

The Importance of a Nitrogen Atom in Modulators of Multidrug Resistance

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ABSTRACT

The presence of a nitrogen atom, charged at physiological pH, has frequently been considered to be a hallmark of P-glycoprotein (PGP) inhibitors, although certain steroids, such as progesterone, lack a nitrogen atom and still are active modulators of PGP. The present study was aimed at investigating the role the nitrogen atom plays in the activity of PGP inhibitors. Propafenone-related amines, anilines, and amides that cover a broad range of pK_a values, as well as an ester, were synthesized and tested for multidrug resistance-reverting activity. The sum of the hydrogen bond acceptor strengths was calculated

and correlated with EC_{50} values for PGP inhibition. For the complete set of 12 compounds, an excellent correlation between these two parameters was found; this included the ester GP570, which lacks a nitrogen atom but contains the strong hydrogen bond-accepting ester unit. The interaction of the nitrogen atom with PGP therefore is nonionic and is determined by the sum of the hydrogen acceptor strengths of the region. The high predictivity of the obtained model is demonstrated in a leave-one-out cross-validation procedure.

The important role of ATP binding cassette transporters in multidrug resistance (MDR) has been widely documented in both eukaryotic and prokaryotic systems (Higgins, 1992; Doige and Ames, 1993; van Veen, 1997). One of the most intensively studied members of the class of energy-dependent efflux pumps is P-glycoprotein (PGP; for a review, see Gottesman and Pastan, 1993; Germann, 1996; Stein, 1997). A variety of naturally occurring toxins, which enter cells via passive diffusion, are pumped out of the cell by PGP and related transporters (van Veen, 1997). Substrate toxins are structurally and functionally diverse. Special efforts have been devoted to the design of inhibitors, which overcome MDR by blocking PGP-mediated efflux. The interaction of substrates/modulators with PGP has been subject of several structure-activity relationship studies (Ford et al., 1989, 1990; Nogae et al., 1989; Pearce et al., 1989; Klopman et al., 1992; Ramu and Ramu, 1992; Chiba et al., 1995, 1996; Dodic et al., 1995; Toffoli et al., 1995; Dhainaut et al., 1996; Ecker et al., 1996; Mazerska et al., 1996; Klopman et al., 1997; Etievant et al., 1998; Pajeva and Wiese, 1998), and pharmacophoric substructures and physicochemical properties for both substrates and modulators have been defined; among them are aromatic ring structures, a basic nitrogen atom, and high lipophilicity (Zamora et al., 1988). Nevertheless, substances lacking a nitrogen atom, such as steroid hormones, still interact with PGP (Ueda et al., 1992; Schinkel et al., 1996). A

recent report by Seelig (1998) compares 100 different substances previously tested as PGP substrates. Substrate binding to PGP is proposed to increase with the number of the hydrogen bonding acceptor units of the compounds. However, data are based on a count of the number of hydrogen bond acceptor units per molecule rather than on quantification of the hydrogen bond acceptor strength.

Based on the hypothesis put forward in this report, we designed and synthesized a set of 12 analogs of the lead molecule propafenone. Among them are the four tertiary amines (GP05, GP29, GP31, and GP62), four anilines (GP240, GP339, GP358, and GP359), two amides (GP360 and GP366), one compound containing both an amide and an amine moiety (GP388), and one ester, which lacks a nitrogen atom (GP570). The sum of the hydrogen bond acceptor strengths was calculated and correlated with the EC_{50} values for PGP inhibition using two different fluorochrome substrates. The results clearly demonstrate a strong correlation between hydrogen bond acceptor strength and pharmacological activity within this set of compounds. The nitrogen atom does not interact with PGP in a charged form but functions as an electron donor group, which can be replaced by other hydrogen bond acceptor groups.

Materials and Methods

Design and Synthesis of Compounds

The phenylpropiophenone moiety of the compounds was kept structurally identical in the complete set of compounds to keep the

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influence of this part of the molecule on biological activity constant. Amines GP05 and GP31 (Chiba et al., 1995), GP29 (Chiba et al., 1997a), and GP62 (Chiba et al., 1997b); anilines GP240 and GP339 (Chiba et al., 1997b); and the ester GP570 (Ecker et al., 1994) were synthesized according to previously published procedures.

