH_3), 3.30 (br, 1 H, OH), 3.96–4.04 [m, 3 H, PhCH_2CH(O)—], 4.55 [m, 1 H, PhCH(O)—], 6.86–7.38 (m, 13 H, arom H)
8a	$C_{23}H_{29}NO_3 \cdot HCl$	93–95	EtAc	1.44–1.62 (m, 6 H, —$CH_2CH_2CH_2$—), 2.38–2.62 [m, 6 H, —CH_2N(CH_2)$_2$—], 3.03–3.08 (m, 2 H, PhCH_2—), 3.25–3.30 (m, 2 H, —CH_2CO), 3.40–4.00 (br, 1 H, —OH), 3.99–4.10 [m, 3 H, ArOCH_2CH(O)—], 7.11–7.55 (m, 9 H, arom H)
8b	$C_{28}H_{31}N_2O_3F \cdot 2HCl$	89–92	EtAc	2.60–2.67 [m, 4 H, —N(CH_2)$_2$—], 2.82–2.89 (m, 2 H, —CH_2N—), 3.04–3.15 [m, 6 H, —(CH_2)$_2$NAr, PhCH_2—], 3.27–3.32 (m, 2 H, —CH_2COPh), 3.50 (br, 1 H, —OH), 4.04–4.17 [m, 3 H, PhCH_2CH(O)—], 6.86–7.57 (m, 13 H, arom H)
9a	$C_{23}H_{29}NO_3 \cdot HCl$	134–138	EtAc	1.25–1.80 (m, 6 H, —$CH_2CH_2CH_2$—), 2.20–2.85 [m, 6 H, —CH_2N(CH_2)$_2$—], 2.90–3.40 (m, 5 H, PhCH_2CH_2—, —OH), 3.96–4.25 (m, 3 H, OCH_2CH—), 6.98 (d, 2 H, *J* = 9.6 Hz, arom H), 7.13–7.31 (m, 5 H, arom H), 7.95 (d, 2 H, *J* = 9.6 Hz, arom H)
9b	$C_{28}H_{31}N_2O_3F \cdot 2HCl$	Amorph.		2.57–2.67 [m, 4 H, —*N*(CH_2)$_2$—], 2.82–2.89 (m, 2 H, —CH_2N—), 3.03–3.17 [m, 6 H —(CH_2)$_2$NAr, PhCH_2—], 3.23–3.42 (m, 2 H, —CH_2COPh), 3.55 (br, 1 H, —OH), 4.06–4.17 [m, 3 H, PhCH_2CH(O)—], 6.86–7.00 (m, 6 H, arom H), 7.20–7.33 (m, 5 H, arom H), 7.93–7.96 (m, 2 H, arom H)

respective ED_{50} values for inhibition of PGP. The latter were determined in rhodamine 123 and daunomycin efflux experiments as well as in daunomycin cytotoxicity assays. Lipophilicity of MDR-modulating agents has been established as one predictive parameter for activity. LogP values of the substances were calculated based on the incremental method described by Ghose *et al.* (25) (Table 2). Figs. 3–5 correlate lipophilicity (logP) with potency of the modulators. In addition to the PGP-expressing CCRF-CEM vcr1000 cell line, a transfectant mouse lymphoma cell line L5178Y VMDR C.06 was used in these experiments to rule out additional mechanisms of drug resistance in the vincristine selected CCRF-CEM line. Fig. 3 (CCRF-CEM vcr1000, rhodamine 123 efflux), Fig. 4 (CCRF-CEM vcr1000, daunomycin efflux), and Fig. 5 (L5178Y VMDR C.06, rhodamine 123 efflux) show that for the *o*-oxyphenone analogs (compounds **1a–l**, ●), an excel-

