CYP3A4. For this reason, a comprehensive framework for the prediction of drug interactions would be of significant clinical importance. In addition, pharmaceutical companies are encouraged to carry out many *in vivo* drug interaction studies during the drug development process and the cost of these studies is increasing. Consequently, it is important to prioritize significant drug interactions to be confirmed as early as possible during the course of development. A reliable method for the prediction of CYP3A4 drug interactions would be advantageous in such circumstances.

A great deal of effort has already been devoted to establish a method for the accurate prediction of *in vivo* drug interactions using *in vitro* experimental data.[6-11] These predictions in principle rely on the $[I] / Ki$ ratio, that is a ratio of free concentration of the inhibitor at the interaction site to the *in vitro* inhibition constant. The results of these studies have increased our understanding of the mechanisms of drug interactions. Nowadays, both human liver specimens and expressed human P450 enzymes are commercially available and it is not difficult to determine a profile of metabolic drug interactions *in vitro*. However, the proper interpretation and quantitative extrapolation of *in vitro* data to *in vivo* situations require a detailed understanding of the overall pharmacokinetics of the drugs involved. Consideration should be given to the site of interaction, the time-courses of the unbound drug concentration at the site, the effects of drug transporters on the pharmacokinetics, and the possible contribution of metabolites to the interaction.[12]

Moreover, the quantitative prediction of drug interactions is difficult because of the following reasons. Firstly, the intestinal CYP3A4 plays a significant role in the first-pass metabolism of orally administered drugs. For example, several human *in*

vivo studies have shown that midazolam, felodipine, cyclosporine, and buspirone are extensively metabolized in the intestine.[13] Although Caco-2 cells are used in predicting the extent of intestinal absorption, it is difficult to predict the intestinal metabolism due to the very low expression of CYP3A4 in this cell line.[14] It is also known that CYP3A4 does not distribute uniformly along the length of the intestine: it is expressed more in the jejunum than in the ileum.[15] In addition, quantitative prediction of oral bioavailability is difficult due to the synergistic role of CYP3A4 and efflux transporters, such as MDR1, in reducing the intestinal absorption of substrate drugs.[16-20] The MDR1 is expressed more in the ileum than in the jejunum.[21] Although the intestine is also considered as an important site of drug interactions, the extent of intestinal metabolism is unpredictable in many cases. Secondly, CYP3A4 recognizes a wide range of substrates and some structural flexibility has been suggested at the substrate recognition site.[22] Consequently, the enzyme kinetics of CYP3A4 is sometimes complicated. Indeed, simple competitive inhibition theory has often failed to explain interactions *via* CYP3A4.[23] Thirdly, a series of CYP3A4 substrates apparently act as mechanism-based inhibitors which covalently bind to the enzyme. For these drugs, the recovery of CYP3A4 activity depends on regeneration of the enzyme at the target site. For this reason, predictions of the mechanism-based interactions from *in vitro* data require more complicated kinetic models compared with reversible inhibitors.[7,8,13,24-26] Considering these complex factors, it is reasonable to conclude that, by using only *in vitro* experimental data, precise prediction of *in vivo* drug interactions is not easy for the variety of drugs that are metabolized by CYP3A4. One of the practical methods to overcome this problem is to use *in vivo* information on interaction data of probe drugs of CYP3A4. This approach would enable the prediction

of various drug interactions from results of a small number of drug interaction studies carried out early in the course of drug development.

The objective of the present study is to construct a framework for the prediction of various drug interactions mediated by CYP3A4 using minimum *in vivo* information on drug interactions. We selected midazolam as a standard substrate and ketoconazole or itraconazole as a standard inhibitor. We have aimed to keep the method as simple as possible from a practical viewpoint while, at the same time, remaining theoretically accurate.

METHODS

Literature search

The analysis is based on 113 *in vivo* studies reported in 78 articles published over the period 1983 to 2006 (Tables I and II). Based on information from a comprehensive review[9], we added some new data from the literature. Some substrates and inhibitors were removed from the original information due to the small contribution or low selectivity for CYP3A4. All studies were used if the report included information on the dosage regime and the increase in AUC. A survey of a series of articles revealed that, in general, inhibitor drugs were administered consecutively for more than several days to attain steady-state conditions and substrate drugs were given as a single dose administration.

