

P-Glycoprotein and Cytochrome P-450 3A Inhibition: Dissociation of Inhibitory Potencies¹

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ABSTRACT

Many P-glycoprotein (P-gp) inhibitors studied *in vitro* and *in vivo* are also known or suspected to be substrates and/or inhibitors of cytochrome P-450 3A (CYP3A). Such overlap raises the question of whether CYP3A inhibition is an intrinsic characteristic of P-gp inhibitors, a matter of concern in the development and rational use of such agents. Thus, the purpose of the present study was to determine whether the ability to inhibit P-gp and CYP3A is, in fact, linked and whether specific P-gp inhibitors with limited ability to inhibit CYP3A can be identified. Therefore, the potency of a series of 14 P-gp inhibitors was assessed by measuring their inhibition of the transepithelial flux across Caco-2 cells of digoxin, a prototypical P-gp substrate. CYP3A inhibition was determined from the impairment of nifedipine oxidation by human liver microsomes. Determination of the apparent K_i values for CYP3A inhibition and the IC_{50} s for P-gp and CYP3A inhibition allowed comparison of the relative inhibitory potency of the compounds on the two proteins' function. The IC_{50} s for P-gp inhibition ranged from 0.04 to 3.8 μ M. All compounds inhibited CYP3A with apparent K_i values of between 0.3 and 76 μ M and IC_{50} s between 1.5 and 50 μ M. However, no correlation was found between the extent of P-gp inhibition and CYP3A inhibition, and the ratio of the IC_{50} for CYP3A inhibition to the IC_{50} for P-gp inhibition varied from 1.1 to 125. These results demonstrate that, although many P-gp inhibitors are potent inhibitors of CYP3A, a varying degree of selectivity is present. The development and use of P-gp inhibitors with minimal or absent CYP3A inhibitory effects should decrease the impact of drug interactions on the therapeutic use of such compounds.

INTRODUCTION

P-gp³ is an ATP-dependent efflux drug transporter that is constitutively expressed in normal tissues including the gastrointestinal epithelium, the canalicular membrane of the liver, the kidney (1, 2), and capillary endothelial cells in the central nervous system (3, 4). Because of such tissue localization and its broad substrate specificity, P-gp appears to play a key role in absorption, distribution, and elimination of many drugs including anticancer agents (5–7). In addition, overexpression in malignant cells has been associated with the development of the multidrug resistance phenomenon for many anticancer drugs (8, 9). *In vitro*, the efficacy of such chemotherapeutic agents can be restored by pharmacological inhibition of P-gp (10–12), and many chemicals have been shown to possess such an effect. Clinically, however, definitive benefit of P-gp inhibition in anticancer drug treatment still remains to be established and is complicated by the fact that many chemomodulators also result in interactions with drug-metabolizing enzymes (13–19). For example, plasma concentrations of doxorubicin almost doubled during PSC833 administration

(18), and similar increases in plasma concentration of etoposide are also produced by this P-gp inhibitor (19).

In addition to their effects as P-gp inhibitors, many chemomodulating agents also inhibit cytochrome P-450 3A (CYP3A) activity (20, 21), which is the most abundant cytochrome P-450 enzyme present in human liver and intestine and is known to be involved in the metabolism of a large number of drugs including anticancer agents (22). Some anticancer drugs such as taxol are metabolized by CYP3A to products that lack antitumor activity (23); in contrast, CYP3A-mediated metabolism of tamoxifen, methoxymorpholinylidoxorubicin, cyclophosphamide, and ifosfamide (CYP3A/2B) produce metabolites, some of which have antitumor activity greater than the parent compound (24–26). For drugs requiring activation, the administration of P-gp inhibitors, which are also CYP3A inhibitors, may result in a reduced therapeutic effect, despite the P-gp inhibition-mediated, enhanced intracellular accumulation of the parent drug. Conversely, for drugs, the elimination of which is dependent on CYP3A, inhibition of CYP3A by P-gp inhibitors may cause excessive drug accumulation and increased toxicity, resulting in the need to reduce the dose of chemotherapeutic agent (13–19). Accordingly, an understanding of the likelihood of CYP3A interactions with P-gp inhibitors is essential for their rational use.

