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The drug efflux–metabolism alliance: biochemical aspects ☆

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Abstract

The considerable overlap in the substrate selectivity and tissue localization of CYP3A and P-glycoprotein has led to the hypothesis that this transporter and enzyme pair act as a coordinated absorption barrier against xenobiotics. A historical perspective on the investigation of this interactive alliance is given, starting from the understanding of the role of intestinal metabolism in explaining cyclosporine clinical data. Several animal studies using *mdr*1*a*(−/−) knockout mice have demonstrated P-glycoprotein's importance in limiting drug absorption and decreasing bioavailability. Human clinical studies investigating the importance of intestinal CYP3A and P-glycoprotein through inhibition or induction of these proteins have provided further evidence of this interaction. Recent in vitro studies using CYP3A4-expressing Caco-2 cells are reported. These studies reveal that the role of P-glycoprotein in the intestine extends beyond simply limiting parent drug absorption but also includes increasing the access of drug to metabolism by CYP3A through repeated cycles of absorption and efflux.

Keywords: Cytochrome P450 3A; P-glycoprotein; Intestine; Metabolism; Drug efflux

Contents

1. Introduction

Until recently, when a drug exhibited poor oral bioavailability, it was generally assumed that this was due to either (a) physico-chemical problems, i.e. poor solubility in the gastrointestinal fluids or inability to diffuse through the intestinal membrane, or alternatively due to (b) significant first-pass hepatic

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metabolism. Our laboratory was among the first to hypothesize that for many drugs poor oral bioavailability could be due to biochemical processes in the intestines rather, and often in addition to, the physico-chemical problems [1,2]. Based on a series of cellular, animal and human studies, we hypothesized that intestinal metabolic enzymes and efflux transporters, working coordinately as a protective mechanism, may be responsible for the poor bioavailability of a number of drugs.

Cytochrome P450 (CYP) 3A, the major phase one drug metabolizing enzyme family in humans [3], and the multidrug efflux pump, P-glycoprotein, are present at high levels in the villus tip of enterocytes in the gastrointestinal tract, the primary site of absorption for drugs administered orally. The importance of CYP3A and P-glycoprotein in limiting oral drug delivery was suggested to us by: (a) their joint presence in small intestinal enterocytes, (b) the significant overlap in their substrate specificities and (c) the poor oral bioavailability of drugs that are substrates for both CYP3A and P-glycoprotein [4]. These enzymatic and drug transporter proteins are induced or inhibited by many of the same compounds [5].

In this manuscript we review prior studies with immunosuppressive agents that led to the development of our hypothesis, more recent animal and human studies, and then describe our most recent cellular studies to probe the interactive nature of the drug efflux–metabolism alliance with four different drug substrates.

2. Human studies of the oral bioavailability of immunosuppressive agents

When we began our studies in this area at the beginning of the 1990s it was generally believed that the poor and variable absorption of cyclosporine was due to the drug's poor solubility in gastrointestinal fluids, resulting from the compound's large molecular weight and high lipid/water partition coefficient. Our first study of the effects of high fat meals on the pharmacokinetics of cyclosporine in healthy subjects [6] led us to believe that the unusual effects resulting from a high fat meal, i.e. no change in absorption rate but a significant increase in the extent of absorption [7] and an increase in the clearance of the drug [8] could be explained by a lipid effect in the liver. We pursued this hypothesis through studies with isolated rat hepatocytes [9] and liver perfusions [10]. However, these biochemical and in vitro studies could not provide a rational explanation for the apparently anomalous results of our human clinical studies. In our 1992 paper [11], we began to realize that the accepted dogma concerning cyclosporine's bioavailability might not be correct. In that study, we evaluated the effects of concomitant rifampin administration on cyclosporine bioavailability. We noted that the increase in hepatic metabolism quantitated by intravenous dosing could not explain the marked decrease in cyclosporine oral bioavailability observed with concomitant rifampin administration. We speculated, based in part on the innovative work of Watkins and co-workers [12,13], that gut metabolism of cyclosporine might account for the differences observed in the presence of rifampin, a known inducer of CYP3A in humans. The proposed model suggested that the correct evaluation of oral bioavailability required assessment of first-pass gut extraction, in addition to the first-pass liver extraction and the fraction of the dose absorbed. Our subsequent analysis of the rifampin data [14] led to a surprising conclusion. That is, cyclosporine in the then commercially available Sandimmune® formulation did not have an absorption problem, but rather a marked first-pass gut extraction, which approximated 60%. As depicted in Fig. 1 [2], our analysis suggests that cyclosporine was absorbed from the Sandimmune® formulation on average about 86% and that just over half of the oral dose is metabolized in the gut wall, while only 8% is lost to hepatic first-pass metabolism, resulting in a bioavailability of 27% in healthy volunteers.

