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# Structure–Activity Relationship Studies of Propafenone Analogs Based on P-Glycoprotein ATPase Activity Measurements

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**ABSTRACT.** Propafenone analogs (PAs) were previously identified as potent inhibitors of P-glycoprotein (Pgp)-mediated toxin efflux. For this as well as other classes of Pgp inhibitors, lipophilicity as well as hydrogen bond acceptor strength are important determinants of biological activity. The question as to whether a direct interaction between PA-type modulators and Pgp takes place was addressed by means of Pgp ATPase measurements and transport studies. Propafenone-type modulators stimulated ATPase activity up to 2-fold over basal activity in a concentration-dependent biphasic manner. Within a series of structural homologs, $K_a$ values of ATPase stimulation strongly correlated with lipophilicity. Analogs containing a quaternary nitrogen stimulated Pgp ATPase activity with lesser efficacy, while $K_a$ values were somewhat higher when compared to corresponding tertiary analogs. Transport studies performed in inside-out plasma membrane (I/O) vesicles demonstrated that analogs containing a tertiary nitrogen rapidly associated with the biomembrane. Quaternary analogs, which are restricted by a permanent positive charge in transiting the plasma membrane by diffusion, accumulated in Pgp containing I/O vesicles in an ATP-dependent and cyclosporin A-inhibitable manner, which identified them as Pgp substrates. Identical structure–activity relationships were found in either Pgp ATPase stimulation experiments in I/O vesicles or in toxin efflux inhibition studies using intact cells. Therefore, differences in membrane transit are not responsible for the observed structure–activity relationships. BIOCHEM PHARMACOL **58**;9:1447–1456, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** P-glycoprotein; propafenone analogs; Pgp ATPase; structure–activity relationships; CCRF-CEM cells

Pgp§ is a member of the ATP-binding cassette [1]. This integral plasma membrane protein mediates the energy-dependent extrusion of a wide variety of natural toxins, thereby rendering cells multidrug-resistant [2, 3]. Several substance groups have been described over the past 15 years which are able to revert the resistance phenotype of Pgp-expressing cells by inhibition of this efflux transporter [4]. These Pgp inhibitors include channel blocking agents (e.g. verapamil, quinidine, amiodarone and dihydropyridine analogs), steroids, antipsychotic drugs, and cyclic peptides including cyclosporins. Most of these substances contain a nitrogen atom, which is positively charged at physiological pH. Another common property of active modulators is high lipophilicity.

We previously identified propafenone, which is in clinical use as a class 1c antiarrhythmic drug, as an inhibitor of Pgp-mediated drug resistance and efflux [5]. In addition, we demonstrated that PAs enhance the cytoxicity of daunorubicin in multidrug-resistant cell lines. Modulator potencies ($1/EC_{50}$ values) were determined by combined simultaneous analysis of sigmoidal dose–response curves [6]. The potencies obtained in cytotoxicity assays correlated significantly with $EC_{50}$ values from rhodamine 123 efflux studies. Similar to results obtained by Ford *et al.* for the class of phenothiazines [7], the lead substance propafenone as well as a series of structural analogs exhibited activities which were closely correlated with their octanol/water partition coefficients (logP values) [8]. A carbonyl group next to the central aromatic ring was identified as a pharmacophoric substructure and most likely interacts via formation of a hydrogen bond [8]. This was also shown for the class of thienothiazine inhibitors of Pgp‖. Similarly, in a comparison of one hundred compounds previously tested as Pgp substrates, Seelig reported the basic element of recognition by Pgp to

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§ *Abbreviations:* Pgp, P-glycoprotein; PAs, propafenone analogs; CCRF ADR5000, doxorubicin-resistant CCRF-CEM cells; I/O vesicles, inside-out oriented plasma membrane vesicles; and wt, wild-type.

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‖ Ecker G, Erker T, Schreder M, Schmid D, Richter E, Hitzler M, and Chiba P, unpublished observations.

be electron donor groups, which must be arranged in distinct spatial patterns [9].

The aim of the present study was to establish structure–activity relationships on the basis of Pgp ATPase activity measurements. In contrast to toxin efflux assays, these measurements do not require a plasma membrane transit of the modulators. Therefore, this approach allowed us to test whether the higher activity of certain analogs is due to differences in plasma membrane transit or reflects properties necessary for direct interaction with Pgp.

## MATERIALS AND METHODS

### *Cell Culture*

The T-lymphoblast cell line CCRF-CEM and the doxorubicin-resistant subline CCRF ADR5000 were a gift from V. Gekeler (Byk Gulden, Konstanz, Germany). The cell lines have been characterized previously. For ADR5000 cells, Pgp expression has been shown to be the cause of their multidrug-resistant phenotype [10–13]. Cells were maintained under standard cell culture conditions. The resistant subline was cultured in the presence of 5 μg/mL doxorubicin. One week prior to the experiments, the selective medium was washed out by centrifugation and subsequent resuspension of cells in fresh culture medium.

### *Synthesis of Compounds*

The preparation of the tertiary amines was carried out in analogy to the synthesis of propafenone as described [5, 8]. The quarternary analogs GP03, GP06, GP07, and GP10 were prepared from the corresponding tertiary amines by reaction with methyliodide [14].