The purpose of the present study was, therefore, to determine the quantitative relationship between P-gp and CYP3A inhibition of a series of compounds with established P-gp inhibitory properties.

MATERIALS AND METHODS

Materials. With the exception of PSC833, which was a gift from Novartis (Basel, Switzerland), the remaining 13 compounds studied (Fig. 1) were provided by Pfizer (Groton, CT). All compounds were dissolved in DMSO. [³H]Digoxin (specific activity, 15 Ci/mmol; radiochemical purity, >97%) was obtained from DuPont-New England Nuclear (Boston, MA). Nifedipine and quinidine were purchased from Sigma Chemical Co. (St. Louis, MO). All tissue culture media and reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Transport in Cultured Caco-2 Cells. Caco-2 cells were grown with DMEM (high glucose) supplemented with 10% FCS, 2 mM L-glutamine, 100 units penicillin/ml, 100 μ g of streptomycin/ml, and 1% nonessential amino acids (Life Technologies, Inc.), and incubated at 37°C under 5% CO₂. Caco-2 cells were plated at a density of 1×10^5 cells/12 mm well on 0.4 μ m size polycarbonate membrane filters (Transwell; Costar Corp., Cambridge, MA). Cells were supplemented with fresh media every 2 days and used in the transport studies on the fourth day after plating. Transepithelial resistance was measured in each well using a Millicell ERS ohm meter (Millipore, Bedford, MA); wells registering a resistance of 200 ohms or greater, after correcting for the resistance obtained in control blank wells, were used. Transport experiments were carried out using the same protocol described previously (27). Briefly, 1–2 h before the start of the transport experiments, the medium in each compartment was replaced with a serum-free medium (Optimem; Life Technologies, Inc.). Then, digoxin transport in the basal-to-apical direction as well as the apical-to-basal direction was measured in two corresponding wells by replacing the media with 700 μ l of serum-free medium (Optimem) containing [³H]digoxin (5 μ M) in either the apical compartment of the first well or in the basal compartment of the corresponding second well. The digoxin concentration corresponded to about one-third of the reported K_M for P-gp-mediated

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³ The abbreviation used is: P-gp, P-glycoprotein.