Theory

The oral clearance (CL_{oral}) of drugs is given by Eq. 1 where CL_{tot}, CL_h, CL_r, F_a, F_g

and F_h are the total body clearance, hepatic clearance, renal clearance, absorption ratio, and the gastrointestinal and hepatic availabilities, respectively. The reason why F_a is located at the left-side in Eq. 1 is that the current analysis (right-side terms) should focus on events after absorption.

$$CL_{oral} \cdot F_a = \frac{CL_{tot}}{F_g \cdot F_h} = \frac{CL_h + CL_r}{F_g \cdot F_h} \qquad \text{Eq. 1}$$

Assuming the well-stirred organ model,[27] Eq. 1 can be converted to Eq. 2 where fu and $CL_{int,h}$ are the unbound fraction of the drug in the blood, and the intrinsic clearance of the liver, respectively. This equation represents a general relationship between the oral clearance and the intrinsic clearance of the liver.

$$CL_{oral} \cdot F_a = \frac{fu \cdot CL_{\mathrm{int},h}}{F_g} + \frac{CL_r}{F_g \cdot F_h} \qquad \text{Eq. 2}$$

In the present study, two simplifications were made with respect to Eq. 2. Firstly, we assumed that CL_r can be ignored, which is frequently the case for lipophilic CYP3A4 substrates. Secondly, we assumed that F_g can be regarded as 1.0 hypothetically. This simplification does not necessarily mean that the gastric metabolism is insignificant. Rather, it means that the gastric metabolism is assumed to occur in proportion to the hepatic metabolism as if it is a part of the liver. This may allow some overestimation of $CL_{\mathrm{int,h}}$ as a consequence. Significant gastric first-pass effects are well established facts for some CYP3A4 substrates; Fg values of midazolam, felodipine, cyclosporine and buspirone are reported to be 0.57, 0.45, 0.39 and 0.21, respectively.[13,16] In the future, it may be advantageous to distinguish between gastric and hepatic metabolism by CYP3A4. At present, however, we have no alternative but to accept this simplification, since we do not know the extent of the *in vivo* gastric metabolism for all of the drugs analyzed in the present study. In this connection, it is worthy of note that

incorporation of gut wall CYP3A4 inhibition did not result in a general improvement in drug interaction predictions in a recent report.[28] Overall, Eq. 2 becomes Eq.3 with these simplifications.

$$CL_{oral} \cdot F_a \cong fu \cdot CL_{\mathrm{int},h} \qquad \text{Eq. 3}$$

Then, we considered a relationship between the intrinsic clearance and metabolic drug interactions under an assumption of the rapid equilibrium of the drug concentration between the blood and the hepatocyte. It is often the case that a drug is metabolized by more than two pathways. In Eq.4, we assume two intrinsic metabolic clearances, $CL_{\mathrm{int,3A4}}$ and $CL_{\mathrm{int,others}}$, which represent the metabolism mediated by CYP3A4 and the sum of other metabolic pathways, respectively.[11,29]

$$CL_{\mathrm{int},h} = CL_{\mathrm{int},3A4} + CL_{\mathrm{int},others} \qquad \text{Eq. 4}$$

In the following equations, asterisks denote parameters altered by a drug interaction. When the metabolism of CYP3A4 is inhibited by a drug interaction, the altered clearance is given by Eq. 5, where I is the unbound concentration of the inhibitor at the interaction site, and K_i is the inhibition constant.

$$CL^{*}_{\mathrm{int},3A4} = \frac{CL_{\mathrm{int},3A4}}{1+\dfrac{I}{K_i}} \qquad \text{Eq. 5}$$

Equation 5 is applicable to both competitive and non-competitive inhibitions, since the drug concentration *in vivo* is usually much lower than the K_m value.