Melting points were determined with a Kofler melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian Unity plus 300 system with tetramethylsilane as internal standard. Elemental analyses were made by J. Theiner (Institute of Physical Chemistry, University of Vienna, Vienna, Austria). Satisfactory carbon, hydrogen, and nitrogen analyses ($\pm 0.4\%$) were obtained for all compounds.

1-(2-(2-Hydroxy-3-(4-trifluoromethylphenylamino)propoxy)-phenyl)-3-phenyl-1-propanone (GP358). An appropriate epoxide [1-(2-(2,3-epoxy-propoxy)phenyl)-3-phenyl-1-propanone (Chiba et al., 1995); 1.04 g, 3.7 mmol] was dissolved in 10 ml of methanol, and 0.5 ml (4.0 mmol) of 4-trifluoromethylaniline was added. The reaction mixture was heated under reflux for 8 h, the solvent was evaporated, and the resulting yellow oil was purified via column chromatography (silica gel, diethyl ether/petroleum ether) to yield 0.41 g (25%) GP358 as colorless crystals: m.p. 73–76°C (isopropanol); ^1H NMR (chloroform-*d*) 1.64 (broad, 2 H, $-\text{OH}$, $-\text{NH}$), 3.05 (t, 2 H, $J = 7.8$ Hz, $\text{Ph}-\text{CH}_2-$), 3.23–3.49 (m, 4 H, CH_2-CO , $-\text{CH}_2-\text{NH}-$), 4.07–4.22 [m, 3 H, $-\text{O}-\text{CH}_2-\text{CH}(\text{O})-$], 6.66–7.63 (m, 13 H, aromatic H); analysis ($\text{C}_{25}\text{H}_{24}\text{F}_3\text{NO}_3$) C, H, N.

1-(2-(2-Hydroxy-3-(4-nitrophenylamino)propoxy)phenyl)-3-phenyl-1-propanone (GP359). 1-(2-Hydroxyphenyl)-3-phenyl-1-propanone (1.6 g, 7.0 mmol) was dissolved in 50 ml of methanol and 0.28 g (7.0 mmol) solid sodium hydroxide, and 1.4 g (7.2 mmol) of *N*-2,3-epoxypropyl-4-nitroaniline was added. The reaction mixture was heated under reflux for 8 h, the solvent was evaporated, and the resulting oil was purified via column chromatography (silica gel, dichloromethane/methanol/ammonia) to yield 0.26 g (8.8%) GP359 as yellow crystals: m.p. 102–103°C (toluene); ^1H NMR (chloroform-*d*) 3.05 (t, 2 H, $J = 7.5$ Hz, $\text{Ph}-\text{CH}_2-$), 3.26–3.51 (m, 4 H, $-\text{CH}_2-\text{CO}$, $-\text{CH}_2-\text{NH}-$), 3.71 (d, 1 H, $J = 4.2$ Hz, $-\text{OH}$), 4.09–4.23 [m, 3 H, $-\text{O}-\text{CH}_2-\text{CH}(\text{O})-$], 5.38 (broad, 1 H, NH), 6.62–8.07 (m, 13 H, aromatic H); analysis ($\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_5$) C, H, N.

***N*-(3-(2-(1-Oxo-3-phenyl-propyl)phenoxy)-2-hydroxypropyl)-*N*-propyl-benzoic acid amide (GP360).** Propafenone (Chiba et al., 1995; 1.02 g, 3.0 mmol) was dissolved in 20 ml of pyridine, and 0.35 ml (3.0 mmol) benzoylchloride was added. The reaction mixture was stirred for 2 h, the solvent was concentrated on an evaporator, and the residual oil was dissolved in diethyl ether and washed twice with 0.1 N HCl. The organic phase was dried over Na_2SO_4 , evaporated, and purified via column chromatography (silica gel, petroleum ether/diethyl ether) to give 0.43 g (32%) GP360 as a yellowish solid: ^1H NMR (chloroform-*d*) 0.63 (t, 3 H, $J = 7.5$ Hz, $-\text{CH}_3$), 1.42 (sx, 2 H, $J = 7.5$ Hz, $-\text{CH}_2-\text{CH}_3$), 2.96 (t, 2 H, $J = 7.5$ Hz, $\text{Ph}-\text{CH}_2-$), 3.11 (qu, 2 H, $J = 7.5$ Hz, $-\text{N}-\text{CH}_2-$), 3.23 (t, 2 H, $J = 7.5$ Hz, $-\text{CH}_2-\text{CO}$), 3.58 (d, 1 H, $J = 13.2$ Hz, CH_a-N), 3.72 (dd, 1 H, $J = 7.5/13.2$ Hz, CH_b-N), 3.99–4.20 [m, 3 H, $-\text{O}-\text{CH}_2-\text{CH}(\text{O})-$], 4.78 (s, 1 H, OH), 6.93–7.55 (m, 14 H, aromatic H); analysis ($\text{C}_{28}\text{H}_{31}\text{NO}_4$) C, H, N.