We reasoned that if cyclosporine did in fact have no absorption problems from the Sandimmune® formulation, then we should be able to achieve significant oral bioavailability by carrying out a study with an inhibitor of CYP3A rather than an inducer. Such a study [15] demonstrated that in the presence of concomitant ketoconazole dosing, the oral bioavailability of cyclosporine from the Sandimmune® formulation averaged $56 \pm 12\%$ versus $22 \pm 5\%$ found in the same healthy subjects adminis-

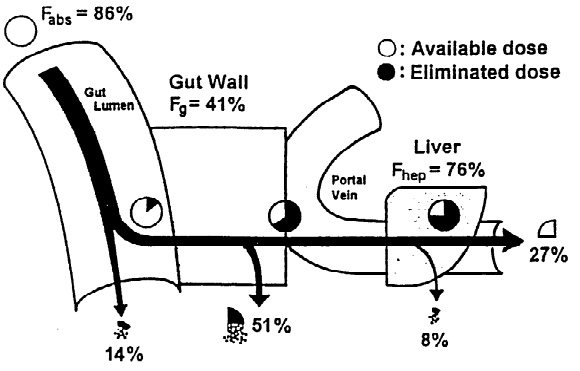

Fig. 1. Schematic diagram depicting the various processes leading to 27% bioavailability following an oral dose of cyclosporine Sandimmune® in healthy volunteers [14]. The values at the bottom of the figure indicate the average fraction of the dose lost in each of the processes, that is, 14% of the dose is either unabsorbed, counter-transported irreversibly by P-glycoprotein, or degraded in the gut lumen, 51% of the drug is metabolized in the enterocytes of the gut wall and only 8% is lost to hepatic first-pass metabolism (reproduced with permission from Ref. [2]).

tered oral and intravenous cyclosporine without ketoconazole [15].

As our studies progressed, we realized that our results could not be explained based only on changes of gut CYP3A enzymes by drug inducers or inhibitors. We were the first to note and publish the striking overlap of substrate specificity and tissue distribution for CYP3A and P-glycoprotein [4]. We suggested that CYP3A and P-glycoprotein might play complimentary roles in drug absorption, distribution, metabolism and excretion by biotransformation and countertransport, especially in the villi of the small intestine. This had been hinted at by Zhao et al. [16], who noted that drugs such as quinidine, nifedipine and cyclosporine that were able to reverse multidrug resistance of vinca alkaloids, were themselves metabolized by CYP3A. Zhao et al. [16] hypothesized that metabolic drug interactions could be involved in the appearance of multidrug resistance. They suggested that ongoing studies would allow a better understanding of the possible coregulation between multidrug resistance and the metabolism of anticancer drugs. Shortly following our *Molecular Carcinogenesis* paper [4], Schuetz et al. [5] demonstrated that modulators and substrates of P-glycoprotein and CYP3A coordinately upregulated these proteins in human colon carcinoma cells and that P-glycoprotein was a major determinant of rifampicin-inducible expression of CYP3A in mice and humans [17]. Most recently, this proposed coregulation of CYP3A and P-glycoprotein has been confirmed by the identification of the nuclear receptor SXR, which has been shown to coordinately regulate drug metabolism and efflux via these two proteins [18].