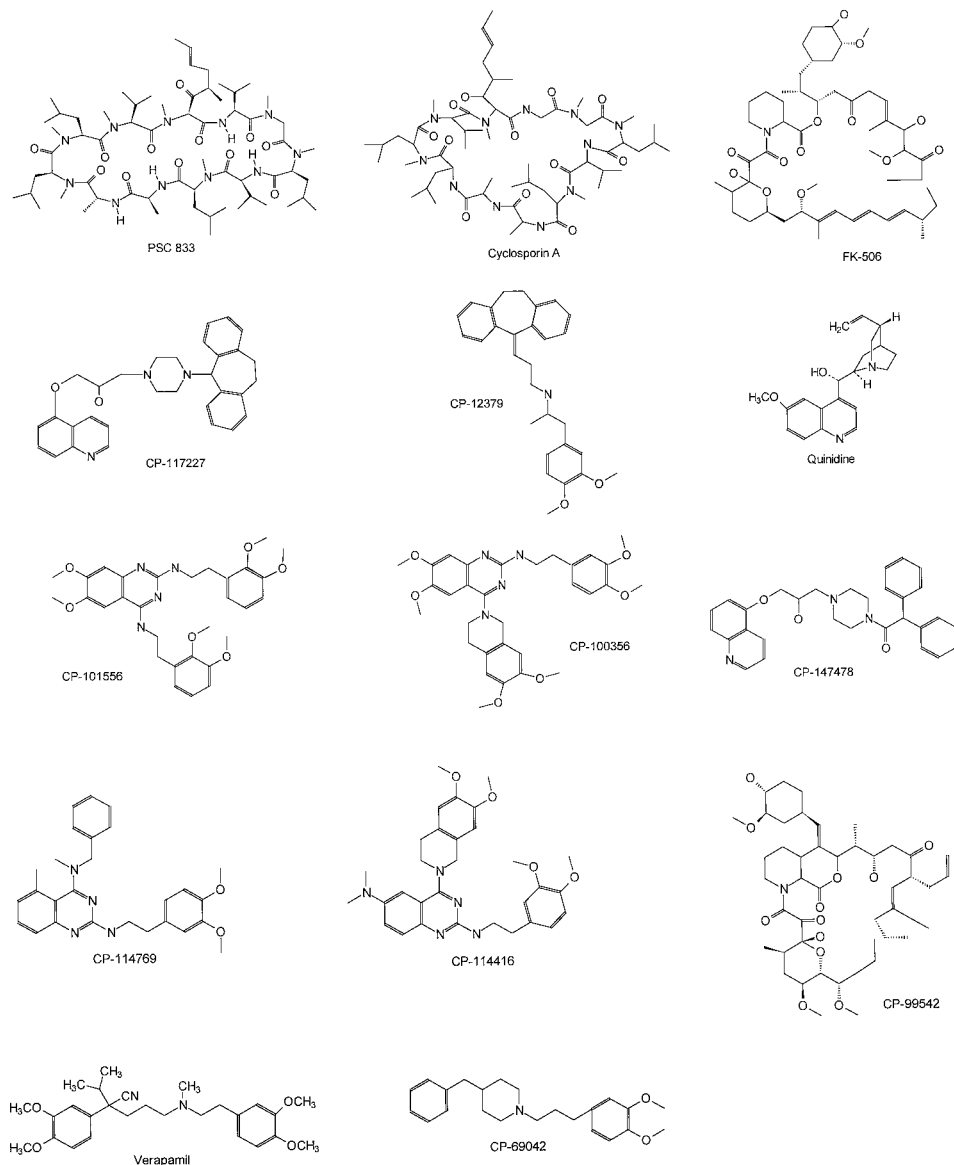


Fig. 1. Chemical structures of the various compounds examined.

transport (28). [^3H]Digoxin appearing in the opposite compartment after 0.5, 1, 2, and 2.5 h was quantified in 25- μl aliquots taken from each compartment by liquid scintillation counting, following the addition of 5 ml of scintillation fluid (ScintiVerse BD; Fisher Scientific, Fair Lawn, NJ), and expressed as percentage of added radioactivity. Inhibition of digoxin translocation was determined after the addition of the test compound in equal concentrations to both the apical and basal compartments. The DMSO concentration in both incubations with and without inhibitor was 10%; preliminary experiments demonstrated that 10% DMSO in Caco-2 cells did not affect the translocation process (data not shown). The extent of inhibition was estimated using the assumption that complete inhibition of P-gp-mediated digoxin transport would result in the loss of net basal-to-apical *versus* apical-to-basal translocation differences. Accordingly, the degree of inhibition at each time point was determined from the relationship:

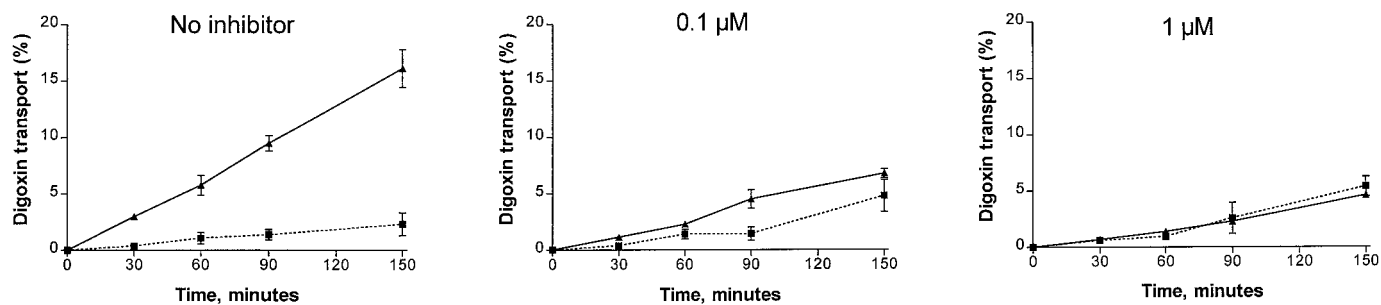
$$\% \text{ inhibition} = 1 - \frac{(i_{\text{basal-to-apical}} - 1_{\text{apical-to-basal}})}{a_{\text{basal-to-apical}} - a_{\text{apical-to-basal}}} \times 100$$