Here, we define the ratio of the contribution of CYP3A4 to oral clearance (CR_{3A4}) by

$$CR_{3A4} = \frac{CL_{oral} - CL_{oral,-3A4}}{CL_{oral}} \qquad \text{Eq. 6}$$

where $CL_{oral,-3A4}$ is an altered *in vivo* oral clearance when $CL_{int,3A4}$ is blocked completely. $CL_{oral,-3A4}$ is given by Eq. 7 based on Eqs. 3 and 4.

$$CL_{oral,-3A4} \cdot F_a = fu \cdot (CL_{\text{int},h} - CL_{\text{int},3A4}) \qquad \text{Eq. 7}$$

From Eqs. 6 and 7, CR_{3A4} is given by Eq. 8, which indicates that the ratio of the *in vivo* contribution of CYP3A4 to the oral clearance has the same value as the fraction metabolized by CYP3A4 to inhibition (fm_{CYP3A4}), which is determined by examining the effect of CYP3A4 selective inhibitors / antibodies on drug metabolism by human liver microsomes when the urinary excretion is very low and rapid equilibrium is achieved in the liver. These relationships have been widely used for prediction of *in vivo* clearances in the presence of drug interactions or CYP enzyme polymorphisms, as reported by other groups.[11,29]

$$CR_{3A4} = \frac{CL_{\text{int},3A4}}{CL_{\text{int},h}} \qquad \text{Eq. 8}$$

Equation 5 can be converted to Eq. 9 using Eqs. 6 and 8.

$$CL^*_{\text{int},h} = \frac{CR_{3A4} \cdot CL_{\text{int},h}}{1+\frac{I}{K_i}} + (1 - CR_{3A4}) \cdot CL_{\text{int},h}$$

$$= (1 - CR_{3A4} \cdot \frac{I}{I+K_i}) \cdot CL_{\text{int},h} \qquad \text{Eq. 9}$$

To estimate AUC from Eq. 9, the equation needs to be integrated with time. For this purpose, the time-averaged apparent inhibition ratio (IR_{3A4}) is defined by Eq. 10.

$$IR_{3A4} = \frac{I_{app}}{I_{app} + K_i} \qquad \text{Eq. 10}$$

where I_{app} is the time-averaged apparent unbound concentration of the inhibitor in the liver. The increase in the ratio of AUC caused by a drug interaction (Eq. 11) is derived from Eqs. 9 and 10. We assumed here that the value of IR_{3A4} for an inhibitor is the same for any substrate.

$$\frac{AUC^{*}_{oral}}{AUC_{oral}} = \frac{CL_{oral} \cdot F_a}{CL^{*}_{oral} \cdot F_a} = \frac{CL_{\mathrm{int},h}}{CL^{*}_{\mathrm{int},h}} = \frac{1}{1 - CR_{3A4} \cdot \dfrac{I_{app}}{I_{app} + K_i}}$$

$$= \frac{1}{1 - CR_{3A4} \cdot IR_{3A4}} \qquad \text{Eq. 11}$$

Eq.11 indicates that an AUC increase by a drug interaction between any oral drug *via* CYP3A4 can be predicted if the CR_{3A4} of the substrate and the IR_{3A4} of the inhibitor are available.

It needs to be mentioned that the above theory may not be applicable directly to mechanism-based inhibitors. However, the final form of Eq. 11 can be accepted even for mechanism-based inhibitors by regarding the IR_{3A4} values as an overall inhibition ratios of CYP3A4 at the equilibrium state between inactivation and generation of the metabolizing enzyme. From this viewpoint, the definition of IR by Eq. 10 is invalid for mechanism-based inhibitors.

Calculation of CR_{3A4} and IR_{3A4} values

The values of CR_{3A4} and IR_{3A4} for various substrates and inhibitors were calculated sequentially according to the following steps based on AUC increases in the 53 interaction studies which are indicated by underlines in Table I. (1) We assumed that the CR_{3A4} value of simvastatin is 1.0, since it has been reported that simvastatin is a selective substrate of CYP3A4[30] and a search of the literature showed that the plasma AUC of simvastatin tends to be increased most markedly following inhibition of CYP3A4. It was confirmed that a reduction of the CR_{3A4} value of simvastatin to 0.95 did not affect the overall outcomes of the present analysis. (2) Once we assumed the CR_{3A4} value for simvastatin, the IR_{3A4} value of itraconazole, a typical inhibitor, was