Similar studies to those described above for cyclosporine were recently reported for tacrolimus and sirolimus. Tacrolimus oral bioavailability was increased from 14±5% to 30±8% with concomitant ketoconazole [19], while in a second study rifampin decreased tacrolimus bioavailability from 14±6% to 7±3% [20]. Since no commercially available intravenous dosage form of sirolimus is available, inhibition and induction comparisons were made using measures of area under the curve (AUC). Ketoconazole dosed together with sirolimus caused a 10-fold increase in drug AUC [21], while unpublished results demonstrate more than a fivefold decrease with concomitant rifampin dosing.

3. The interactive nature of CYP3A and P-glycoprotein in the intestine

Fig. 2 depicts our conception of the interaction between CYP3A and P-glycoprotein in the intestine. Drug is absorbed by passive processes into the enterocyte where it may be metabolized by the enzyme. However, drug is also subject to active back transport into the intestine allowing further access to the enzyme upon subsequent absorptions.

Such an effect is depicted in Fig. 3, where drug molecules not subject to P-glycoprotein will pass through the enterocyte only once, while molecules subject to P-glycoprotein efflux may be continually cycled between the enterocyte and the gut lumen, thus allowing the enzyme repeated access to the drug molecule, or leading to non-absorption due to efflux.

We began to realize, based on Figs. 2 and 3, that intestinal metabolism of drugs could be changed as a function of P-glycoprotein activity without either inhibiting or inducing CYP3A enzymes. That is, if P-glycoprotein efflux is inhibited, the drug molecule passes through the intestine in a single pass as depicted in Fig. 3 (far right pathway), and intestinal

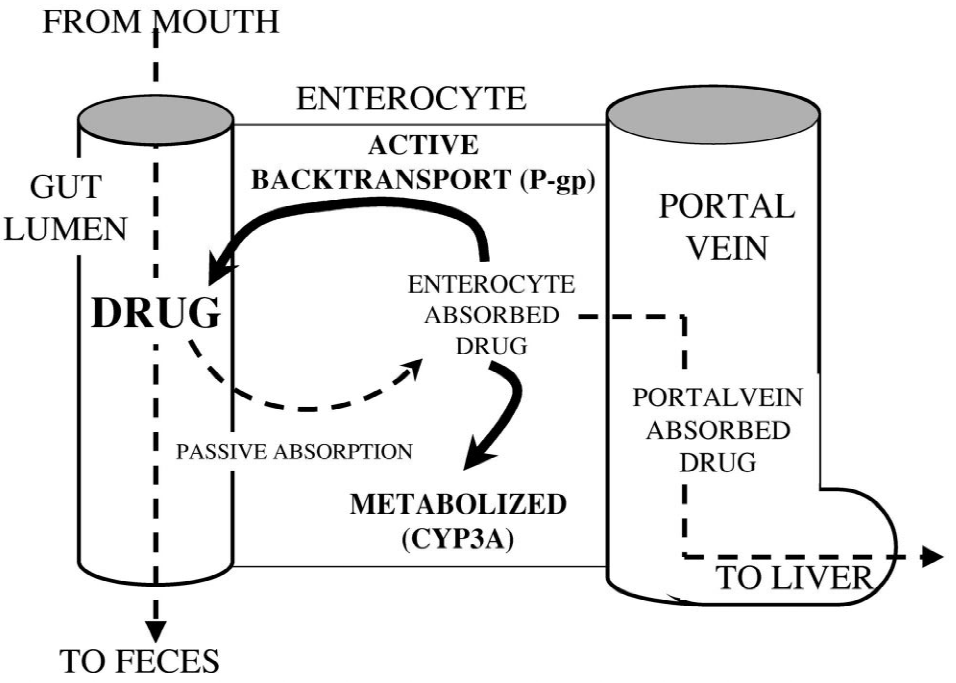

Fig. 2. Schematic depiction of the enteral cycling of a drug that is a substrate for P-glycoprotein and CYP3A and the potential for the transporter controlling access to the enzyme.