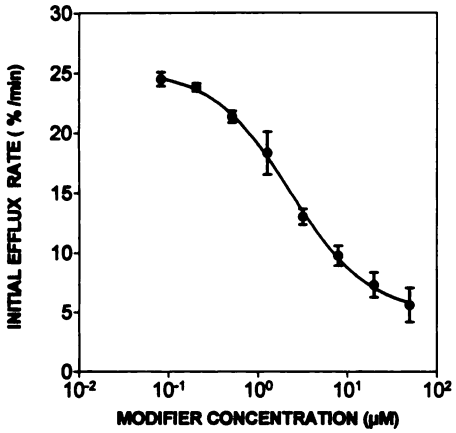


Fig. 2. Dose-response curve for the parent compound propafenone is given as a representative example, showing the effect of different concentrations of propafenone on rhodamine 123 initial efflux rates in CCRF-CEM vcr1000 cells. Similarly shaped dose-response curves were obtained for all compounds tested. Initial efflux rates were determined from the slope of a linear regression line fitted to mean fluorescence intensity values determined after 30, 60, 90, and 120 sec. Initial rhodamine 123 efflux rates are given as a percentage of the fluorescence value determined for the zero time point. Data points represent mean ± standard error of two independently performed experiments. ED_{50} values were calculated from the dose-response curves and are summarized in Table 2. See Materials and Methods and Results for details.

lent correlation was found between the logP and the log1/ED_{50} (log potency). A linear least-squares fit yielded r values of >0.95. Data indicate that, within the structurally homologous series of *o*-acylphenoxypropanolamines, lipophilicity is a main determinant for biologic activity of the compounds; highly lipophilic substances have high potency. A good correlation was obtained between ED_{50} values obtained in either daunomycin cytotoxicity assays or daunomycin efflux experiments (Fig. 6). This indicates that the predominant mechanism of resistance in the CCRF-CEM vcr1000 cells is indeed the overexpression of PGP. This is also supported by the analogy between the rhodamine 123 efflux data obtained in the CCRF-CEM vcr1000 cell line and those obtained in the transfectant L5178Y VMDR C.06 line.

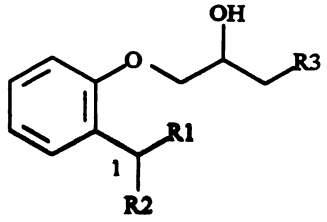
Influence of the carbonyl group on activity. It has been indicated that a hydrogen bond acceptor near an aromatic ring system is an important feature of active modulators (12). Propafenone and its derivatives contain an arylcarbonyl substructure that shows these characteristics. The influence of a modification of this part of the molecule on modulator potency was therefore studied. Two hydroxy (**6a** and **6b**) and two methoxy derivatives (**7a** and **7b**) (Figs. 3–5, △, ▽) were synthesized, and their biological activity was tested. The determined ED_{50} values were markedly lower than those predicted based on the activity-lipophilicity curves in Figs. 3–5.

It has been reported in the literature that both the reduction of carbonyl groups and the conversion of carbonyl groups to methoxy groups lead to a decrease in the hydrogen bond acceptor free energy factor (C_a) in a variety of substances (27). The loss of activity with decreasing hydrogen bond acceptor strength suggests that in addition to lipophilicity, the H-bond acceptor characteristics of the oxygen determine biological activity of the molecule.

TABLE 2

Chemical structure, logP, logK_w, and ED_{50} values of different structural analogues of propafenone

Values represent the mean of at least two independent experiments. Nonhomologous compounds were tested in at least three independent experiments. The interexperimental coefficient of variation was <20%.