***N*-Benzyl-*N*-(3-(2-(1-oxo-3-phenyl-propyl)phenoxy)-2-hydroxypropyl)-propionic acid amide (GP366).** 1-(2-(3-Benzylamino-2-hydroxy-propoxy)phenyl)-3-phenyl-1-propanone (Ecker et al., 1996; 0.59 g, 1.5 mmol) was dissolved in 25 ml of pyridine, and 0.14 ml (1.6 mmol) propionylchloride was added. The reaction mixture was stirred for 2 h and concentrated on an evaporator. The resulting oil was dissolved in ethyl acetate and washed twice with 0.1 N HCl. The organic phase was dried over Na_2SO_4 and evaporated to dryness, and the resulting oil was purified via column chromatography (silica gel, diethyl ether/ethyl acetate) to yield 0.29 g (42.7%) GP366 as colorless oil: ^1H NMR (chloroform-*d*) 1.16 (t, 3 H, $J = 7.5$ Hz, $-\text{CH}_3$), 2.41 (qu, 2 H, $J = 7.5$ Hz, $-\text{CH}_2-\text{CH}_3$), 2.95 (t, 2 H, $J = 7.8$ Hz, $\text{Ph}-\text{CH}_2-$), 3.18–3.24 (m, 2 H, $-\text{CH}_2-\text{CO}$), 3.53 (dd, 1 H, $J = 3.0/14.4$ Hz, CH_a-N), 3.64 (dd, 1 H, $J = 7.2/14.4$ Hz, CH_b-N), 4.00–4.16 [m, 3 H, $-\text{O}-\text{CH}_2-\text{CH}(\text{O})-$], 4.51 (d, 1 H, $J = 14.9$ Hz, $\text{Ph}-\text{CH}_a-\text{N}$), 4.58 (d,

1 H, $J = 14.9$ Hz, $\text{Ph}-\text{CH}_b-\text{N}$), 4.72 (broad, 1 H, OH), 6.93–7.62 (m, 14 H, aromatic H); analysis ($\text{C}_{28}\text{H}_{31}\text{NO}_4$) C, H, N.

1-(2-(3-(4-Benzoyl-1-piperazinyl)-2-hydroxy-propoxy)phenyl)-3-phenyl-1-propanone (GP388). 3-Phenyl-1-(2-(2-hydroxy-3-(1-piperazinyl)-propoxy)phenyl)-1-propanone (0.62 g, 1.7 mmol) was dissolved in a mixture of 5 ml dichloromethane and 25 ml triethylamine, and 0.2 ml (1.7 mmol) benzoylchloride was added. The reaction mixture was stirred for 1 h at 70°C and filtered off. The filtrate was diluted with dichloromethane, washed twice with water, dried over Na_2SO_4 , and evaporated to dryness. The resulting brown oil was purified via column chromatography (silica gel, dichloromethane/methanol/ammonia) to yield 0.35 g (45%) GP388 as yellow oil, which solidifies slowly: ^1H NMR (chloroform-*d*) 2.16–2.57 [m, 6 H, $-\text{CH}_2-\text{N}(\text{CH}_2)_2-$], 3.02 (t, 2 H, $J = 7.8$ Hz, $\text{Ph}-\text{CH}_2-$), 3.29–3.74 [m, 7 H, $-(\text{CH}_2)_2-\text{N}-\text{CO}$, CH_2-CO , OH], 4.02–4.07 [m, 3 H, $-\text{O}-\text{CH}_2-\text{CH}(\text{O})-$], 6.95–7.72 (m, 14 H, aromatic H); analysis ($\text{C}_{29}\text{H}_{32}\text{N}_2\text{O}_4$) C, H, N.