where i and a represent the percentage of digoxin transport in the presence and absence of an inhibitor, respectively. Because digoxin transport during the studied time points was linear, the percentage of inhibition calculated at each time point was averaged. The extent of inhibition at various inhibitor concen-

trations (0.001, 0.01, 0.1, 1, 5, 10, 20, and 100 μM) was used to determine the IC_{50} according to the Hill equation (Prism program; Graphpad, San Diego, CA). Experiments were performed in triplicate, with each experiment carried out on a different day.

Measurement of CYP3A Activity. CYP3A activity was determined in human liver microsomes prepared from human liver sample HL110 (Nashville Regional Organ Procurement Agency, Nashville, TN) as described (29) with the total cytochrome P-450 content measured as described by Omura and Sato (30). CYP3A activity was assessed in duplicate by the formation rate of the nifedipine metabolite (dehydronifedipine). Briefly, the incubation medium consisted of microsomes containing 100 pmol cytochrome P-450, 1.5 mM NADPH, nifedipine at various concentrations (15, 20, 30, 60, and 200 μM), and 10 μl of various inhibitor concentrations diluted in DMSO, resulting in final inhibitor concentrations between 0.5 and 40 μM in a total volume of 500 μl 0.1 M phosphate buffer (pH 7.4). Control incubations without addition of inhibitor were also performed in the presence of 2% DMSO. At this concentration of DMSO, nifedipine oxidation was reduced by 25% (data not shown). To avoid variability in CYP3A activity due to differing DMSO concentrations, 2% DMSO was used in all incubation procedures. Incubations were performed at 37°C and stopped after 10 min by the addition of 1 ml of CH_2Cl_2 . The nifedipine metabolite was measured by HPLC-UV as described elsewhere (31)

CP 114416



CP 99542

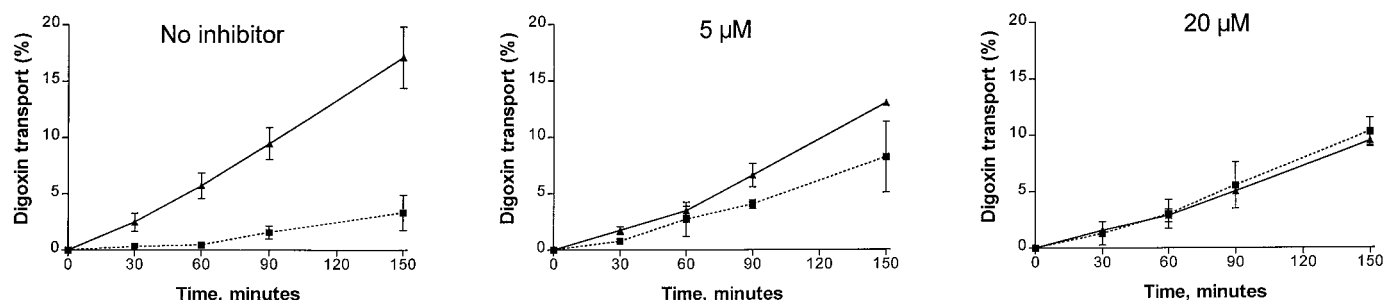


Fig. 2. Transepithelial translocation of [3 H]digoxin ($5 \mu\text{M}$) across a Caco-2 cell culture monolayer in the absence or presence of varying concentrations of CP114416 and CP99542. \blacktriangle and solid line, translocation from basal to apical compartments; \blacksquare and dotted line, translocation from apical to basal compartments. Data are means from three or more experiments; bars, SD.

with an interassay variability of $<5\%$. The Michaelis-Menten kinetics (apparent V_{max} , apparent K_m) were calculated using a nonlinear regression computer program ("kcat"; BioMetallics, Princeton, NJ). The type of inhibition and the inhibition constant (apparent K_i) were derived from appropriate reploting of the Lineweaver-Burk plot (32). IC_{50} s were calculated by the same procedure as for P-gp inhibition, using a nifedipine concentration of $20 \mu\text{M}$.