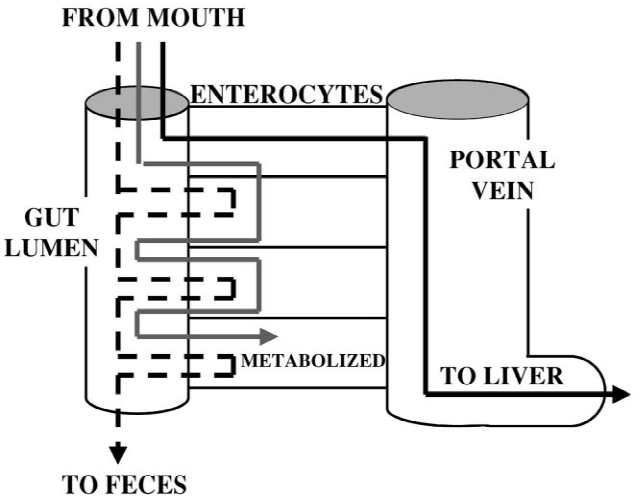

Fig. 3. Schematic depicting the potential effects of P-glycoprotein efflux on access of drugs to intestinal CYP3A enzymes.

metabolism would decrease since there is less access of the drug to the enzymes. We attempted to test this hypothesis by carrying out studies with a substance that had negligible effects on CYP3A but could affect P-glycoprotein. We carried out a study in healthy volunteers where cyclosporine was dosed as the oral Sandimmune® formulation with and without water-soluble vitamin E (TPGS, tocopheryl poly-

ethylene glycol 1000 succinate) [22]. This commercially available additive was shown to have no effect on CYP3A metabolism [22], but to be an inhibitor of P-glycoprotein [23]. In 10 healthy volunteers, oral cyclosporine AUC was increased 58±24% when the Sandimmune® formulation was given concomitantly with TPGS [22].

4. Recent animal and human studies addressing the interactive nature of CYP3A and P-glycoprotein

Within the last 3 years our laboratory has reviewed the role of P-glycoprotein and CYP3A in limiting oral drug absorption [24–26]. A series of studies in animal models have demonstrated that inhibition or induction of intestinal CYP3A and/or P-glycoprotein has marked effects on the bioavailability of paclitaxel [27], HIV-1 protease inhibitors [28,29] and digoxin [30,31]. We recently demonstrated a marked increase in bioavailability in rats of an investigational cysteine protease inhibitor with a concomitant oral dose of ketoconazole [32]. Furthermore, the complete loss of P-glycoprotein from the gut in *mdr*1*a*(−/−) knockout mice supports the important role for intestinal P-glycoprotein in the excretion of drugs into the intestine and in limiting the uptake of drugs from the intestine. For example, the oral bioavailability of paclitaxel is threefold higher in *mdr*1*a*(−/−) mice compared with *mdr*1*a*(+/+) mice [33]. Higher plasma concentrations of the HIV-1 protease inhibitors indinavir, nelfinavir and saquinavir were observed in *mdr*1*a*(−/−) mice at 4 h after oral administration [34].

Palkama et al. [35] demonstrated that saquinavir markedly increased the oral bioavailability of midazolam from 41 to 90% in healthy volunteers. Using these data, Zhang and Benet [26] calculated the hepatic and intestinal bioavailability, as previously done in our laboratory for cyclosporine [14] and tacrolimus [21]. We calculated that saquinavir treatment increased the hepatic bioavailability of midazolam from 61 to 83%, whereas the intestinal bioavailability increased from 67 to nearly 100%. In other words the hepatic extraction ratio decreased from 0.39 to 0.17 and the intestinal extraction ratio decreased from 0.33 to close to zero. This study clearly demonstrates that saquinavir inhibited both intestinal and hepatic CYP3A-mediated first-pass extraction of midazolam after oral administration. A similar observation was made by Gorski et al. [36] in their drug–drug interaction study of midazolam and clarithromycin.

