Altern. Animal Test. EXperiment. 7 (1),23-29 (2000)

Quantitative Prediction of *In Vivo* Drug-drug Interactions from *In Vitro* Data: Effects of Active Transport of Inhibitors into the Liver

Shin-ichi Kanamitsu[1], Yutaka Shinozaki[2], Kiyomi Ito[3], Takafumi Iwatsubo[4], Hiroshi Suzuki and Yuichi Sugiyama

Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

[1]Current address: Section of Drug Metabolism Research, Naruto Research Institute, Otsuka Pharmaceutical Factory, Inc.

[2]Current address: Central Research Laboratories, Zeria Pharmaceutical Co., Ltd.

[3]Current address: School of Pharmaceutical Sciences, Kitasato University

[4]Current address: Drug Metabolism Laboratories, Institute for Drug Development Research, Yamanouchi Pharmaceutical Co., Ltd.

Abstract

The degree of *in vivo* drug-drug interactions caused by competitive or non-competitive inhibition of drug metabolism can be predicted using the *in vitro* inhibition constant (Ki) and the unbound concentration of inhibitor in the liver (Iu). Although it can be assumed for most inhibitors that the value of Iu is equal to the unbound concentration in the liver capillary (sinusoid) (Iout,u), Iu is larger than Iout,u if the inhibitor is actively taken up by the liver. In the present study, the possibility of active transport of inhibitors into the liver was evaluated using isolated rat hepatocytes. An uptake study using FCCP, an ATP-depletor, showed that unbound quinidine, erythromycin, sulfaphenazole, ketoconazole, and omeprazole are concentrated 2.2-, 1.4-, 1.2-, 1.2-, and 1.0-fold, respectively, in hepatocytes due to active transport. This value of the unbound concentration ratio of each inhibitor was multiplied by the unbound concentration at the inlet to the liver (Iin,u) to estimate the maximum value of Iu, and the *in vivo* increase in the AUC of the corresponding substrates (sparteine, cyclosporine, tolbutamide, terfenadine, and diazepam, respectively) was predicted based on the Iu/Ki ratio. Because none of the investigated inhibitors was found to be highly concentrated in the liver, the predicted *in vivo* interaction was not greatly affected by taking account of active transport of the inhibitor.

Keywords: *drug interaction, active transport, isolated rat hepatocyte*

Correspondence: Yuichi Sugiyama,
Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
Tel: 81-3-5689-8094 Fax: 81-3-5800-6949 E-mail: sugiyama@seizai.f.u-tokyo.ac.jp

Introduction

In order to prevent toxic drug-drug interactions, it is very important to be able to quantitatively predict pharmacokinetic changes caused by co-administration of drugs which are known to inhibit the hepatic metabolism of the study drug (Ito *et al.*, 1998a, 1998b). In the case of a competitive or non-competitive inhibition of the enzyme, the degree of *in vivo* interaction can be predicted using the values of the inhibition constant (Ki) and the unbound concentration of the inhibitor in the liver (Iu). Although the value of Ki can be estimated from *in vitro* studies using human liver microsomes etc., the value of Iu cannot usually be measured in humans. Therefore, it has been assumed in previous studies that the unbound concentration of the inhibitor in the liver capillary is equal to Iu (Ito *et al.*, 1998a; Kanamitsu *et al.*, 2000). In order to avoid false-negative predictions of *in vivo* interactions, the unbound concentration at the inlet to the liver (Iin,u) has been used as the maximum value of Iu. However, the above assumption is not valid in the case of inhibitors which are concentrated in the liver by active transport and may lead to the underestimation of the *in vivo* interaction. In the present study, the active transport of some P450 inhibitors into the liver was evaluated in *in vitro* studies using isolated rat hepatocytes and an attempt was made to improve the predictability of the *in vivo* interaction by taking account of the active transport.