obtained based on Eq. 11, using the result of a drug interaction study involving simvastatin and itraconazole. (3) The CR_{3A4} value of midazolam, a typical substrate, was calculated from studies with midazolam and itraconazole using the calculated IR_{3A4} value of itraconazole with Eq. 11. An algebraic mean of the AUC increase was used for the calculation, whenever the results of multiple studies are reported for an interaction set of interest. (4) The IR_{3A4} values of the other inhibitors including ketoconazole, another typical inhibitor, were calculated from interaction studies between the inhibitor and midazolam, using the calculated CR_{3A4} value of midazolam with Eq. 11. (5) The CR_{3A4} values of other substrates were calculated from interaction studies between the substrate and itraconazole or ketoconazole whenever possible, using the calculated IR_{3A4} value of itraconazole or ketoconazole, respectively, with Eq. 11. (6) For nifedipine, no interaction study with itraconazole or ketoconazole has been reported. Accordingly, the CR_{3A4} value of nifedipine was calculated from the study with nifedipine and diltiazem, using the calculated IR_{3A4} value of diltiazem, with Eq.11. Diltiazem was selected, since the AUC of nifedipine was most significantly increased by the administration of diltiazem.

RESULTS

We surveyed 113 *in vivo* drug-drug interaction studies published in 78 articles shown in Tables I and II. The CR_{3A4} values of 14 substrates and the IR_{3A4} values of 18 inhibitors were calculated using Eq. 11 based on the results of 53 clinical studies (the estimation set), which are indicated by the underlined article numbers in Table I. As shown in Table III, high CR_{3A4} values of more than 95% were calculated for simvastatin,

lovastatin, buspiron, and nisoldipine, 85-94% for triazolam, midazolam, felodipine, and 70-84% for cyclosporin, nifedipine, and alprazolam. High IR_{3A4} values of more than 95% were estimated for ketoconazole (daily dose 200-400 mg), voriconazole (400 mg) and itraconazole (100-200 mg), 85-94% for telithromycin (800 mg), clarithromycin (500-1,000 mg), saquinavir (3,600 mg) and nefazodone (400 mg), and 70-84% for erythromycin (1,000-2,000 mg), diltiazem (90-270 mg), and fluconazole (200 mg), and verapamil (240-480mg).

The current method enabled predictions of the AUC increase caused by drug-drug interactions of any combination of the substrates and inhibitors. In order to validate the suitability of the present method, the extent of increase in AUC by drug interaction was predicted for the remaining 60 clinical studies (the validation set), which are indicated by the article numbers without an underline in Table I, and the results were compared with the observed values (Fig. 1B). This prediction was performed by substituting the values of CR_{3A4} and IR_{3A4} shown in Table III and IV, respectively in Eq. 11. As shown in Fig. 1B, with the current method, we could predict the increase in AUC within 67-150% of the observed AUC increase for 50 clinical studies (83%), and within 50-200% for 57 clinical studies (95%).

In the calculation of CR_{3A4} and IR_{3A4} values, the algebraic mean of the increase in AUC was used when more than one article was available for the same interaction set (Table I). In these cases, however, significant deviation was observed in the AUC increase between or among reports. For the analysis, we often combined the data of clinical studies with different doses of the inhibitor. Since the lower doses frequently gave more AUC increase of a substrate, it is possible that the deviation of the inhibitor dose may not largely affect the results, as far as the dose of inhibitor is set within the

therapeutic range. The extent of this deviation is shown in Fig. 1A, which was prepared in the same style as Fig. 1B. Each circle and vertical bar in Fig. 1A represents the mean + S.D. reported in each article shown by the underlines in Table I. If the S.D. values were not reported in articles listed in Tables I and II, the reported mean values are shown by squares. As shown by the dotted lines in Fig. 1A, for most of the articles, the increase in AUC of substrate drugs caused by drug inhibition deviated by 67-150% of the algebraic mean values. The predictions within 50-200% of the observed AUC increase in Fig. 1B were regarded as successful, since the corresponding variation of AUC in Fig. 1A (the estimation set) was within this range.

Then, we reorganized the data shown in Fig. 1 to indicate the relationships between the IR_{3A4} values and the increase in AUC of each substrate (Fig. 2). It was found that the AUC increased steeply as the IR_{3A4} value increased for highly CYP3A4-dependent substrates, such as simvastatin, lovastatin and buspirone, whereas only minimal increases were observed for poor CYP3A4 substrates, such as zolpidem and cerivastatin (Fig. 2). In the same manner, potent inhibitors, such as azole antifungals, increased the blood levels of a number of CYP3A4 substrates markedly, whereas no or only very minor increases were observed for weak inhibitors, such as azithromycin, gatifloxacin and fluoxetine (Fig. 3).