### *Preparation of Plasma Membrane Vesicles*

The preparation of plasma membrane vesicles was performed as described by Schlemmer and Sirotnak [15], with minor modifications. In brief, $3–4 \times 10^9$ cells were washed with PBS by centrifugation at 500 *g* for 7 min. The cell pellet was resuspended in 10 mL of cavitation buffer containing 20 mM TRIS–HCl, pH 7.4, 250 mM sucrose, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). All subsequent steps were performed in an ice-water bath. The cells were equilibrated with nitrogen gas at a pressure of 1000 pounds per square inch in a Parr 540 cell disruption bomb (Parr Instruments). After 20 min the cells were passed through the opening valve. In this step, cell membrane fragments vesiculate spontaneously, and approx. 75% of vesicles were shown to be oriented in an inside-out (I/O) fashion as checked by concanavalin A precipitation and subsequent measurement of Na/K-ATPase activity as described below. One millimole of EDTA was immediately added to the homogenate. Nuclei were pelleted by centrifugation at 2000 *g* for 6 min. The supernatant was centrifuged at 7500 *g* for 12 min. The pellet was discarded and the supernatant was spun in an ultracentrifuge (Beckman Instruments) at 48,000 *g* for 30 min. The membrane pellet was resuspended at a concentration of 3 mg/mL total protein and layered over a 16%/45% (w/v) discontinous sucrose gradient and spun at 48,000 *g* for 30 min. The 16/45 interface was washed from excess sucrose by addition of buffer and an additional ultracentrifugation step. Pellets were resuspended in ATPase assay buffer and the protein concentration was adjusted to 0.5 mg/mL as determined by the method of Bradford [16] using bovine gamma globulin as a standard.

Na/K-ATPase and Ca-ATPase were measured as marker enzymes for plasma membranes and contaminating endoplasmic reticulum, respectively. Azide-sensitive ATPases were used to detect potentially contaminating mitochondria. Purity of the plasma membrane preparation was confirmed by electron microscopy, which proved the absence of endoplasmic reticulum and mitochondria. Vesicles ranged in size from 50 to 200 nm. For estimation of the proportion of inside-out to rightside-out vesicles, we employed concanavalin A precipitation and subsequent measurement of Na/K-ATPase activity in the presence of different SDS concentrations both in the precipitated and non-precipitated fractions [17]. In brief, 100 μL of concanavalin A-Sepharose (Sigma) in 0.1 M Na acetate puffer pH 6.0 was dried by vacuum centrifugation and reconstituted with distilled water. One hundred micrograms of the membrane vesicle preparation was washed free from sucrose by ultracentrifugation at 48,000 *g* for 10 min in a TL-100 ultracentrifuge (Beckman Instruments). The supernatant was removed by suction and the pellet resuspended in 20 mM Tris–HCl, pH 7.4, 250 mM cellobiose, and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. One hundred microliters of concanavalin A-Sepharose beads was added and the mixture was agitated for 1 hr at 4°. The precipitate was sedimented by centrifugation at 2000 *g* for 5 min. The supernatant was collected, and the pellet was washed three times with Tris-cellobiose buffer and subsequently resuspended in ATPase buffer.

### *Measurement of P-Glycoprotein ATPase Activity*

These assays were performed according to a modification of a method originally described by Chifflet *et al.* [18] for the determination of Na/K-ATPase activity. The protocol is based on the colorimetric determination of inorganic phosphate released by the hydrolysis of ATP. The assay mixture contained 20 mM Tris–HCl, pH 7.4, 64 mM sucrose, 4.8 mM $MgCl_2$, 0.2 mM EGTA, 10 mM $NaN_3$, 1 mM ouabain, and 5 μg of plasma membrane protein for each time point. A serial dilution of the respective Pgp modulator spanning a concentration range from 10 nM to 1.5 mM was added. Lipophilic modulators were dissolved in DMSO and diluted into the assay mixture to obtain a final DMSO concentration of 1% (v/v). The specific inhibitors ouabain, EGTA, and $NaN_3$ were added to block Na/K-, $Ca^{2+}$-, and mitochondrial ATPases. The difference between parental and Pgp-expressing cells was termed basal (unstimulated) Pgp

ATPase activity. The assay mixtures were preincubated at 37° and the reactions were started by the addition of 2 mM ATP. After 10, 20, 30, and 40 min, the reaction was stopped by pipetting aliquots of the incubation mixture into an equal volume of 12% (w/v) SDS. The same volume of a mixture of freshly prepared 6% ascorbic acid in 1 N HCl and 1% ammoniumheptamolybdate was added. Samples were incubated at room temperature for 5 min. The color development was stabilized and enhanced by addition of 2% (w/v) trisodium citrate and 2% (w/v) sodium *meta*-arsenite in 2% (v/v) acetic acid. The optical density was measured at 630 nm in an EL311 Bio-Tek microtiter plate reader (Bio-Tek Instruments). ATPase activity in wt membranes in the presence of 1 mM ouabain was defined as residual ATPase activity. Blanks were measured in the absence of plasma membranes. An assay mixture containing 10 μM verapamil was used as a positive control for Pgp stimulation. The ATPase activities were plotted versus modulator concentration, and a hyperbolic function was fitted to the data points defining the ascending parts of the curve using least squares. $K_a$ values were either related to lipophilicity of the compounds or compared to $EC_{50}$ values obtained in rhodamine123 efflux inhibition experiments. For each modulator at least three independent experiments were performed. Experiments were repeated until a coefficient of variation of ≤20% was obtained.