Materials and Methods

Chemicals

[^{3}H]quinidine(555 MBq/μmol) and [^{14}C]erythromycin (2.04 MBq/μmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Sulfaphenazole and carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP) were purchased from Sigma Chemical Co. (St. Louis, MO). Ketoconazole was obtained from Research Biochemicals International. Omeprazole was kindly donated by Astra-Japan, Ltd. All other chemicals were of reagent grade.

Cell preparation

Hepatocytes were isolated from male Sprague-Dawley rats (7-9 weeks old) by the procedure of Baur *et al.* (1975). After isolation, the hepatocytes were suspended (1 mg protein/mL for quinidine uptake and 2 mg protein/mL for others) at 0℃ in albumin-free Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.3). Cell viability was routinely checked by the trypan blue [0.4% (w/v)] exclusion test. We used more than 90% as a viability criterion. Protein concentrations were determined by the method described by Bradford (1976), using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Uptake study

Drug uptake was initiated by adding the drug solution (50 μL) to the cell suspension (950 μL) preincubated at 37℃ for 3 min in the presence or absence of an ATP-depletor, FCCP (2 μM). The final concentration of drugs in the uptake medium was 0.1 μM for [^{3}H]quinidine, 20 μM for sulfaphenazole, and 5 μM for [^{14}C]-erythromycin, omeprazole, and ketoconazole. At designated times, the reaction was terminated by separating the cells from the medium using a centrifugal filtration technique (Schwenk, 1980). Briefly, in the uptake study of quinidine and erythromycin, 200 μL aliquots were placed into centrifuge tubes containing 50 μL 2N NaOH, covered by 100 μL of a mixture (density 1.011) of silicone and mineral oil. The samples were then centrifuged for 10 sec in a tabletop microfuge (10,000 x g, Beckman Instruments Inc., Fullerton, CA). Centrifugation pelleted the hepatocytes through the oil layer into the alkaline solution. After the cells had dissolved in the alkaline solution, the tube was sliced into two and each compartment was transferred to a scintillation vial. The alkaline

compartment was neutralized with 50 μL 2N HCl. Then, after addition of scintillation cocktail (Atomlight, Packard Instrument Co., Meriden, CT) to the vials, the radioactivity in the medium and cells was determined using a liquid scintillation spectrophotometer (LS 6000SE, Beckman Instruments Inc.).

In the uptake study of sulfaphenazole, ketoconazole, and omeprazole, the drugs in both the medium and cells were determined by HPLC with UV detection, after centrifugal filtration as described above, except that 250 mM sucrose in 50 mM sodium phosphate buffer (pH7.3) was used instead of 2N NaOH in the lowest layer of the tube. Acetonitrile (80 μL) was added to the medium (40μL) and cell solution (40μL) to precipitate protein. After centrifugation at room temperature for 5 min, the supernatant was subjected to HPLC. The HPLC system consisted of a model L-7100 pump (Hitachi Ltd., Tokyo, Japan), a model L-7200 sample injector (Hitachi), a model L-4250 UV absorbance detector (Hitachi), a model D-7500 Chromato-Integrator (Hitachi), and a TSKgel ODS-80Ts reversed-phase column (250 x 4.6 mm internal diameter, Tosoh, Tokyo, Japan). Sulfaphenazole was detected at 270 nm with a mobile phase consisting of a 7/3 (v/v) mixture of 0.1 M acetic acid and acetonitrile delivered at 1.0 mL/min. Ketoconazole was detected at 254 nm with a mobile phase consisting of a 2/2/1 (v/v/v) mixture of methanol, acetonitrile, and 20 mM potassium phosphate buffer (pH 6.8) delivered at 1.0 mL/min. Omeprazole was detected at 302 nm with a mobile phase consisting of a 35/65 (v/v) mixture of acetonitrile and distilled water delivered at 1.0 mL/min.