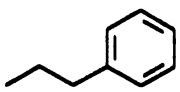
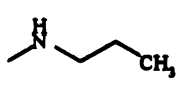
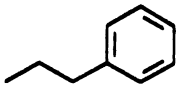
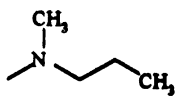
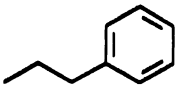
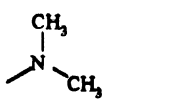
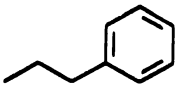
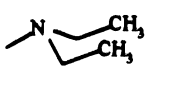
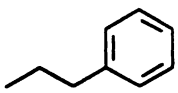
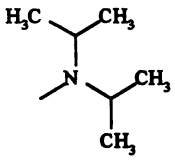
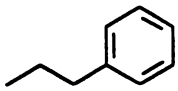
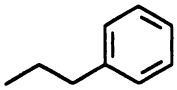
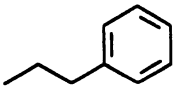
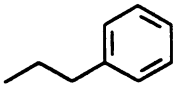
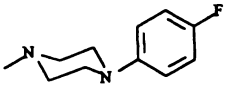
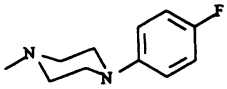
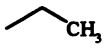
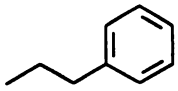
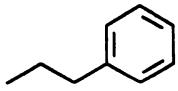
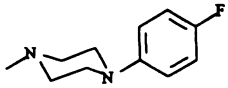
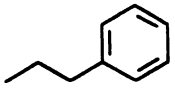
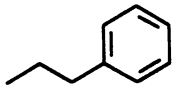
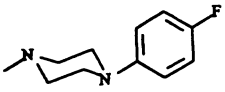
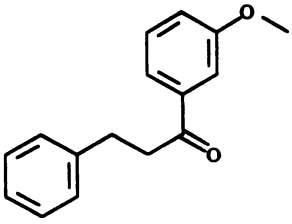
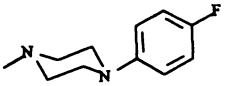
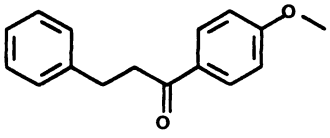
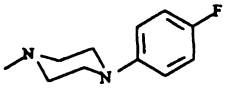
Compound	R^1	R^2	R^3	Calculated logP	Logk_w	ED_{50} efflux			ED_{50} MTT vcr1000
						CCRF-CEM vcr1000		L5178Yt	
						rhodamine 123	Dauno	rhodamine 123	
1a	—O			3.36	4.18	2.30	1.08	0.50	0.46
1b	—O			3.77	4.82	0.79	0.42	0.54	n.d.
1c	—O			2.94	4.16	5.01	2.66	1.44	3.67
1d	—O			3.62	4.48	n.d.	0.83	n.d.	

TABLE 2—*Continued*

Compound	R^1	R^2	R^3	Calculated logP	Logk_w	ED_{50} efflux			ED_{50} MTT vcr1000
						CCRF-CEM vcr1000 rhodamine 123	Duano	L5178Yt rhidamine 123	
1e	=O			4.25	5.22	0.32	0.17	0.26	0.44
1f	=O			3.26	4.24	1.59	1.77	0.95	n.d.
1g	=O			3.67	4.60	0.98	0.53	0.60	0.28
1h	=O			2.54	3.85	10.30	3.76	3.81	6.19
1i	=O			4.93	5.54	0.07	0.09	0.17	0.23
1k	=O	—CH_3		2.67	3.83	6.19	3.84	2.81	n.d.
1l	=O			2.07	3.39	39.81	6.55	13.95	11.89
6a	—OH			3.94	4.69	3.05	2.80	1.23	2.06
6b	—OH			5.20	5.67	0.68	0.67	0.80	0.69
7a	—OCH_3			4.30	4.97	0.99	1.14	0.50	0.55
7b	—OCH_3			5.56	6.21	0.23	0.22	0.26	0.85
8a				3.67	4.53	1.99	0.39	0.71	0.85
8b				4.93	5.70	2.24	1.12	0.77	0.87
9a				3.67	4.43	6.61	1.01	2.70	2.40
9b				4.93	5.47	6.17	2.78	4.09	1.39

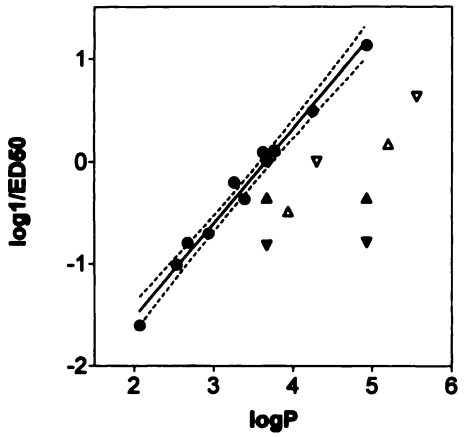


Fig. 3. Correlation between calculated logP and log1/ED_{50} (log potency) tested in the CCRF-CEM vcr1000 cell line with the rhodamine 123 efflux assay. Propafenone (●), hydroxy (△), methoxy (▽), *meta* (▲), and *para* derivatives (▼). *Solid line* obtained through linear regression of logP versus log potency values of the homologous propafenone derivatives; *dotted lines*, 95% confidence interval for the regression line. For homologous propafenone derivatives, r = 0.99 (11 substances). See Results for details.