### *Rhodamine123 Efflux Studies*

Rhodamine123 efflux studies were performed as previously described [8], except that CCRF ADR5000 cells were used in the experiments. In brief, the cells were pelleted, the supernatant was removed by aspiration, and the cells resuspended at a density of $1 \times 10^6$/mL in RPMI1640 medium containing 0.2 mg/L rhodamine123. Cell suspensions were incubated at 37° for 30 min after which time a steady state of rhodamine123 accumulation was reached. Tubes were chilled on ice and cells were pelleted at 500 *g*. Cells were washed once in RPMI1640 medium to remove extracellular fluorochrome. Subsequently, cells were resuspended in medium prewarmed to 37° and containing either no modulator or a chemosensitizer at various concentrations ranging from 3 nM to 500 μM, depending on the solubility and expected potency of the modifier. Generally, 8 serial dilutions were tested for each modulator. After 30, 60, 90, and 120 sec, aliquots of the incubation mixture were drawn and pipetted into 4 volumes of ice-cold stop solution (RPMI1640 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 rates observed in resistant cells. Samples drawn at the respective time points were kept in an ice-water bath and measured within 1 hr on a Becton Dickinson FACS Calibur flow cytometer as described. Hyperbolic dose–response curves were fitted to the data points using non-linear least squares, and $EC_{50}$ values were calculated as described [8].

### *Transport studies*

GENERAL PROCEDURE FOR THE SYNTHESIS OF $^3$H-LABELED COMPOUNDS. To a solution of 0.9 mmol of the corresponding secondary or tertiary amine in methanol, 0.30 mL of a 140 μM solution of [$^3$H]$CH_3I$ in toluene (71.4 Ci/mmol, 10 m Ci/mL, Amersham) was added. After stirring for 1 hr in a closed reaction vessel, the reaction was completed by addition of unlabeled $CH_3I$ (TLC-control). For isolation of the desired compounds, the procedure described for the corresponding unlabeled derivatives was used [8].

TRANSPORT STUDIES IN WHOLE CELLS. The uptake of [$^3$H]daunomycin was performed as described by Kraupp *et al.* [19] using a rapid centrifugation filtration method. Briefly, cells were suspended in RPMI1640 medium containing 2% fetal bovine serum at a density of $5 \times 10^6$/mL. 0.4 μCi of [$^3$H-G]daunomycin (0.4 μCi; 3.6 Ci/mmol, 1 mCi/mL, New England Nuclear) and unlabeled daunomycin were added to the cell suspension to give a final concentration of 1 μmol/L. Modifier stock solutions were prepared in pure DMSO at 100 × the final concentration and added 10 min prior to the addition of radioactivity. Samples were incubated at 37°. At the respective time points, 1-mL aliquots were transferred to 1.8-mL polypropylene tubes containing a bottom layer of dibutylphtalate/dioctylphtalate 4 + 1 (w/w) (density 1.035 g/mL), and cells were separated from the incubation medium by centrifugation. After centrifugation, medium was removed from the tubes by suction and residual radioactivity was washed off with water. After the second washing step, the water layer was removed together with most of the oil and cell pellets were solubilized in 0.5 M NaOH/1% (w/v) SDS. Cell-associated radioactivity was then determined in a liquid scintillation counter. For experiments with [$^3$H]GP02 and [$^3$H]GP06, cells were incubated with 1 μCi of labeled chemosensitizer at a concentration of 24 μmol/L. Uptake was measured over a time period of 30 min and 1-mL aliquots were drawn at the respective points.

TRANSPORT STUDIES IN INSIDE-OUT VESICLES. ATP-dependent uptake of substrates into inside-out vesicles was determined as described by Schlemmer and Sirotnak [16]. Briefly, 50 μL of plasma membranes was resuspended in transport buffer (100 mM sucrose, 100 mM MOPS (3-(*N*-morpholino)propanesulfonic acid), 5 mM $MgCl_2$, 40 mM KOH, pH 7.0) and preincubated at 37° in siliconized glass tubes for 5 min. Transport was initiated by the addition of 5 mM ATP simultaneously with 100 nM [$^3$H]vinblastine (11 Ci/mmol, 0.25 μCi/mL, Amersham) or 3 μM [$^3$H]GP06. To correct for non-specific filter binding, samples for zero time points were kept on ice for 10 sec in the presence of radioactivity prior to centrifugation, and were subtracted as a blank. For each time point a separate sample was used. After the desired time intervals, 10 mL of ice-cold transport buffer was added and samples were immediately filtered through HAWP 0025 filters (Millipore) using a

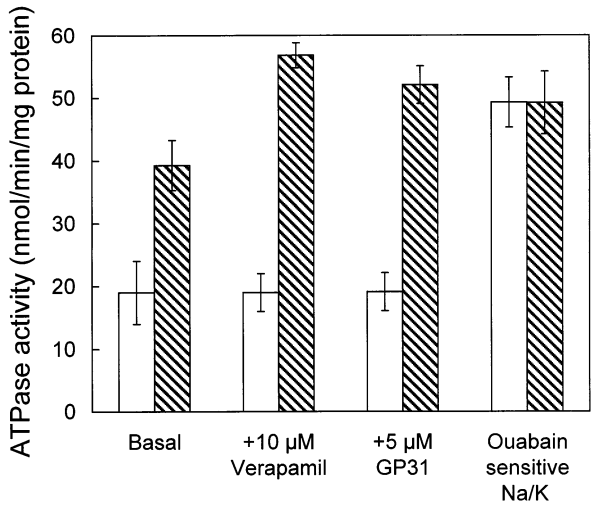



**FIG. 1. ATPase activity in membrane vesicles from CCRF-CEM/wt cells (open bars) and P-glycoprotein-containing CCRF ADR5000 cells (hatched bars). The plasma membrane-specific, ouabain-sensitive Na/K-ATPase activity was equal in both preparations. In the presence of 1 mM ouabain wt membranes had an activity of 19 ± 5 nmol/min/mg protein compared to 39.3 ± 5 nmol/min/mg protein in Pgp-containing membranes. The difference was basal Pgp ATPase activity. In the presence of 10 µM verapamil, the Pgp ATPase activity was stimulated 1.9-fold. At 5 µM, the propafenone analog GP31 stimulated Pgp ATPase activity approximately 1.6-fold. Verapamil and GP31 did not have any effect in vesicles prepared from parental cells.**

negative pressure of 100 mm Hg. The filter disks were washed three times with 10 mL of ice-cold transport buffer. Filters were air-dried, liquid scintillation cocktail was added, and radioactivity was counted in a PACKARD CA2000 liquid scintillation counter (Packard Instruments).