Data analysis

The time-courses of the cellular uptake of the drugs were plotted as an uptake value (μL/mg protein) obtained by dividing the amount taken up by their concentration in the medium. The initial uptake velocity of quinidine and erythromycin was calculated from linear regression of data points taken at 15, 30 and 45 sec and at 10, 20 and 60 sec, respectively. Assuming both active transport and passive diffusion for the influx into hepatocytes and only passive diffusion for the efflux from the hepatocytes, the initial uptake velocity in the presence of an adequate concentration of ATP-depletor represents the uptake by passive diffusion, because active transport of the drug is completely inhibited. The cell-to-medium unbound concentration ratio (C/M ratio) at steady-state can be described by Eq.(1):

$$\begin{aligned} &\text{C/M ratio} \\ &= (\text{PSactive} + \text{PSpassive}) / \text{PSpassive} \\ &= \nu o / \nu \text{passive} \end{aligned} \quad (1)$$

where PSactive and PSpassive represent the membrane permeation clearance by active transport and passive diffusion, respectively; νo and ν passive represent the initial uptake velocity obtained in the absence and presence of ATP-depletor, respectively. This C/M ratio can also be calculated by measuring the steady-state drug concentration (sum of the bound and unbound forms) in the cell and that in the medium in the absence and presence of the ATP-depletor as follows:

$$\begin{aligned} &\frac{C_{cell}/C_{medium}(\text{control})}{C_{cell}/C_{medium}(+\text{ATP-depletor})} \\ &= \frac{C_{cell,free}/f_T/C_{medium}(\text{control})}{C_{cell,free}/f_T/C_{medium}(+\text{ATP-depletor})} \\ &= \frac{C_{cell,free}(\text{control})}{C_{medium}} = \text{C/M ratio} \end{aligned} \quad (2)$$

where C_{cell} and C_{medium} represents steady-state total drug concentration in the cell and medium, respectively; $C_{cell,free}$ represents the steady-state unbound drug concentration in the cell; and f_T represents the unbound fraction in the cell. It is assumed that f_T is not affected by the ATP-depletor and that $C_{cell,free}$ equals C_{medium} in the presence of the ATP-depletor. Eq.(2) was used to calculate the C/M ratio of sulfaphenazole, ketoconazole, and omeprazole