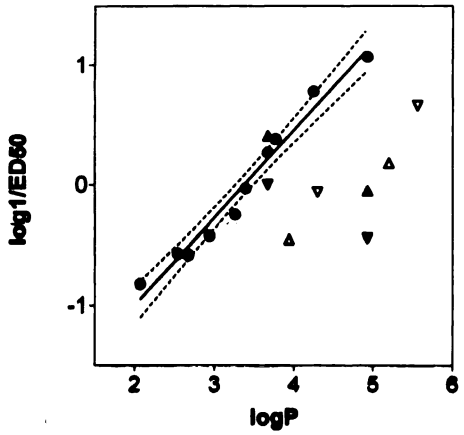


Fig. 4. Correlation between calculated logP and log1/ED_{50} (log potency) tested in the CCRF-CEM vcr1000 cell line with the daunomycin efflux assay. For homologous propafenone derivatives, r = 0.99 (10 substances). Propafenone (●), hydroxy (△), methoxy (▽), *meta* (▲), and *para* derivatives (▼). *Solid line* obtained through linear regression of logP versus log potency values of the homologous propafenone derivatives; *dotted lines*, 95% confidence interval of the regression line.

Influence of the relative positioning of the hydrogen bond acceptor and the nitrogen-bearing lipophilic side chain on activity. In addition to the *ortho*-substituted derivatives (**1g** and **1i**), we synthesized compounds with the phenylpropionyl side chain in *meta* (**8a** and **8b**) and *para* (**9a** and **9b**) position. All *para* derivatives (**9a** and **9b**) (Figs. 3–6, ▼) as well as the *meta* derivative **8b** (Figs. 3–6, ▲) had much higher ED_{50} values than the corresponding *ortho* derivatives (**1g** and **1i**). The *meta* derivative of **1g** (**8a**) did show ~2-fold lower activity in rhodamine 123 efflux assays in the CCRF-CEM vcr1000 cells. Also, in the transfectant cell line, a slight, although significant, decrease in activity could be observed with the use of rhodamine 123 as PGP substrate. In daunomycin efflux experiments, this decrease could, however, only be observed with compound **8b**. Remarkably, for both the *meta* and *para* compounds, an increase in activity could not be achieved through an increase in the lipophilicity of the molecule (**8b** versus **8a**, **9b** versus **9a**).

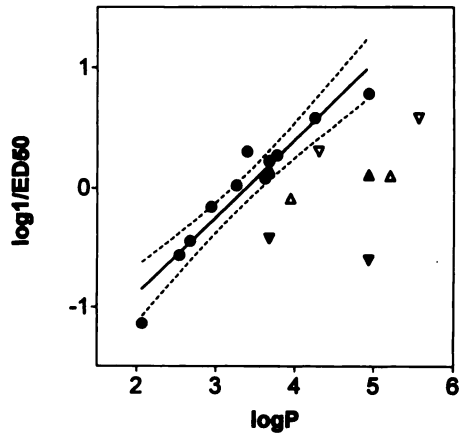


Fig. 5. Correlation between calculated logP and log1/ED_{50} (log potency) tested in the transfectant L5178Y VMDR C.06 cell line with the rhodamine 123 efflux assay. For homologous propafenone derivatives, r = 0.96 (11 substances). Propafenone (●), hydroxy (△), methoxy (▽), *meta* (▲), and *para* derivatives (▼). *Solid line* obtained through linear regression of logP versus log potency values of the homologous propafenone derivatives; *dotted lines*, the 95% confidence interval of the regression line.