## RESULTS

### *Effect of Propafenones on P-Glycoprotein ATPase Activity*

In the presence of ouabain, EGTA, and Na-azide, which are potent inhibitors of the membrane-bound Na/K-, $Ca^{2+}$-, and mitochondrial ATPases, respectively, the Pgp-containing vesicles exhibited an ATPase activity of 39.3 ± 5 nmol/min/mg protein, which was approximately twofold higher than the activity in wt membranes. This difference is termed basal Pgp ATPase activity according to Garrigos *et al.* [20] and represents the sum of unstimulated Pgp ATPase and residual ATPase activities of unknown origin. The average of ouabain-sensitive Na/K-ATPase activity was 49.2 ± 4 nmol/min/mg protein in wt cells and 49.2 ± 5 nmol/min/mg protein in Pgp-expressing cells (Fig. 1). Verapamil (10 µM) and 5 µM of the most active propafenone analog, GP31, stimulated ATPase activity 1.9- and 1.6-fold in P-glycoprotein-containing CCRF ADR5000 membrane vesicles, but not in vesicles from wt cells (Fig. 1). Ouabain-sensitive (Na/K)-ATPase activity was unaltered in the presence of PAs at concentrations exceeding $K_a$ values by a factor of up to 100 (data not shown).

Figure 2 shows the concentration dependencies of stimulation of Pgp ATPase activity for analogs GP62, GP45, and GP17 as representative examples of PAs with high, intermediate, and low activity. Dose–response curves revealed a biphasic characteristic. At lower concentrations, propafenone and its tertiary analogs stimulated Pgp ATPase activity up to twofold in a concentration-dependent manner. When exceeding a critical concentration, a gradually lower stimulation of ATPase activity was observed. For comparison, dose–response curves for cyclosporin A, a known inhibitor of Pgp, and of daunomycin, a known substrate of Pgp, are included in Fig. 2. Cyclosporin A showed a monophasic inhibition characteristic with a half-maximal inhibitory concentration of approximately 100 nmol/L. The Pgp substrate daunomycin led to a small, though measurable stimulation, and at higher concentrations to a gradually increasing inhibition of Pgp ATPase activity.

After subtraction of the basal Pgp ATPase activity, hyperbolic dose–response curves were fitted to data points defining the ascending parts of the curves [21]. Activation constants and efficacies were used as variable parameters of the fitting equation, whereby efficacies of the lead compound propafenone and its tertiary analogs were comparable (1.8- to 2.1-fold). A synopsis of structure and calculated logP and $K_a$ values is given in Table 1. The average $K_a$ value for the lead compound propafenone was determined to be 3.34 ± 0.15 µM in independent experiments using different plasma membrane preparations. The most active

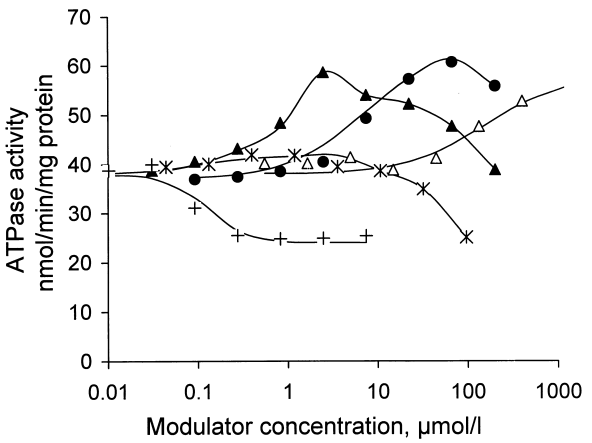
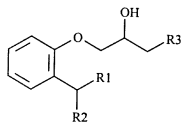
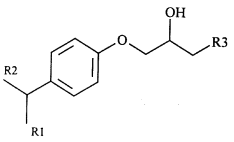



**FIG. 2. Effect of tertiary PAs on P-glycoprotein ATPase activity in Pgp-containing plasma membrane vesicles. Concentration dependencies are given for GP62(▲), GP45(●) and GP17(Δ) as representative examples of PAs with high, intermediate, and low activity. The rate of ATP hydrolysis was measured as described in the Methods section. Concentration-dependent modulation of Pgp ATPase activity by cyclosporin A (+) and daunomycin (*) is shown for comparison. For each substance, data from one representative experiment are shown. Hyperbolic dose–response curves were fitted to the data points by non-linear regression analysis. A synopsis of chemical structure, logP, and $K_a$ values is given in Table 1.**