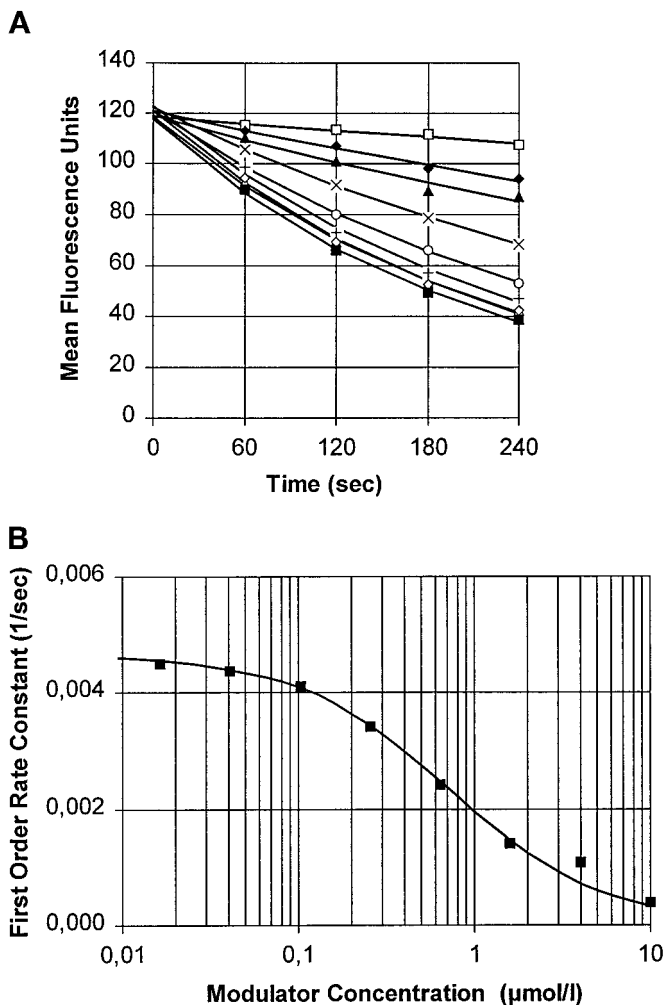
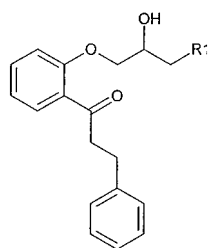


Fig. 1. Time course of a typical daunomycin efflux experiment in the absence of modulator and in the presence of increasing concentrations of GP05 (A). Curves are shown for the control (no modulator; ■) and GP05 at a concentration of 0.016 μM (Δ), 0.041 μM (\diamond), 0.102 μM (+), 0.256 μM (\circ), 0.640 μM (\times), 1.60 μM (\blacktriangle), 4.00 μM (\blacklozenge), and 10.0 μM (\square), respectively. Exponential curves are fitted to data points obtained after 1, 2, 3, and 4 min by the method of least-squares, and apparent efflux first order rate constants are determined for each modulator concentration. These efflux first order rate constants are plotted on the ordinate-versus-modulator concentration on the abscissa (B). The solid line represents a dose-response curve fitted to the data points by the method of least-squares using the EC_{50} value as adjustable variable.

TABLE 1

Chemical structure, physicochemical parameters, and PGP-inhibitory activity of compounds GP05 to GP570



General structure of compounds GP05 to GP388

Compound	R1	log <i>P</i>	p <i>K</i> _a	C _a	EC ₅₀ Rhodamine 123	EC ₅₀ Daunomycin	
						Observed	Predicted
GP05		3.67	8.44	5.90	1.45	0.65	1.49
GP29		4.43	7.45	6.70	0.62	0.38	0.49
GP31		4.93	7.33	7.24	0.26	0.14	0.26
GP62		3.98	7.56	7.45	0.11	0.06	0.23
GP240		4.23	3.25	4.72	17.61	6.47	6.71
GP339		4.05	1.70	5.87	4.57	1.54	1.43
GP358		5.15	1.73	4.75	17.87	8.49	6.05
GP359		4.26	0.23	5.21	5.89	2.57	3.62
GP360		4.60	-1.46	6.48	2.57	1.65	0.58
GP366		4.60	N.A.	6.55	2.92	2.04	0.51
GP388		3.87	6.67	8.07	0.36	0.13	0.06
GP570		3.35	N.A.	3.50	44.45	30.80	36.25

N.A., not applicable.