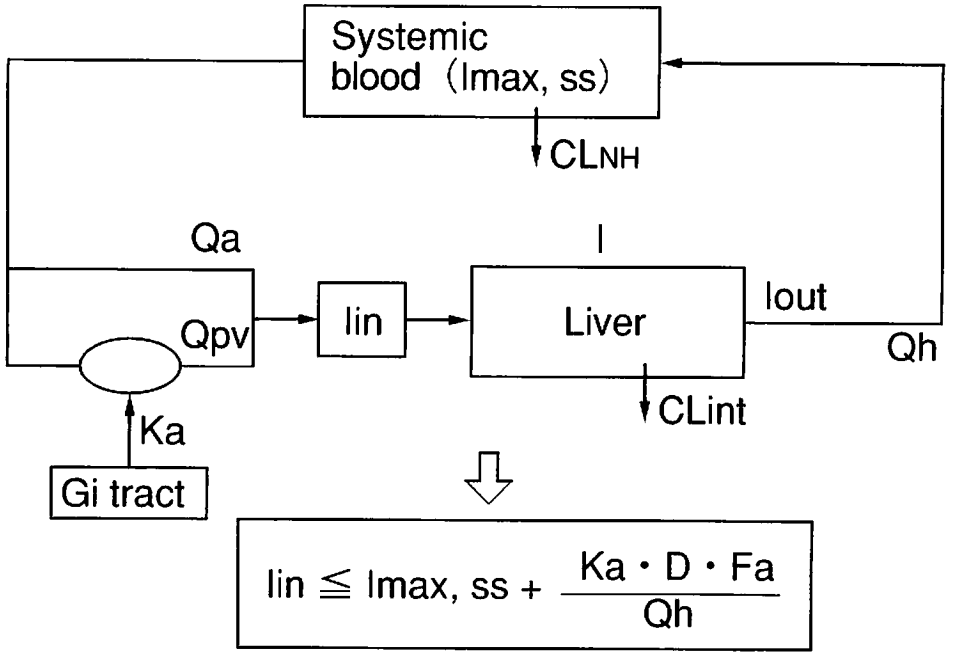

Fig. 1.
Model for estimating the concentration of inhibitor at the inlet to the liver after oral administration (Iin).
Iout, I, and Imax,ss represents the inhibitor concentration at the exit of the liver (hepatic vein side), the inhibitor concentration at the liver capillary, and maximum inhibitor concentration in the systemic circulation, respectively. Qa, Qpv and Qh (=Qa + Qpv) represents the blood flow at the hepatic artery, portal vein, and hepatic vein, respectively.

because no initial linear phase was detected in the uptake study involving these drugs.

Prediction of in vivo drug-drug interactions

The degree of *in vivo* drug-drug interactions (Rc) in humans involving the investigated inhibitors was predicted by the following equation (Ito *et al.*, 1998a):

$$Rc = \frac{1}{fh \cdot fm \cdot \frac{1}{1+Iu/Ki} + 1 - fh \cdot fm} \qquad (3)$$

where fh and fm represent the fraction of hepatic clearance (CLh) in the total body clearance and the fraction of the metabolic process subject to inhibition in CLh, respectively, for the affected drug. Values of both fh and fm were estimated using pharmacokinetic data from the literature. The Ki values of the inhibitors were taken from the report of *in vitro* inhibition studies using human liver microsomes.

In order to avoid underestimating the *in vivo* interaction, the maximum value of Iin,u (Iin,max,u) after oral administration of the inhibitor was estimated as follows (Fig. 1; Ito *et al.*, 1998a) and used as Iu in Eq.(3):

$$Iin,max,u = (Imax + ka \cdot D \cdot Fa / Qh) \cdot fu \qquad (4)$$

where Imax is the maximum concentration of the inhibitor in the systemic circulation, ka is the first-order absorption rate constant, D is the dose, Fa is the fraction absorbed from the gastrointestinal tract into the portal vein, Qh is the hepatic blood flow rate, and fu is the unbound fraction in the blood. The product of Iin,max,u and the C/M ratio of the inhibitor obtained in the uptake study was also used as Iu in Eq.(3) to calculate Rc.

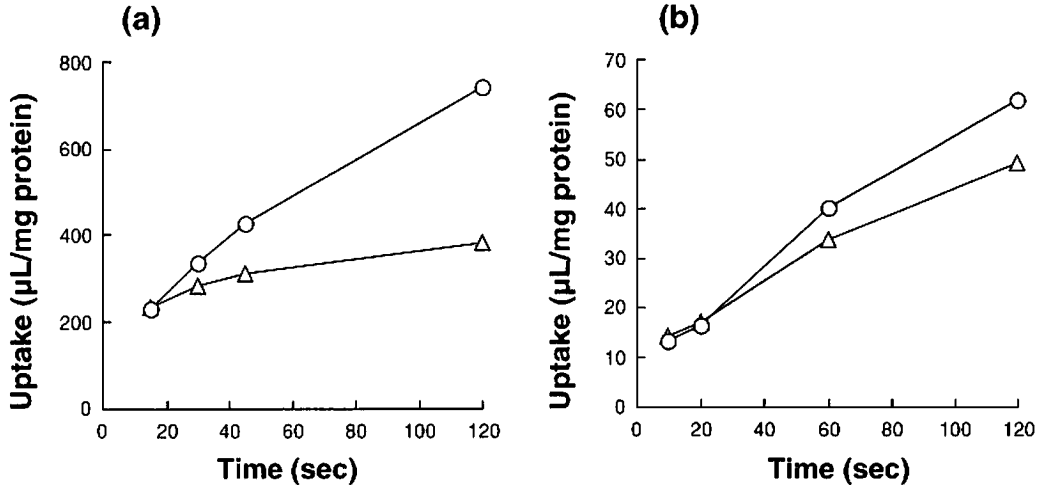

Fig. 2.
Time courses of the uptake of (a) [^{3}H]quinidine and (b) [^{14}C]erythromycin into isolated rat hepatocytes.
○: Control (in the absence of FCCP) △: in the presence of FCCP (2 μM).