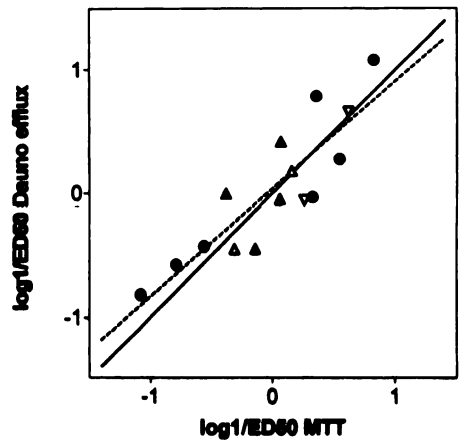


Fig. 6. Correlation between ED_{50} values determined in daunomycin cytotoxicity assay and daunomycin efflux experiments in CCRF-CEM vcr1000 cells. *Solid line*, ideal 1:1 correlation; *dotted line*, regression line calculated with the data points (r = 0.85, 15 substances). Propafenone (●), hydroxy (△), methoxy (▽), *meta* (▲), and *para* derivatives (▼).

TABLE 3

Distances between carbonyl group and nitrogen atom as well as ether oxygen and nitrogen atom of substances 1g, 8a, and 9a

Substance	Distance	
	C=O/N	—O—/N
	Å	
1g	5.757	4.901
8a	8.830	4.905
9a	10.904	4.898

Distances between the carbonyl oxygen and the nitrogen were calculated as described in Materials and Methods (Table 3). An increased distance was found in the order *para* > *meta* > *ortho*, which seems to correspond to pharmacological activity of the compounds. To exclude differences in the con-

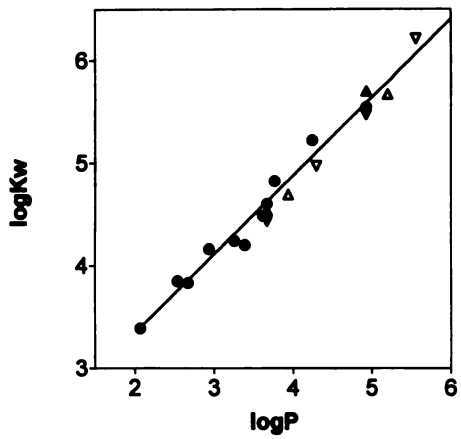


Fig. 7. Correlation between calculated logP values and experimentally determined logK_w values. An r value of 0.99 was obtained (19 substances). Propafenone (●), hydroxy (△), methoxy (▽), *meta* (▲), and *para* derivatives (▼). *Solid line* obtained through linear regression of logP versus log potency values of the homologous propafenone derivatives. See Methods for experimental details and logP calculation.

formation of the propanolamine side chain, the distance between the ether oxygen and the nitrogen was also calculated and found to be unchanged (4.901 ± 0.004 Å).

Because calculated lipophilicity might not be accurate for nonhomologous propafenone analogs, a reverse-phase high performance liquid chromatographic method was established for the determination of logK_w values. Results are shown in Table 2 and Fig. 7. An excellent correlation between calculated logP values and experimentally determined logK_w values could be observed for all compounds, including the nonhomologous derivatives **6a–9b**.

Discussion

There is accumulating evidence that PGP represents one factor contributing to clinical treatment failure in human malignancies. Although its involvement in clinical resistance of solid tumors is still unclear, there is good evidence that PGP has a role in the therapeutic outcome in certain types of hematological neoplasia (9). Several clinical trials have been initiated that use standard chemotherapy regimens in conjunction with chemosensitizing agents (for a review, see Ref. 10). A better characterization of the interaction of PGP with modulators on a molecular basis seems necessary to support clinical application of these drugs as trials in patients with cancer have not been highly successful (7–10, 28).

It has been demonstrated that PGP has broad substrate specificity. Drug binding has been shown to be strongly influenced by the physicochemical properties of modulators (29). Our data show a significant correlation between lipophilicity and biological activity for the group of propafenone analogs. However, this correlation is observed only in structurally homologous series of analogs. Modifications at critical sites of the substrate molecule lead to a decrease in activity, which can no longer be traced to a change in lipophilicity of the molecule alone.

Our data indicate that within the extended set of analogs, modifications in the *ortho* position of the ether oxygen lead to a decrease in activity of the modulators. The hydroxy (**6a** and **6b**) and methoxy derivatives (**7a** and **7b**) have ED_{50} values that clearly differ from the ED_{50} values predicted by the activity-lipophilicity curves of Figs. 3–5. It can be concluded from this set of data that the type of oxygen (carbonyl, alcohol, ether) influences the interaction with PGP via a mechanism independent of lipophilicity. This interaction may be mediated by a hydrogen bond formation, in which a hydrogen bond acceptor close to C1 seems to be required. This is in accordance with a model derived from a computer search of a National Cancer Institute database containing 40,000 structurally diverse compounds that proposes that a hydrogen bond acceptor near an aromatic ring is required to make a substance a modulator for PGP (12).