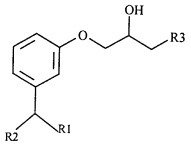
**TABLE 1. General formulas for propafenone analogs**




para




meta



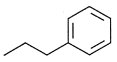
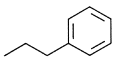
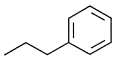
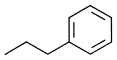
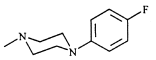
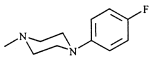
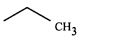
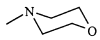
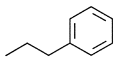
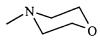
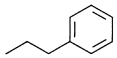
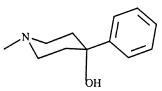
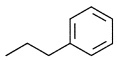
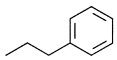
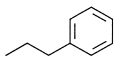
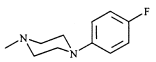
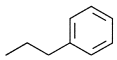
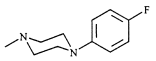
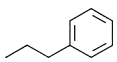
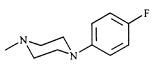
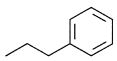
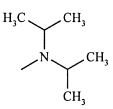
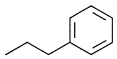
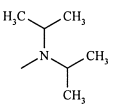
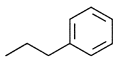
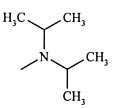
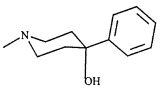
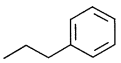
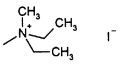
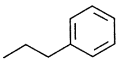
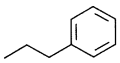
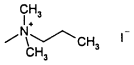
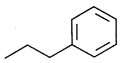
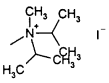

| GP Code* | $R^1$ | $R^2$ | $R^3$ | calcd. logP (Molgen) | Ka (μmol/L) ATPase | $EC_{50}$ (μmol/L) Rh123 efflux |
|---|---|---|---|---|---|---|
| | |  |  | | | |
| 01 | =0 |  |  | 3.39 | 3.34 | 2.11 |
| 02 | =0 |  |  | 3.62 | 5.3 | 2.59 |
| 05 | =0 |  |  | 3.67 | 2.59 | 2.12 |
| 17 | =0 |  |  | 1.42 | 122 | 278 |
| 31 | =0 |  |  | 4.93 | 0.36 | 0.64 |
| 45 | =0 |  |  | 2.67 | 6.13 | 10.9 |
| 46 | =0 |  |  | 0.94 | 120 | 2219 |
| 57 | =0 |  |  | 2.54 | 18.5 | 17.7 |
| 62 | =0 |  |  | 3.98 | 1.01 | 0.307 |
| 88 | —OH |  |  | 3.94 | 12.8 | 3.28 |
| 90 | —$OCH_3$ |  |  | 4.3 | 1.53 | 0.891 |
| 134 | =0 |  |  | 4.93 | 1.47 | 21.5 |
| para | | | | | | |
| 155 | —OH |  |  | 5.2 | 0.55 | 9.96 |
| 157 | =0 |  |  | 4.93 | 4.15 | 5.62 |
| meta | | | | | | |
| 159 | =0 |  |  | 4.25 | 7.64 | 10.7 |
| para | | | | | | |
| 163 | —OH |  |  | 4.52 | 12.2 | 4.13 |
| 164 | —$OCH_3$ | | | 4.88 | 2.26 | 0.63 |

**TABLE 1. Continued**

| GP Code* | $R^1$ | $R^2$ | $R^3$ | calcd. logP (Molgen) | Ka (μmol/L) ATPase | $EC_{50}$ (μmol/L) Rh123 efflux |
|---|---|---|---|---|---|---|
| 381 | =0 |  |  | 2.38 | 10.5 | 3.19 |
| 389 | =0 |  |  | 1.42 | 1160 | 1950 |
| para | | | | | | |
| 03 | =0 |  |  | ND† | 6.7 | 916 |
| 06 | =0 |  |  | ND | 8.4 | 31.0 |
| 07 | =0 |  |  | ND | 4.4 | 943 |
| 10 | =0 |  |  | ND | 6.0 | 103 |

*Unless otherwise noted, the substitution pattern on the central aromatic ring is in the *ortho*-position.
†ND, not determined.

analog GP31 ($K_a$ = 0.36 ± 0.076 μM) was 9.3-fold more active than propafenone itself. In comparison with tertiary analogs, quaternary PAs showed a somewhat different picture. Figure 3 shows the concentration dependence of Pgp ATPase modulation for the quaternary analog GP03 (■) in comparison with its corresponding tertiary analog GP02 (▲). The maximal stimulation by the quaternary analog was lower than that of its tertiary counterparts (2-fold vs 1.5-fold). A similar picture was obtained for the substance pair GP05 (tertiary) and GP06 (quaternary) (1.9-fold vs 1.2-fold) and the lead compound propafenone (GP01) and its quaternary analog GP07 (2-fold vs. 1.6-fold) (data not shown). $K_a$ values were in a comparable order of magnitude for both species (Table 1). Lower maximal stimulation of Pgp ATPase activity had been reported to be a feature of a variety of well-transported Pgp substrates such as daunomycin [21], which at this point suggested an active transport of quaternary analogs.