Greiner et al. [37] investigated the role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. Rifampin treatment increased intestinal P-glycoprotein levels (determined from Western blot analysis of P-glycoprotein in duodenal biopsies) about 3.5-fold, which correlated well with the decrease in AUC of oral digoxin, but not intravenous digoxin. In humans, digoxin is not subject to metabolism by CYP3A enzymes, but it is a well-documented P-glycoprotein substrate.

5. Investigating the interaction using cellular systems

At present, the Caco-2 (colon carcinoma) cell line is frequently used to model the human intestinal absorption of drugs [38]. Although Caco-2 cells express the P-glycoprotein efflux transporter [39], they lack CYP3A. Very recently, we characterized the expression of CYP3A4 and efflux transporters, P-glycoprotein as well as MRP1 and MRP2, in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [40]. We demonstrated that CYP3A4 protein levels were significantly increased when cells were incubated with the inducers alone or in combination, as measured by Western blot. The increase obtained with combined sodium butyrate and TPA was 40-fold over the control levels and was greater than that obtained with either inducer alone (sodium butyrate, 10-fold, and TPA, fivefold) [40]. The functional activity of these cells was evaluated by measuring the extraction ratio (ER) of midazolam during transit through the polarized cell system. The extraction ratio in an in vitro system refers to the fraction of the parent drug that was metabolized as it crossed the monolayer. Calculation of the extraction ratio was carried out using a variation of the equation proposed by Fisher et al. [41]. The equation here incorporates in the denominator the intracellular levels of unchanged

midazolam, since we reason that if the drug is inside the cell, it could interact with CYP3A4

$$ER = \frac{\sum \text{metabolites}_{(\text{apical, basolateral, intracellular})}}{\sum \text{parent}_{(\text{basolateral, intracellular})} + \sum \text{metabolites}_{(\text{apical, basolateral, intracellular})}}. \quad (1)$$

The extraction ratio of midazolam when 3 μM was applied to the apical chamber and was incubated for 30 min at 37°C was 0.9±0.1% in non-induced cells. In contrast, the extraction ratio was 32±1% in cells induced with both sodium butyrate and TPA [40]. This functional increase of 36-fold closely correlates with the 40-fold increase in protein level of CYP3A4 as determined by densitometry.

The role of P-glycoprotein in modulating the extent of intestinal drug metabolism was examined in vitro using the CYP3A4-transfected Caco-2 cells described above, grown as monolayers. The transport and metabolism of several model P-glycoprotein and CYP3A4 compounds (listed in Table 1) were examined when dosed on the apical side of the cells (mimicking human intestinal absorption) alone or in combination with the P-glycoprotein inhibitor GG918 (200 nM) or the dual P-glycoprotein and CYP3A4 inhibitor cyclosporine (10 μM). We hypothesized that for compounds that were substrates of both P-glycoprotein and CYP3A4, effective inhibition of intestinal P-glycoprotein would not only increase absorption by blocking countertransport but also decrease total metabolism, resulting in a significantly enhanced intestinal bioavailability.

The compounds tested were from a variety of drug classes including K77, an investigational cysteine protease inhibitor currently being developed to treat Chagas' disease; rapamycin, an immunosuppressive drug; midazolam, an anesthetic agent; and felodipine, a Ca^{2+}-channel blocker. Each of these drugs was a known substrate of CYP3A4 having a K_m for metabolite formation close to the dose administered (shown in brackets, Table 1). K77 was found to be the best P-glycoprotein substrate tested and had a ninefold greater (B)asolateral to (A)pical flux compared with its A to B flux. Rapamycin transport exhibited only a 2.5-fold efflux ratio across the cells and was considered a weaker P-glycoprotein substrate. Midazolam and felodipine are not P-glycoprotein substrates, as indicated by their efflux ratios of 1, and acted as negative controls for these studies. Negative controls were important to ensure that the specific P-glycoprotein inhibitor GG918 was not affecting the cells in any way other than to inhibit P-glycoprotein.