In addition, repositioning the *ortho* acyl substituent at the aromatic ring, thereby obtaining a *meta* and *para* analog, led to a decrease in PGP-blocking activity in the order *ortho* > *meta* > *para*. A comparison of the *meta* and *para* derivatives of higher (**8b** and **9b**) with those of lower lipophilicity (**8a** and **9a**) shows that an increase in overall lipophilicity does not result in a further increase in potency for *meta* and *para* derivatives. Our data suggest that the interaction of these substrate molecules with PGP is restricted.

Further studies will focus on quantification of the hydrogen bond acceptor properties of the substituent in position 1 and the influence of the distance between relevant substructures.

The propafenone molecule is small but has distinct structural features. This makes possible a broad range of chemical modifications. Therefore, propafenone analogs represent an excellent model system for the extension of studies on quantitative structure-activity relationships of substances that are able to inhibit PGP.

Acknowledgments

We acknowledge the technical assistance of Manuela Hitzler, Susanne Prets, and Anita Jungreithmeier. Dedicated to Prof. Dr. Wilhelm Fleischhacker on the occasion of his 65th birthday.

References

1. Biedler, J. L., T. Chang, M. B. Meyers, R. F. H. Peterson, and B. A. Spengler. Drug resistance in Chinese hamster lung and mouse tumor cells. *Cancer Treatment Rep.* **67**:859–867 (1983).
2. Bradley, G., P. F. Juranka, and V. Ling. Mechanism of multidrug resistance. *Biochim. Biophys. Acta* **948**:87–128 (1988).
3. Gollapudi S., M. Reddy, P. Gangadharam, T. Tsuruo, and S. Gupta. *Mycobacterium tuberculosis* induces expression of P-glycoprotein in promonocytic U1 cells chronically infected with HIV type 1. *Biochem. Biophys. Res. Commun.* **199**:1181–1187 (1994).
4. Riordan, J. R., and V. Ling. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J. Biol. Chem.* **254**:12701–12705 (1979).
5. Roninson, I. B., J. E. Chin, K. Choi, P. Gros, D. E. Housman, A. Fojo, D. W. Shen, M. M. Gottesman, and I. Pastan. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA* **83**:4538–4542 (1986).
6. Tsuruo, T. Reversal of multidrug resistance by calcium channel blockers and other agents, in *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells* (I. B. Roninson, ed.). Plenum Publishing, New York, 349–371 (1991).
7. Leyland-Jones, B., W. Dalton, G. A. Fisher, and B. I. Sikic. Reversal of multidrug-resistance in cancer chemotherapy. *Cancer* **72**:3484–3488 (1993).
8. Beck, W. T. Modulators of P-glycoprotein-associated multidrug resistance, in *Molecular and Clinical Advances in Anticancer Drug Resistance* (R. F. Ozols, ed.). Boston, Kluwer Academic Publ., 151–170 (1991).
9. Sikic, B. I. Modulation of multidrug resistance: at the threshold. *J. Clin. Oncol.* **11**:1629–1635 (1993).
10. Raderer, M., and W. Scheithauer. Clinical trials of agents that reverse multidrug resistance: a literature review. *Cancer* **72**:3553–3563 (1993).
11. Klopman, G., S. Srivastava, I. Kolossvary, R. F. Epand, N. Ahmed, and R. M. Epand. Structure-activity study and design of multidrug-resistant reversal compounds by a computer automated structure evaluation methodology. *Cancer Res.* **52**:4121–4129 (1992).