Finally, the data were reorganized to show that the increase in AUC in 251 kinds of drug interactions between 14 substrate drugs and 18 inhibitors could be predicted (Fig. 4, note that telithromycin is included both among the substrates and inhibitors). The nomogram in Fig. 4 indicates that a very marked increase in the AUC is anticipated when substrate drugs with high CR_{3A4} values were administered with potent inhibitors with high IR_{3A4} values.

DISCUSSION

CYP3A4 is the most important drug-metabolizing enzyme, which oxidizes preferentially relatively large, lipophilic, and neutral to basic molecules. Therefore, CYP3A4 is recognized as a key enzyme which determines the clearance of various drugs and, in some cases, has a major effect on their safety and efficacy. Although no major polymorphism in the CYP3A4 gene has been identified, marked inter-individual differences have been reported in the activity of CYP3A4.[31] One possible reason for such differences in the activity is that CYP3A4 is inducible by various diets and drugs, such as rifampicin and carbamazepin, *via* the mechanism mediated by PXR.[32-34] Furthermore, CYP3A4 is the predominant metabolizing enzyme not only in the liver but also in the intestine. It has been reported that intestinal metabolism is the major factor determining the bioavailability of some drugs.[16,35-37] However, as far as we know, nobody has succeeded in predicting the extent of the first-pass effect on metabolism by intestinal CYP3A4 from *in vitro* data. Although there are some established methods to determine the activity of CYP3A4 *in vivo,* including evaluation of the metabolic ratio of selective substrates (midazolam, testosterone and cortisol) and the erythromycin breathe test, it has been reported that these methods do not offer consistent results,[38] possibly due to differences in the organ of metabolism (liver or intestine) and/or the presence of multiple recognition sites in the CYP3A4 molecule.[39]

In spite of these issues regarding the *in vivo* evaluation of CYP3A4 activity, the current rather simple method gave satisfactory predictions in most cases. The following issues may contribute to this success. Firstly, uncertain factors were avoided

since the current method primarily relies on an overall *in vivo* evaluation. For example, the present method satisfactorily predicted drug interactions with mechanism-based inhibitors such as azithromycin, clarithromycin, diltiazem, erythromycin, and roxythiromycin (Fig.3), which frequently exhibit complicated kinetics. Accurate predictions have been achieved recently from *in vitro* data for mechanism-based inhibitors by sophisticated analysis. For the successful prediction, it has been reported that evaluation of the unbound fraction of the drug in the incubation medium is important.[13,40] Moreover, the analysis requires a turnover rate of the metabolizing enzyme and a rate constant for the irreversible reaction, both of them are not easy to estimate from *in vitro* experiments.

Secondly, we used simvastatin as a selective substrate and ketoconazole and itraconazole as selective inhibitors of CYP3A4, although these drugs are not absolutely specific for CYP3A4. For example, although we assumed that the CR_{3A4} value of simvastatin is 1.0, this drug is also metabolized by CYP2C8 to a minor extent.[27] Ketoconazole is a well known selective inhibitor of CYP3A4, but this drug also inhibits the activities of CYP2C8,[41] 2C9[42] and MDR1,[43] which may also affect the disposition of substrate drugs analyzed in the present study. In spite of these defects, the success in the prediction of drug interactions with the present method (Fig. 1) suggests that CYP3A4 plays a crucial role in most of the drug interactions analyzed in the present study.

A number of probe drugs have been used to study the activity of CYP3A4, including midazolam, nifedipine, simvastatin, and erythromycin.[44] Among them, it is generally recognized that the most reliable probe drug is midazolam for CYP3A4.[45,46] The plasma AUC of midazolam is increased significantly by coadministration of various

CYP3A4 inhibitors (Figs. 1, 2 and 4). In our preliminary analysis, we found that the rank order of the AUC increase of typical substrates, such as simvastatin, lovastatin and buspirone, by a series of inhibitors was generally in good agreement with the rank order of the AUC increase of midazolam produced by these inhibitors. These results suggest that the extent of CYP3A4 inhibition after administration of each inhibitor is almost the same among substrates. From this analysis, we hypothesized that calculation of AUC increases from IR_{3A4} values should be possible.