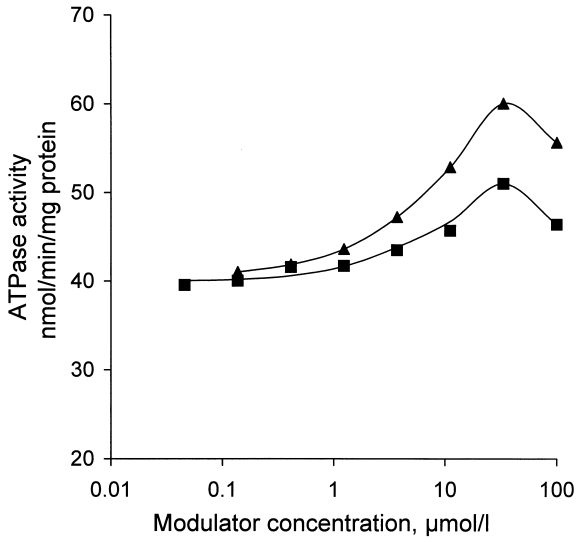



**FIG. 3. Comparison of the modulation of P-glycoprotein ATPase activity by tertiary and quaternary PAs. The ATP hydrolysis rate was measured as described in the Methods section. Concentration dependencies of the tertiary analog GP02 (▲) and its corresponding quaternary analog GP03 (■) are shown as an example. Maximal stimulation was lower for the quaternary than for the tertiary analog.**

A linear correlation between the activation potencies ($\log(1/K_a)$) and lipophilicities (logPs) of structurally related tertiary compounds (shown as filled triangles in Fig. 4) was observed ($r^2$ = 0.91), whereby analogs with higher octanol/water partitioning coefficients (logPs) showed higher activities. The correlation was similar to that found earlier between lipophilicities and potencies determined in rhodamine123 efflux inhibition studies [8]. Reduction of the carbonyl group to an alcohol or an ether results in a decrease in electron donor strength (hydrogen bond acceptor strength) of the analogs. The hydroxy compounds GP88 and GP155, GP163 (open circles in Fig. 4) and the methoxy analogs GP90 and GP164 (inverted open triangles in Fig. 4) had lower ATPase stimulation potencies than predicted by lipophilicity alone, indicating that the hydrogen bond acceptor property is a prerequisite for high activity.

In addition to hydrogen bond acceptor strength, the distance between the nitrogen atom and carbonyl group was shown previously to influence activity of propafenone-type modulators in efflux experiments [8]. Therefore, $K_a$ values of compounds with *meta-* or *para*-substitution on the central aromatic ring (GP134, GP157, GP159, and GP389) were determined in ATPase stimulation experiments.

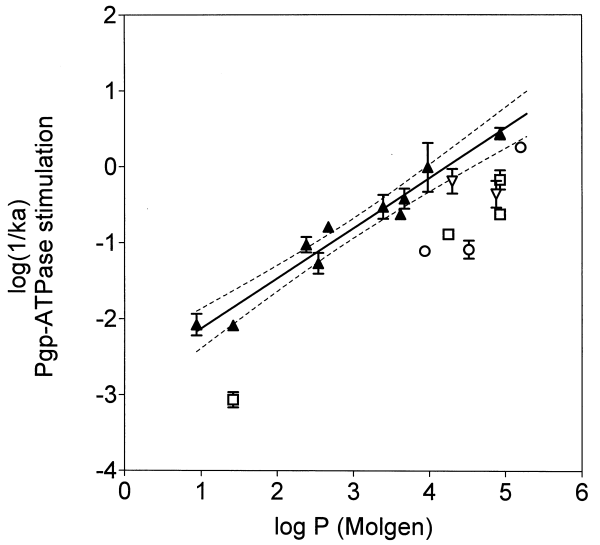



**FIG. 4. Correlation between calculated logP and log(1/$K_a$) values (Pgp ATPase activation potency) for CCRF ADR5000 cells. A linear correlation between logP and log(1/$K_a$) values was found for PAs (▲). Dotted lines indicate the 95% confidence interval of the linear regression line ($r^2$ = 0.91). Error bars indicate the standard deviation of at least 3 independent determinations of log(1/$K_a$) values. Hydroxy (○), methoxy (▽), and meta- and para-derivatives (□) show lower potency than predicted by their respective lipophilicity.**

These analogs are represented by open squares in Fig. 4. All four compounds were less active than predicted by their lipophilicities. Again, these data were in agreement with those obtained in toxin efflux inhibition assays [8]. A correlation between rhodamine123 efflux inhibition potencies (log1/$EC_{50}$) and ATPase stimulation potencies (log(1/$K_a$)) is given in Fig. 5. Propafenone itself as well as its tertiary analogs, depicted as filled triangles (▲), are located close to the dotted line, which represents an ideal 1:1 correlation. Quaternary analogs (open squares in Fig. 5) showed a tendency to be less active in whole-cell assays than in ATPase stimulation experiments performed in I/O vesicles.

### *Uptake of Quaternary and Tertiary Analogs in CCRF-CEM wt Cells*

Compounds GP02 (tertiary amine) and GP06 (quaternary) were obtained in radioactive form by reacting the corresponding tertiary amine with [$^3$H-$CH_3$]methyliodide (see Methods). Subsequently, uptake into wt cells was evaluated. Parental CCRF-CEM cells did not accumulate the quaternary analog [$^3$H]GP06 in measurable amounts (Fig. 6). In contrast, an extremely rapid association of tertiary [$^3$H]GP02 to identical steady-state levels was demonstrable in both sensitive and resistant cells, whereby steady-state levels were the same. Daunomycin uptake was included as a control and was approximately 3-fold higher in sensitive than in resistant cells (Fig. 6).