Statistics. Significance of inhibition for CYP3A and P-gp were determined using a Student's t test or Mann-Whitney U test, with $P < 0.05$ being taken as the minimum level of significance accepted. Data are expressed as means \pm SD.

RESULTS

P-gp Inhibition. The effect of P-gp inhibition on the basal-to-apical and apical-to-basal digoxin translocation across Caco-2 cells is shown in Fig. 2, which also illustrates the extremes of potency found for P-gp inhibition with CP114416 and CP99542. The IC_{50} s for inhibition of digoxin transport for the various compounds are presented in Fig. 3A.

CYP3A Inhibition. The apparent K_m and apparent V_{max} values for nifedipine oxidation in the presence of 2% DMSO without any inhibitor were $30 \mu\text{M}$ and $7 \text{ nmol/nmol cytochrome P-450/min}$, respectively [which are similar to data previously reported in the literature (31)]. All of the compounds except the cyclosporine derivatives acted as mixed-function inhibitors, whereas the cyclosporine derivatives were competitive inhibitors. The apparent K_i values ranged from 0.32 to $76 \mu\text{M}$ (Table 1), and the IC_{50} values ranged from 1.5 to $50 \mu\text{M}$ (Fig. 3A).

Except for CP117227, which produced hyperbolic inhibition, all compounds exhibited linear inhibition. CP117227 inhibited CYP3A by 35% at $40 \mu\text{M}$. Because of its weak CYP3A inhibitory effects and limited solubility in DMSO, the IC_{50} for verapamil could not be determined, but at concentrations up to $40 \mu\text{M}$, the maximal inhibition by verapamil of CYP3A-mediated nifedipine oxidation was 30%. On the basis of the inhibition produced by the range of verapamil con-

centrations, the concentration required to produce 25% inhibition was calculated.

Comparison of P-gp with CYP3A Inhibition. The relative inhibition ratio of IC_{50} (CYP3A): IC_{50} (P-gp) varied widely (1.06–125; Fig. 3B). However, a statistically significant correlation of the CYP3A and P-gp inhibition IC_{50} s was not observed ($r^2 = 0.17$, $P = 0.16$; $n = 12$).

DISCUSSION

P-gp inhibition has a potential for therapeutic benefit in the treatment of drug-resistant cancer. In addition, we have shown recently that the disruption of P-gp at the blood/brain barrier results in the enhanced penetration of HIV protease inhibitors and possibly other drugs into the central nervous system (27). Optimal P-gp inhibitor therapy will require drugs that are sufficiently potent to inhibit P-gp at therapeutically achievable plasma concentrations while producing minimal inhibition of CYP3A at these concentrations. To identify such drugs, it is necessary to compare their relative potency as P-gp and CYP3A inhibitors to define their relative selectivity. Drugs that are relatively selective for P-gp will be less likely to produce metabolically determined drug interactions (15). In the past, little attention has been given to the potential for inhibitors of P-gp to also inhibit CYP3A activity (16), despite the potential for such inhibition to alter the therapeutic efficacy of anticancer drugs in at least two ways: (a) through reduced cytotoxicity of anticancer agents, which are activated by CYP3A; and (b) through increased systemic toxicity of anticancer drugs, the elimination of which is mediated by CYP3A (19, 22). In addition, because of the dual expression of P-gp and CYP3A in the gastrointestinal epithelial cells, P-gp inhibition combined with CYP3A inhibition may produce a substantial increase in oral bioavailability of drugs over and above that produced by P-gp inhibition