It has often been reported that *in vitro Ki* values vary significantly among CYP3A4 substrates used,[10] which contradicts our hypothesis that the IR_{3A4} value is the same for any substrate. For example, nifedipine was allocated to a different group from midazolam and triazolam by a cluster analysis of the victim profile of *in vitro* drug interactions.[47] However, as represented in Fig.2, no clear discrepancy was observed for the predicted AUC increases of any particular substrate assuming a single IR_{3A4} value for each inhibitor. It is therefore possible that the *in vivo Ki* value of each inhibitor is not affected by the substrate drugs analyzed in the present study. This result may be due to the fact that the number of available drug interaction studies is limited for each inhibitor. Accordingly, we should be cautious in predicting the increase in AUC for a novel substrate drug by using the IR_{3A4} values determined in the present study.

In the validation process of the present study, the method provided successful predictions in 57 out of 60 cases. Telithromycin is a particular example of an accurate prediction. The CR_{3A4} value of telithromycin was calculated to be 0.49 from the results of an interaction study with ketoconazole. The AUC increase produced by interaction with itraconazole, which has an IR_{3A4} value of 0.95, was predicted to be 1.85, which was

in good agreement with the observed increase of 1.60. Telithromycin also acts as an inhibitor of CYP3A4. The IR_{3A4} value of telithromycin was 0.91 and an AUC increase of simvastatin produced by interaction of telithromycin was predicted to be 11.1, which was also in good agreement with the observed increase of 10.8.

In contrast, we had difficulties in predicting 3 reports of drug interactions; i.e., cyclosporin-voriconazole, triazolam-itraconazole, and one of two reports for a triazolam-erythromycin interaction. In article #63 in Table II, it was reported that the AUC of cyclosporin was increased 1.70-fold by the administration of voriconazole, whereas we predicted a 4.61-fold increase (Figs. 1B and 4). In the same manner, although the AUC of triazolam was reported to be increased 27.1-fold by the administration of itraconazole (article #71 in Table II), we predicted an 8.85-fold increase (Figs. 1B and 4). Concerning the interaction between triazolam and erythromycin, there was a deviation in the increase in AUC of triazolam by erythromycin. In article #31 in Table II, a 3.65-fold increase was reported whereas a 2.06-fold increase was reported in article #61. Our prediction was 4.32-fold (Figs. 1B and 4) and was in accord with the former article. The reason for these deviations is unknown. Further studies will help to investigate whether there was some mechanistic reason or whether there was simply some unavoidable variability. The factors that need to be considered include the contributions by other metabolizing enzymes and transporters, and the variety of enzyme kinetics of CYP3A4 inhibition.

In the present study, midazolam, itraconazole and ketoconazole were used as a standard substrate or an inhibitor because they are used most commonly in interaction studies. As a result, overall AUC increases were successfully predicted, indicating that the standard drugs were selected appropriately. It may be possible to use other

commonly used substrates of CYP3A4 such as simvastatin, lovastatin and buspirone to calculate IR_{3A4} values because no deviation was observed in the predictability of AUC increase for these substrate drugs when coadministered with wide range of inhibitors (Fig. 2).

To prioritize drug interaction studies during the course of drug development, Obach et al. have recently proposed a rank-order approach in which the mechanism of possible interactions is explored by *in vitro* experiments and then the most probable interactions are evaluated *in vivo* using typical substrates or inhibitors.[48] The results of the present study support their approach. If a drug interaction study is carefully designed using the appropriate standard drugs, significant interactions *via* CYP3A4 will not be missed. In addition, the extent of CYP3A4-mediated interactions between many other drugs will be able to be predicted using the current method, as suggested by the results in Fig. 4.

In conclusion, we have constructed a general framework for prediction of the increase in AUC which is mediated by CYP3A4. The precision and robustness of the method have been demonstrated satisfactorily. Several standard substrates and inhibitors are proposed for the evaluation of drug interactions *via* CYP3A4. This method would be applicable (1) to prioritize clinical trials to investigate drug interactions during the course of drug development, and (2) to estimate the clinical significance of unknown drug interactions.

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