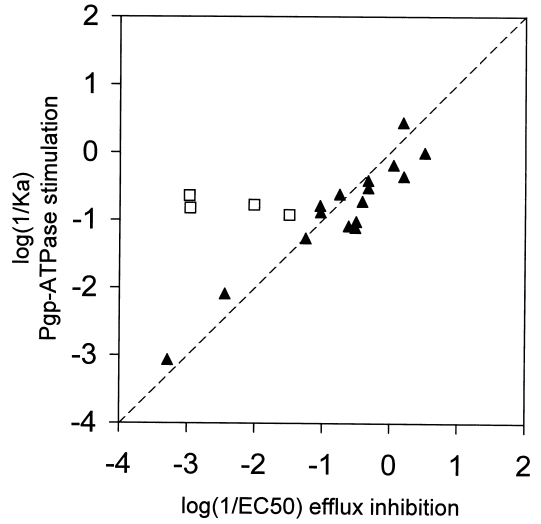



**FIG. 5. Correlation between log(1/$EC_{50}$) values obtained in rhodamine123 efflux experiments in intact cells and log(1/$K_a$) values for Pgp ATPase stimulation. Tertiary propafenones (▲) are located close to the dotted line, which represents the ideal 1:1 correlation. Quaternary analogs (□) were more active in Pgp ATPase stimulation experiments than in whole-cell-based fluorochrome efflux experiments. Data points represent the means of at least triplicate determinations. The coefficient of variation was below 20%.**

### *Transport Studies in Inside-out Vesicles*

Since quaternary analogs were not taken up by CCRF-CEM cells in significant amounts, the question as to whether these substances are transported by Pgp remained unre-

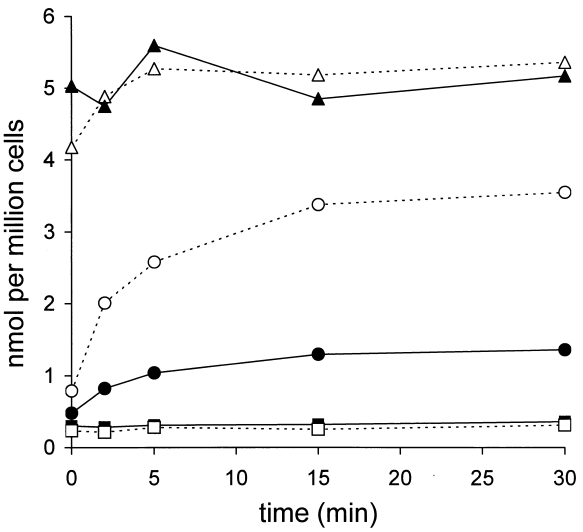



**FIG. 6. Uptake of [$^3$H] GP02 (tertiary analog) and [$^3$H] GP06 (quaternary analog) into CCRF-CEM wt and Pgp-expressing cells. The time–course of uptake of [$^3$H] GP02 (triangles) and [$^3$H] GP06 (squares) at an equimolar concentration of 24 μmol/L is shown. [$^3$H] Daunomycin uptake at a concentration of 1 μmol/L (circles) is included as a control and is approximately threefold higher in parental than in resistant cells. Open symbols represent data points obtained for CCRF-CEM wt cells. Closed symbols indicate uptake into Pgp-expressing CCRF ADR5000 cells. See the Methods section for details.**

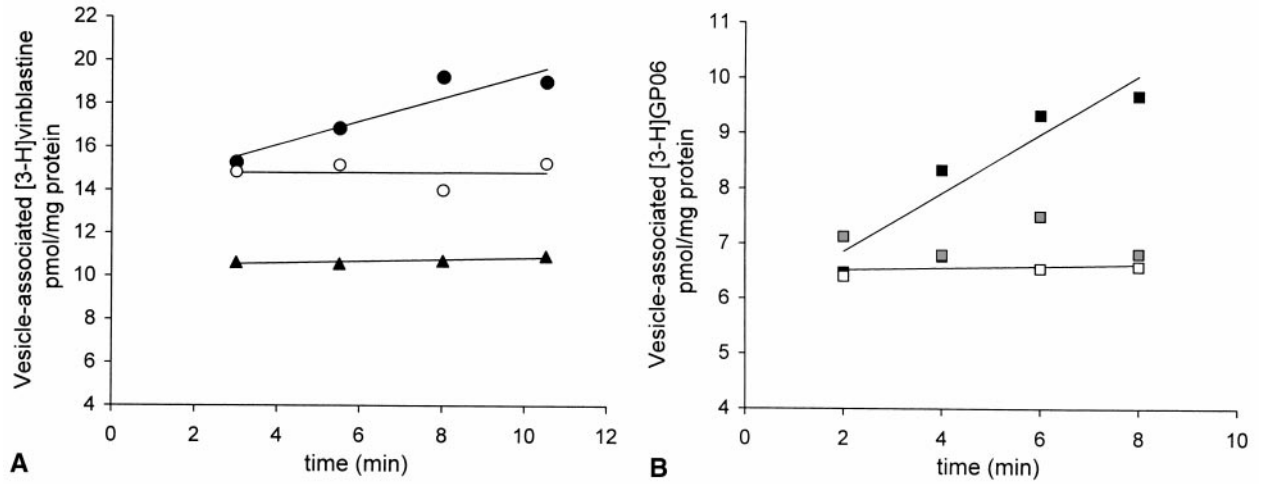



**FIG. 7. Transport of radioactive compounds into inside-out plasma membrane vesicles prepared from CEM ADR5000 cells. (A) Time-dependent accumulation of [$^3$H] vinblastine in I/O vesicles. The concentration of [$^3$H] vinblastine was 100 nM. Data points were obtained in the absence of ATP (○), in the presence of 5 mM ATP (●), and in the simultaneous presence of ATP and 10 μM GP05 (▲). Initial binding of [$^3$H] vinblastine to Pgp vesicles was inhibited by GP05. Non-Pgp-associated binding of [$^3$H] vinblastine to plasma membrane vesicles was approximately 10 pmol/mg membrane protein. (B) Time–course of accumulation of quarternary [$^3$H] GP06 into inside-out vesicles of Pgp-expressing CCRF ADR5000 cells in the absence of ATP (□), in the presence of 5 mM ATP (■), and in the presence of 5 mM ATP and 10 μM cyclosporin A(▨), a known inhibitor of Pgp.**