Calculation of Hydrogen Bond Acceptor Values

The calculation of the hydrogen bond acceptor strength was performed using the software package HYBOTPLUS (pION, Cambridge, MA), which is based on a database of approximately 15,000 experimentally determined values. Only heteroatoms were considered, and in the case of amide and ester moieties, nitrogen/oxygen and carbonyloxygen were considered as mutually exclusive with respect to interaction as hydrogen bond acceptor. In these cases (GP360, GP366, and GP570), the value of the stronger electron donor (C=O) was introduced into the equation. Thus, the sum of the hydrogen bond acceptor strength in the vicinity of the nitrogen atom was determined.

Calculation of logP Values

The logP values were calculated according to the method of Ghose et al. (1988) using the software package MOLGEN (CHERS, Bratislava, Slovakia). As previously demonstrated for a series of propafenone analogs, the calculated values correlate excellently with $\log k_w$ values obtained using two different HPLC methods (Prets et al., 1996). The molecules were generated using the builder function and were energetically minimized using the MM2 algorithm implemented in the optimization tool. Conformationally independent logP values were calculated.

Calculation of pK_a Values

Calculation of the pK_a values were performed using the software package PALLAS (VCH, Weinheim, Germany). In case of piperazine derivatives, the pK_a value of the nitrogen atom with higher basicity was taken for correlation analysis. For compounds GP366 and the ester GP570, pK_a values could not be determined.

Cell Lines

The human T-lymphoblast cell line CCRF-CEM and the MDR CCRF VCR1000 cell line were provided by V. Gekeler (Byk Gulden, Konstanz, Germany). The resistant CCRF VCR1000 line was obtained through stepwise selection in vincristine-containing medium (Gekeler et al., 1992). Cells were kept under standard culture conditions (RPMI 1640 medium supplemented with 10% FBS). The PGP-expressing resistant cell line was cultured in presence of 1000 ng/ml vincristine. One week before the experiments, cells were transferred into medium without selective agents or antibiotics.

Daunomycin and Rhodamine 123 Efflux Studies

Efflux studies were performed as described by Chiba et al. (1996). Briefly, cells were pelleted, the supernatant was removed by aspiration, and cells were resuspended at a density of 1×10^6 /ml in RPMI 1640 medium containing either 3 μ M daunomycin or 0.53 mM rhodamine 123. Cell suspensions were incubated at 37°C for 30 min. After this time, a steady state of accumulation was reached. Tubes were chilled on ice, and cells were pelleted at 500g. Cells were washed once in RPMI 1640 medium to remove extracellular fluorochrome. Subsequently, cells were resuspended in medium prewarmed to 37°C containing either no modulator or chemosensitizer at various concentrations ranging from 3 nM to 500 μ M, depending on solubility and expected potency of the modifier. The latter is a prediction based on lipophilicity of the compound (Chiba et al., 1996). Generally, eight serial dilutions were tested for each modulator. After 1, 2, 3, and 4 min for daunomycin and 30, 60, 90, and 120 s for rhodamine 123, aliquots of the incubation mixture were drawn and pipetted into 4 volumes of ice-cold stop solution (RPMI 1640 medium containing verapamil at a final concentration of 100 μ M). Parental CCRF-CEM cells were used to compensate for simple membrane diffusion, which was less than 3% of the efflux rates observed in resistant cells. Samples drawn at the respective time points were kept in an ice water bath and measured within 1 h on a Becton

Dickinson FACS Calibur flow cytometer as described. Dose-response curves were fitted to the data points using the nonlinear least-squares method, and EC₅₀ values were calculated as described by Chiba et al. (1996). Time courses of daunomycin efflux in the absence and presence of different concentrations of modulator and the corresponding dose-response curve are shown for GP05 as an example (Fig. 1, A and B).