Results and Discussion

Figure 2 shows the time-courses of uptake of quinidine and erythromycin into isolated rat hepatocytes. The uptake of both drugs was reduced by adding 2 μM FCCP to the incubation medium. On the other hand, no initial linear phase was detected in the uptake of sulfaphenazole, ketoconazole, and omeprazole, possibly because of their rapid uptake into hepatocytes. Therefore, the C/M ratios of these drugs were calculated using the drug concentration in the cell and that in the medium at 60 sec, by which time the steady-state was reached (data not shown).

The values obtained for the C/M ratio (mean ± SD; n=4) were 2.2 ± 0.8, 1.4 ± 0.2, 1.2 ± 0.1, 1.2 ± 0.3, and 1.0 ± 0.1 for quinidine, erythromycin, sulfaphenazole, ketoconazole, and omeprazole, respectively. Similar values for the C/M ratio were obtained when 30 μM rotenone was used as an ATP-depletor instead of FCCP (data not shown). It has been reported by Yamazaki et al. (1993) that the cellular ATP content is reduced to about 7% and 20% of the control value after a 3-min incubation with 2 μM FCCP and 30 μM rotenone, respectively. Therefore, the finding in the present study indicates that quinidine, erythromycin, sulfaphenazole, and ketoconazole are transported into rat hepatocytes, at least partly, by an active transport system.

Nakamura et al. (1994) reported a concentration-dependent uptake of [^{3}H]cimetidine into isolated rat hepatocytes. Fitting the initial uptake velocity (v_o) to the following equation yielded Vmax = 648 pmol/min/mg, Km = 32 μM, and PSpassive = 3.2 μL/min/mg:

$$v_o = V_{max} C_{medium} / (K_m + C_{medium}) + PS_{passive} C_{medium} \quad (5)$$

where Vmax is the maximum uptake velocity and Km is the Michaelis constant. On the other hand, the value of Iin,max,u is calculated to be 30.1 μM after oral administration of 200 mg cimetidine. Using the kinetic parameters reported by Nakamura et al. and Eqs. (1) and (6), the C/M ratio after a therapeutic dose of cimetidine was calculated to be about 4.3.

$$PS_{active} = V_{max} / (K_m + C_{medium}) \quad (6)$$

The degree of the predicted *in vivo* drug-drug interactions in humans, where the five

Table 1. Prediction of *in vivo* drug-drug interactions in humans.

Inhibitor (Affected drug)	fh · fm	C/M ratio	-Active Transport[a]		+Active Transport[b]		Observed AUCratio
			Iu/Ki	Predicted AUC ratio	Iu/Ki	Predicted AUC ratio	
Cimetidine (Theophylline)	0.52	x 4.3	0.05	x 1.0	0.2	x 1.1	x 1.5
Quinidine (Sparteine)	0.25	x 2.2	60	x 1.3	132	x 1.3	x 2.9
Erythromycin (Cyclosporine)	0.76	x 1.4	0.6	x 1.3	0.8	x 1.5	x 1.6
Sulfaphenazole (Tolbutamide)	0.80	x 1.2	200	x 5.0	240	x 5.0	x 5.3
Ketoconazole (Terfenadine)	0.58	x 1.2	4	x 1.3	5	x 1.3	>x 5.0
Omeprazole (Diazepam)	0.99	x 1.0	0.03	x 1.0	0.03	x 1.0	x 2.0

a) The Iin,max,u calculated based on the model in Fig. 1 was used as Iu.
b) The product of the Iin,max,u and the C/M ratio was used as Iu.