solved. Thus, inside-out (I/O) vesicles were prepared from CCRF-ADR5000 cells and used in subsequent experiments. In this model system, the cytoplasmic face of the plasma membrane is exposed to the external medium. Vesicles were first incubated with the known substrate [$^3$H]vinblastine to show that they were functionally intact. An ATP-dependent uptake into the vesicles was detectable (Fig. 7A). Vinblastine transport was abolished in the presence of 10 μM GP05. In addition, GP05 eliminated binding of [$^3$H]vinblastine to Pgp, which was illustrated by a lower amount of vinblastine associated with vesicles in the presence of GP05 than in the absence of GP05 and ATP. Similar to vinblastine, the propafenone analog [$^3$H]GP06 was accumulated in I/O vesicles in an ATP-dependent manner (Fig. 7B). A lack of accumulation in I/O vesicles in the absence of ATP is further proof of the inability of quaternary PAs to penetrate the plasma membrane by simple diffusion. Data from these experiments confirm that quaternary analogs are recognized as substrates by Pgp. In contrast, tertiary PAs associated rapidly with I/O vesicles. High background due to high biomembrane distribution precluded the determination of a measurable flux of these analogs into I/O vesicles.

## DISCUSSION

Measurement of Pgp ATPase activity as a function of modulator concentration is a means to obtain quantitative data describing interaction between these substances and Pgp [20]. In such assays, there is no need to simultaneously use a transported toxin such as daunorubicin or vinblastine. Potential Pgp-independent interferences between toxin and modulator, such as fluorescence quench phenomena or membrane displacement, are thus eliminated.

In the present study, propafenone analogs were shown to modulate Pgp ATPase activity. Lower concentrations stimulated up to twofold over basal activity in a dose-dependent manner. At increasingly higher concentrations, a return to, or even below, basal Pgp ATPase activity was observed. Litman *et al.* [22] reported similar data for verapamil, whereby the maximal stimulation was comparable to that observed for PAs. The qualitatively similar biphasic pattern of Pgp ATPase modulation obtained with propafenones and verapamil leads one to assign this class of modulators to the group of verapamil-type substances, which also includes vinblastine and taxol [23].

As for efflux inhibition experiments, a good correlation between logP and $K_a$ values for ATPase stimulation was obtained. The preservation of a lipophilicity dependence in ATPase stimulation experiments using I/O vesicles is evidence for the fact that penetration of tertiary analogs into the plasma membrane is rapid and does not represent a rate-limiting step for the action of propafenone-type modulators. Moreover, accumulation of radiolabeled tertiary analogs is extremely rapid. At the shortest time points which were achievable experimentally (approximately 10 sec), greater than 80% of steady-state levels were reached (see Fig. 6). This indicates that the previously identified pharmacophores, namely a carbonyl group and a basic nitrogen, do not contribute to the ability of tertiary PAs to penetrate the plasma membrane, but rather represent molecular substructures which are important for interaction at recognition site(s) on Pgp. In addition, quaternary analogs showed a tendency to be less active in intact cells. This may in part be due to their permanent positive charge, which impedes their plasma membrane permeation, as shown in radioactive uptake experiments. Since synthesis of quaternary analogs was achieved by reaction of tertiary amines

with [$^3$H]methyliodide, measured flux was exclusively associated with the quaternary compound. The situation may be different for toxin efflux inhibition assays. Despite recrystallization, a small amount of the respective tertiary educt might be present and thus be responsible for the observed residual activity of the quaternary analogs in rhodamine123 efflux inhibition assays.

Dose-dependent modulation of Pgp ATPase activity differed between quaternary and tertiary compounds, whereby maximal stimulation was 40–70% lower for permanently charged substances. A similar observation has been made for substrates of Pgp such as daunomycin. Indeed, quaternary PAs accumulated in inside-out plasma membrane vesicles in an ATP-dependent and cyclosporin A-inhibitable manner. This identified quaternary PAs as substrates for Pgp, which indicates a direct interaction of PAs with Pgp. Because of high background, we were unable to demonstrate an ATP- and time-dependent influx of tertiary analogs into inside-out vesicles. This might be interpreted as evidence that tertiary PAs are not recognized as substrates. On the other hand, these data do not per se exclude the possibility that tertiary compounds are recognized as Pgp substrates. As discussed above, tertiary PAs reach their site of action within fractions of a minute, which indicates that they traverse the plasma membrane rapidly. The consequence of rapid diffusion has previously been discussed by Eytan *et al.* [24], who compared transbilayer movements of different substrates/inhibitors of Pgp. If tertiary analogs moved across the bilayer with rates exceeding their Pgp-mediated extrusion rates, a net flux would not be demonstrable. This might also provide an explanation for higher maximal stimulation of Pgp ATPase activity by tertiary PAs when compared to their quaternary counterparts, since the latter are unable to traverse the plasma membrane by diffusion.

In conclusion, a quantitative approach has been taken to confirm that previously reported structure–activity relationships for PAs are indeed based on Pgp–modulator interaction and do not reflect a limitation in biomembrane permeation based on differences in chemical structure.

*This work was supported by grants from the Austrian Science Fund (P11760MOB) and from the Austrian National Bank (6899). We thank V. Gekeler (Byk Gulden, Konstanz, Germany) for providing the cell lines used in this study. We are indebted to A. Ellinger (Institute of Histology II, University of Vienna, Austria) for preparing electron micrographs of the plasma membrane preparations.*

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