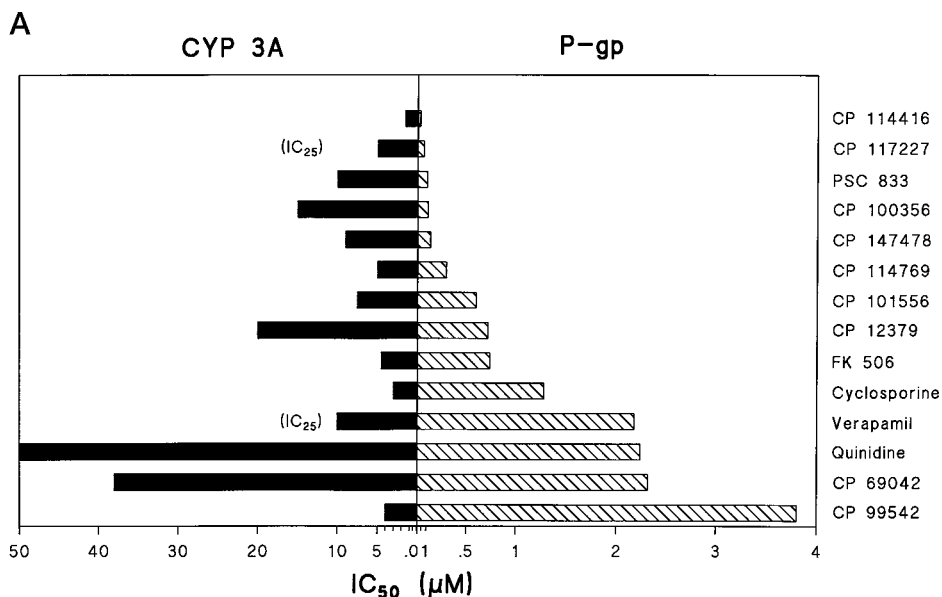
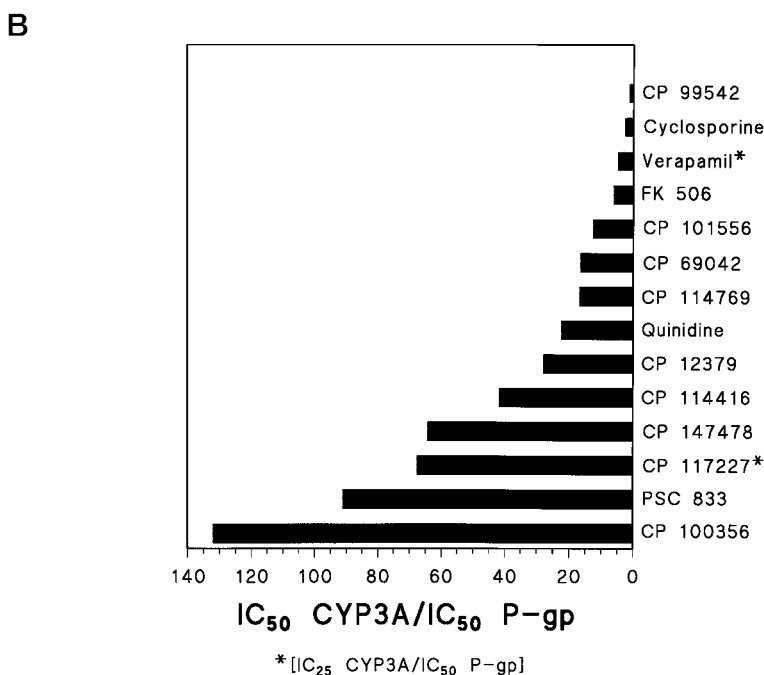


Fig. 3. A, IC₅₀ for P-gp and CYP3A inhibition [for CP117227 and verapamil, inhibitor concentrations that produced 25% CYP3A inhibition (IC₂₅), respectively]. B, the selectivity ratio for CYP3A to P-gp inhibition [IC₅₀ (CYP3A):IC₅₀ (P-gp)]. The higher the ratio, the less risk for CYP3A-mediated drug interactions with a similar P-gp inhibitory effect.



alone. Thus, an understanding of the relative effects of putative P-gp inhibitors on CYP3A activity is essential for rational development and therapy with these compounds.