All compounds tested were significantly metabolized while traversing the cells as indicated by the extraction ratios ranging from 25 to 60% (Table 1, drug alone). As expected, incubation with cyclosporine resulted in decreased extraction ratios for all compounds, as cyclosporine is a known CYP3A4 inhibitor. The percent decrease in extraction ratio that could be attributed to the direct inhibition of CYP3A4 metabolism by cyclosporine was between 46 and 60% (calculated from the exclusive CYP3A4 substrates felodipine and midazolam). The percent decreases in ERs with cyclosporine for K77 and rapamycin were greater than this (74–83%), suggesting an additional factor (likely P-glycoprotein) was involved.

Incubation of felodipine and midazolam with GG918 (a P-glycoprotein inhibitor that does not

Table 1
Extraction ratios and characteristics of CYP3A4 and P-glycoprotein substrates tested across CYP3A4-Caco-2 cells

Drug	Substrate for		Efflux ratio	Extraction ratio % (S.D.)		
	3A4[a]	P-gp[b]	B to A/A to B	Drug alone	Drug + cyclosporine	Drug + GG918
K77[c] (10 μM)	Yes	Yes	9	33(3)	5.7(0.3)	14(1)
Rapamycin (1 μM)	Yes	Yes	2.5	60(5)	15(1)	45(1)
Midazolam (3 μM)	Yes	No	1	25(2)	10(1)	23(2)
Felodipine (10 μM)	Yes	No	1	26(1)	14(1)	24(2)

ER represented as mean (S.D.).
[a] 3A4, CYP3A4.
[b] P-gp, P-glycoprotein.
[c] K77, K11777: *N*-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl.

inhibit CYP3A4) did not change the transport profiles or the extraction ratios for either of the compounds. This was expected as these two drugs were negative controls for P-glycoprotein function and the effects of GG918 should be negligible. K77 and rapamycin transport profiles and ERs, however, were significantly affected by GG918. Consistent with complete inhibition of P-glycoprotein, efflux transport of both K77 and rapamycin was abolished. By inhibiting P-glycoprotein, the ER for K77 went from 33 to 14% (a 58% decrease), indicating that when the transporter was inactivated, there was decreased exposure of K77 to CYP3A4. The ER for rapamycin was decreased 25% in the presence of GG918 (from 60 to 45%) consistent with its moderate interaction with P-glycoprotein, when compared with K77. Therefore, for compounds that were substrates of CYP3A4 and P-glycoprotein, selective inhibition of the transporter yielded significant effects on the extent of metabolism by CYP3A4. These data support a role for P-glycoprotein in increasing the exposure of drugs to CYP3A4 in the intestine by allowing repeated cycling of drug via diffusion and active efflux.

6. Conclusion

Our understanding of the biochemical mechanisms controlling oral drug absorption has progressed significantly in recent years. These advances have been achieved, in part, by investigators showing the importance of CYP3A in the intestine and the generation of P-glycoprotein knock-out mice. This increased knowledge of the intestinal barriers to drug delivery has led to a paradigm shift in the way we consider drug interactions and absorption problems. New drug candidates are routinely screened for their potential to interact with P-glycoprotein and these results can influence the future development of the compound. The availability of in vitro systems mimicking the small intestine will enhance our ability to perform inhibition and drug interaction studies and could, in the future, provide a quantitative model for predicting human intestinal metabolism. The P-glycoprotein–CYP3A alliance has been characterized primarily from the perspective of its role on the disposition of the parent drug, however, considerable work still needs to be completed to determine how metabolites are themselves influenced by transport processes. Although this is one of the first drug efflux–metabolism alliances that has been explored, there will undoubtedly be other drug efflux–metabolism pairs exhibiting similar phenomena that are currently being uncovered.

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