12. Ahkmed, N., A. T. Fojo, S. E. Bates, and S. Scala. Pgp substrate diversity among compounds identified by COMPARE analysis in the NCI drug screen. *Proc. Am. Assoc. Cancer Res.* **36**:339 (1995).
13. Chiba, P., G. Ecker, B. Tell, A. Moser, D. Schmid, and J. Drach. Modulation of PGP-mediated multidrug-resistance by propafenone-analogs. *Proc. Am. Assoc. Cancer Res.* **35**:357 (1994).
14. Chiba, P., S. Burghofer, E. Richter, B. Tell, A. Moser, and G. Ecker. Synthesis, pharmacologic activity and structure-activity relationships of a series of propafenone-related modulators of multidrug-resistance. *J. Med. Chem.* **38**:2789–2793 (1995).
15. Somberg, J. C. Clinical use of class Ic antiarrhythmic drugs, in *Antiarrhythmic Drugs* (E. M. Vaughan Williams, ed.). Springer-Verlag, Berlin/Heidelberg, 258–263 (1989).
16. Gekeler, V., G. Frese, A. Noller, R. Handgretinger, A. Wilisch, H. Schmidt, C. Muller, R. Dopfer, T. Klingebiel, H. Diddens, H. Probst, and D. Niethammer. Mdr1/P-glycoprotein, topoisomerase and glutathione-S-transferase gene expression in primary and relapsed state adult and childhood leukemias. *Br. J. Cancer* **66**:507–517 (1992).
17. Gekeler V., S. Weger, and H. Probst. MDR1/P-glycoprotein gene segments analyzed from various human leukemic cell lines exhibiting different multidrug resistance profiles. *Biochem. Biophys. Res. Commun.* **169**:796–802 (1990).
18. Pastan, I., M. M. Gottesman, K. Ueda, E. Lovelace, A. V. Rutherford, and M. C. Willington. A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc. Natl. Acad. Sci. USA* **85**:4486–4490 (1988).
19. Gilboa, E., M. A. Eglitis, P. W. Kantoff, and F. Anderson. Transfer and expression of cloned genes using retroviral vectors. *Biotechniques* **4**:504–512 (1986).
20. Drach D., S. Zhao, J. Drach, R. Mahadevia, C. Gattringer, H. Huber, and M. Andreeff. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* **80**:2729–2734 (1992).
21. Jancis, E. M., R. Carbone, K. J. Loechner, and P. S. Dannies. Estradiol induction of rhodamine 123 efflux and the multidrug resistance pump in rat pituitary tumor cells. *Mol. Pharmacol.* **43**:51–56 (1992).
22. Lee, J. S., K. Paull, M. Alvarez, C. Hose, A. Monks, M. Grever, A. T. Fojo, and S. E. Bates. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Mol. Pharmacol.* **46**:627–638 (1994).
23. Franke, A., J. Mueller, H. Lietz, W. W. Wiersdorff, H. G. Hege, C. D. Mueller, J. Gries, D. Lenke, G. Von Philipsborn, and M. Raschach. Aminopropanol derivatives of 2'-hydroxy-3-phenylpropio-phenones and therapeutic agents containing them. Eur. Pat. Appl. EP 75:207, March 30 (1983); *Chem. Abstr.* **99**:122033c (1983).
24. Bognar, R., and M. Rakosi. Flavonoids. IV. Reduction of 2'-hydroxychalcone and flavanone. *Acta Chim. Acad. Sci. Hung.* **13**:217–228 (1957).
25. Ghose, A. K., A. Pritchett, and G. M. Crippen. Atomic physicochemical parameters for three dimensional structure directed quantitative structure-activity relationships. III: Modeling hydrophobic interactions. *J. Comput. Chem.* **9**:80 (1988).
26. Belsner, K., M. Pfeifer, and B. Wilffert. Reversed-phase high-performance liquid chromatography for evaluating the lipophilicity of pharmaceutical substances with ionization up to logP = 8. *J. Chromatogr.* **629**:123–134 (1993).
27. Raevsky, O. A., V. Y. Grigor'ev, D. Kireev, and N. S. Zefirov. Complete thermodynamic description of H-bonding in the framework of multiplicative approach. *Quant. Struct. Act. Relat.* **11**:49–63 (1992).
28. Kaye, S. B. P-glycoprotein (P-gp), and drug resistance: time for reappraisal? *Br. J. Cancer* **67**:641–643 (1993).
29. Wadkins, R. M. and P. J. Houghton. The role of drug-lipid interactions in the biological activity of modulators of multi-drug resistance. *Biochim. Biophys. Acta.* **1153**:225–236 (1995).

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