investigated drugs and cimetidine served as inhibitors, was re-calculated using the values obtained for their C/M ratio (Table 1). None of the 6 drugs was found to be concentrated highly enough in rat hepatocytes to affect the estimated degree of *in vivo* drug-drug interactions. Even in the case of cimetidine, whose C/M ratio was calculated to be 4.3, the predicted degree of *in vivo* interaction with theophylline was almost unchanged (1.0- vs. 1.1-fold), irrespective of whether active transport was taken into consideration or not. This finding may be due to the low values of both Iu/Ki (0.05) and fh · fm of theophylline (0.52). In case of the quinidine/sparteine interaction, the *in vivo* interaction was considerably underestimated in spite of large values for the estimated Iu/Ki ratio, possibly due to underestimation of the value of fh · fm. In other words, metabolic pathways other than dehydration of sparteine, which is known to be mediated by CYP2D6, may also be inhibited by quinidine. Furthermore, especially in the case of the ketoconazole/terfenadine interaction, the *in vitro* Ki value may have been overestimated based on the total concentration of inhibitor in the medium without correction for the nonspecific binding to microsomes (Obach, 1997). The estimated Iu/Ki ratio should be increased by using the Ki value based on the unbound concentration of inhibitor in the medium, which is expected to yield a predicted AUC ratio closer to the *in vivo* observation.

In conclusion, none of the investigated inhibitors was found to be highly concentrated in rat hepatocytes and the predicted *in vivo* interaction was not greatly affected by taking account of the active transport of inhibitor into the liver. However, there may be a species difference in the transport system between rat and human hepatocytes. Therefore, evaluation of the active transport of drugs into human hepatocytes or human liver slices in future studies may improve the predictability of *in vivo* drug-drug interactions in humans.

Acknowledgement

This work was supported in part by a grant from the Japanese Society of Alternatives to Animal Experiments.

References

Baur, H., Kasperek, S., and Pfaff, E. (1975) Criteria of viability of isolated liver cells, *Hoppe-Seylers Z. Physiol. Chem.*, 356, 827-838.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 72, 248-254.

Ito, K., Iwatsubo, T., Kanamitsu, S., Ueda, K., Suzuki, H., and Sugiyama, Y. (1998a) Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver, *Pharmacol. Rev.*, 50, 387-411.

Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y., and Sugiyama, Y. (1998b) Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport, *Annu. Rev. Pharmacol. Toxicol.*, 38, 461-499.

Kanamitsu, S., Ito, K., and Sugiyama, Y. (2000) Quantitative prediction of *in vivo* drug-drug interactions from *in vitro* data based on physiological pharmacokinetics: Use of maximum unbound concentration of inhibitor at the inlet to the liver, *Pharm. Res.*, 17, 336-343

Nakamura, H., Sano, H., Yamazaki, M., and Sugiyama, Y. (1994) Carrier-mediated active transport of histamine H_2 receptor antagonists, cimetidine and nizatidine, into isolated rat hepatocytes: Contribution of Type 1 system, *J. Pharmacol. Exp. Ther.*, 269, 1220-1227.

Obach, R. S. (1997) Nonspecific binding to microsomes: impact on scale-up of *in vitro* intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol, *Drug Metab. Dispos.*, 25, 1359-1369.

Schwenk, M. (1980) Transport systems of isolated hepatocytes. Studies on the transport of biliary compounds, *Arch. Toxicol.*, 44, 113-126.

Yamazaki, M., Suzuki, H., Hanano, M., and Sugiyama, Y. (1993) Different relationships between cellular ATP and hepatic uptake among taurocholate, cholate, and organic anions, *Am. J. Physiol.*, 264, G693-G701.