Results

Compounds were designed to cover a broad range of pK_a values ranging from 6.67 to 8.44 for tertiary amines to 0.23 to 3.25 for anilines and -1.46 for amide GP360. The ester served as a molecular probe for the general requirement of a nitrogen atom. As outlined in Table 1, all compounds showed moderate-to-high PGP inhibitory activity. For daunomycin efflux inhibition, EC₅₀ values ranged from 0.06 to 30.80 μ M. These values excellently correlated with those for rhodamine 123 efflux inhibition ($r = 0.995$; Fig. 2). As previously demonstrated for homologous series of amines, lipophilicity is one of the predictive parameters for biological activity. The lack of correlation of calculated lipophilicity values with log potency is shown in Fig. 3 ($r = 0.034$, $P = .916$).

The amines (GP05, GP29, and GP31) are located close to the regression line previously determined for propafenone analogs, whereas anilines, amides, and the ester are located below the line. The hydroxyphenylpiperidine GP62 and the benzoylpiperazine GP388 show higher activity than predicted by lipophilicity alone; therefore, for the entire set of compounds used in the present study, a bias toward lipophilicity does not exist.

No significant correlation between pK_a and potency was found; thus, the basicity of the nitrogen atom does not allow prediction of PGP-inhibitory activity of propafenone analogs.

Calculation of hydrogen bond acceptor strength was performed as detailed in *Materials and Methods* using the software package HYBOTPLUS. C_a values are given in Table 1. As shown in Fig. 4, an excellent correlation between the sum of heteroatomic C_a values and PGP-inhibitory potency

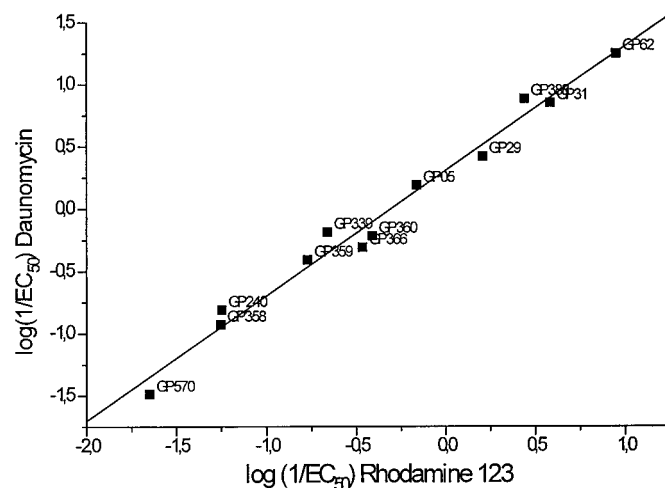


Fig. 2. Correlation between EC₅₀ values obtained in daunomycin efflux experiments and those obtained in rhodamine 123 efflux experiments.

(expressed as daunomycin efflux inhibition) was obtained for 12 compounds (eq. 1).

$$\log(1/EC_{50}) = 0.58(\pm 0.07) C_a - 3.55(\pm 0.44)$$

$$n = 12, r = 0.93, P = 10^{-5}, F = 65.9; Q_{cv}^2 = 0.82 \quad (1)$$

A leave-one-out cross-validation procedure demonstrated the high predictivity of the model ($Q_{cv}^2 = 0.82$; Table 1). A multiple linear regression analysis using both C_a and $\log P$ values as x -descriptors showed that within this set of compounds, lipophilicity does not significantly contribute to the observed variance in activity (eq. 2).

$$\log(1/EC_{50})$$

$$= 0.60(\pm 0.16) C_a - 0.23(\pm 0.38) \log P - 2.68(\pm 1.73) \quad (2)$$

Discussion

One recurring tenet in structure-activity relationship studies on PGP modulators is the requirement of a basic nitrogen