This study demonstrates that there is a large range in the potency of these compounds as both P-gp and CYP3A inhibitors. All of the compounds exhibited some inhibitory effect on CYP3A. However, an important finding was that no significant correlation exists between the ability of the compounds to inhibit P-gp and their ability to inhibit CYP3A. Although some of the most potent P-gp inhibitors were also the most potent CYP3A inhibitors (*e.g.*, CP114416), conversely, some relatively potent CYP3A inhibitors were relatively poor P-gp inhibitors (*e.g.*, CP99542; Fig. 3A). Thus, the molecular recognition sites of P-gp and CYP3A differ in ways that result in differential effects of compounds at the two sites.

Table 1 Mechanism of CYP 3A inhibition and respective apparent K_i of the 14 compounds studied

Compound	Mechanism of CYP 3A inhibition	apparent K_i (µM)
CP114146	Linear, mixed function	0.32
CP100356	Linear, mixed function	13.0
CP114769	Linear, mixed function	2.0
CP101556	Linear, mixed function	5.9
FK506	Linear, competitive	1.1
CP99542	Linear, competitive	1.6
Cyclosporin	Linear, competitive	4.9
PSC833	Linear, competitive	16
Verapamil	Linear, mixed function	76
CP12379	Linear, mixed function	13
CP69042	Linear, mixed function	23
CP117227	Hyperbolic, mixed function	3.0
CP147478	Linear, mixed function	0.75
Quinidine	Linear, mixed function	51

From a scientific standpoint, P-gp inhibitory potency would appear to be the most important factor in developing a P-gp inhibitor. However, with regard to therapeutic utility, this may not be the case if such inhibition is also accompanied by a significant CYP3A interaction. A less potent P-gp inhibitor but one without or with minimal CYP3A inhibitory effect might be more desirable, provided adequate concentrations can be achieved *in vivo*. Such selectivity can be assessed by the ratio of IC₅₀ (CYP3A):IC₅₀ (P-gp). Given that the higher the IC₅₀ the higher the drug concentration required for inhibition, those drugs with the highest ratios will be those with the greatest selectivity for P-gp inhibition. Of the drugs studied, CP100356 has the highest selectivity ratio (Fig. 3B), although it was not the most potent P-gp inhibitor. Interestingly, the cyclosporine derivative PSC833 showed a high selectivity index of about 90, in contrast to the low ratio (2.3) for cyclosporine itself, implying that compounds with similar chemical structures can substantially differ in their ability to inhibit P-gp and CYP3A. By the use of the selectivity index, it should be possible to screen compounds for their relative effects on transport and drug metabolism. This will allow the selection of those compounds that produce the greatest inhibition of drug transport and, hence, reversal of multidrug resistance, minimizing, at the same time, the increase in plasma concentrations due to inhibition of drug metabolism and loss of drug efficacy due to reduction in metabolic activation.

The disappointing therapeutic effects of P-gp inhibitors in cancer therapy thus far may reflect the fact that in many of the studies, the P-gp inhibition strategy used drugs like verapamil and cyclosporine, which are established and readily available. However, as can be seen in Fig. 3, these are drugs with relatively low potency and low selectivity indices, implying that the high concentrations required to inhibit P-gp would also produce CYP3A inhibition. Concentrations of these drugs achieved clinically range up to 10 μM for quinidine, 0.4 μM for cyclosporine, and 0.4 μM for verapamil. The duality of effects of these P-gp inhibitors on both P-gp and CYP3A will result in multiple effects at different tissue sites so that inhibition of P-gp will increase tumor concentration, increase oral absorption, and increase central nervous system penetration. In addition, inhibition of CYP3A will further enhance oral absorption and decrease hepatic metabolism with consequent further increase in plasma concentration. The therapeutic/toxic effects of the changes will depend on whether CYP3A is involved in activation or detoxification of the drug in question (16–19).

In conclusion, this study has demonstrated the considerable overlap in the activity of inhibitors of P-gp on CYP3A. The use of first generation P-gp inhibitors such as verapamil and cyclosporine with poor P-gp selectivity may be complicated by metabolic drug interactions with the anticancer drug regimen. Even small structural modifications, such as cyclosporine to PSC833, can profoundly alter the selectivity of the drug. Therefore, if potent P-gp-selective agents can be developed that minimize the risk of CYP3A-mediated metabolic interactions with the anticancer drug regimen, this should result in improved therapeutic specificity and efficacy.

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