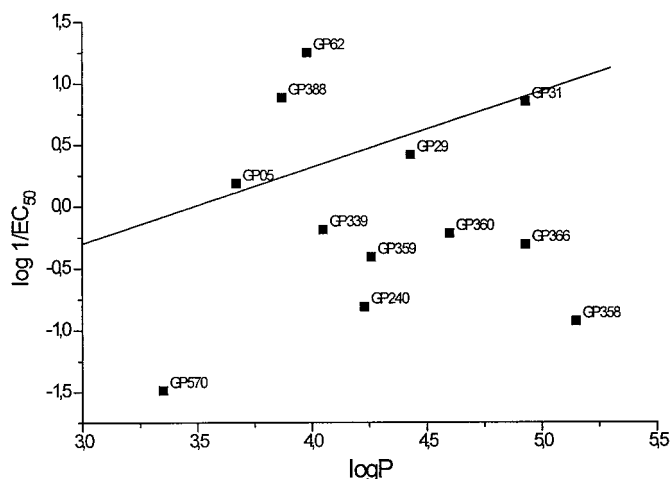


Fig. 3. Correlation between calculated $\log P$ and $\log 1/EC_{50}$ (log potency) tested in the CCRF vcr1000 cell line with the daunomycin efflux assay. The solid line represents the correlation previously obtained for propafenone analogs.

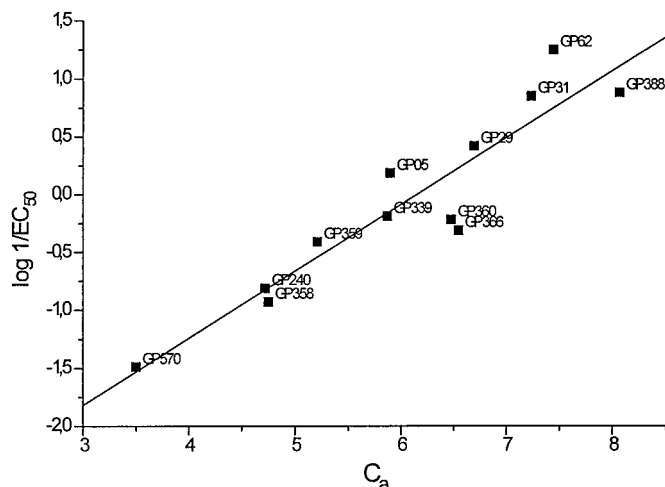


Fig. 4. Correlation between hydrogen bond acceptor strength (C_a) and $\log 1/EC_{50}$ (log potency) tested in the CCRF vcr1000 cell line with the daunomycin efflux assay. Solid line obtained through linear regression of C_a versus log potency values.

atom in the molecule. However, substances lacking a nitrogen atom have been described as being active modulators (Ueda et al., 1992). This discrepancy was recently discussed by Seelig (1998), who showed preliminary evidence that the presence of at least two hydrogen bond acceptor units is required for the interaction of low-molecular-weight substances with PGP. From the literature, the following questions remained unresolved: 1) Does a nitrogen atom contribute to activity of MDR modulators by its alkalinity? 2) Is hydrogen bond acceptor strength a predictor of pharmacological activity?

In this report, a set of 12 compounds was designed, synthesized, and tested in fluorochrome efflux inhibition studies, whereby special attention was given to an even distribution among strongly basic amines, weakly basic anilines, and nonprotonable amides. The compounds covered a calculated pK_a range from -1.46 to 8.44 . To test for the general requirement of a nitrogen atom, the ester GP570 was included in the set. All compounds showed PGP-inhibitory potential, which indicates that a basic nitrogen atom is not an absolute requirement for activity. This is clearly demonstrated for the amides GP360 and GP366, which are not protonable in aqueous solutions.

A bias toward lipophilicity, which was previously defined as being important for activity (Chiba et al., 1996), was not introduced into the data set. This was shown both with linear regression analysis using $\log P$ as the independent variable (Fig. 3) and with multiple linear regression analysis. The latter demonstrated that within the given set of substances, $\log P$ did not significantly contribute to the description of the variance of the pharmacological activity data (eq. 2). This allowed the quantification of the influence of the hydrogen bond acceptor strength on biological activity of the compounds. As shown in Fig. 4, an excellent correlation between C_a values and potency was found for the complete set of substances. This also included GP570, which contains an ester moiety as a strong hydrogen bond acceptor subunit but lacks a nitrogen atom. A leave-one-out cross-validation procedure demonstrated the high predictivity of the obtained model (Table 1).

Data show that the interaction of the nitrogen atom with PGP is nonionic and determined by electron donor capability. In this region of the molecule, a nitrogen atom is not an absolute requirement for activity and only influences activity through its contribution to hydrogen bond acceptor strength.

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