12 *In Vitro* Liver Systems to Study Induction/Inhibition: Prediction of *In Vivo* Metabolism and Drug–Drug Interactions

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12.1 SUMMARY

Cytochrome P450 (CYP) enzymes expressed in liver microsomes are mainly involved in the biotransformation of drugs. CYP enzyme-related metabolic activity is a major determining factor in the pharmacokinetic profile, including bioavailability. A change in the metabolic activity derived from CYP inhibition and induction by comedication often results in adverse events or failure of drug therapy. The metabolic stability, CYP inhibition, and induction are key parameters in the optimization of lead structures and the selection of new molecular entities (NMEs) in drug discovery programs. Regarding these screenings, the advent of various evolutional technologies has facilitated a paradigm shift from the traditional system to a robust moderate to high throughput system that meets the demands of modern drug discovery. Furthermore, the prediction of clearance of NMEs and alteration of the plasma concentration profiles

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associated with drug–drug interactions (DDIs) caused by comedication has become an important task in pharmacokinetic studies. This chapter reviews advanced assay systems for metabolic stability, CYP inhibition, and induction, as well as predictive methods for pharmacokinetic profiles and DDI-associated changes in their profiles in humans.

12.2 INTRODUCTION

Pharmacokinetic profiles and DDIs such as drug-metabolizing enzyme inhibition and induction of NMEs in humans have increasingly become recognized as critical parameters of the developability of drugs [1]. Drug metabolism is a major factor governing pharmacokinetics. CYP enzymes are major drug-metabolizing enzymes and can catalyze the biotransformation of two thirds of commonly used drugs [2] and many other compounds such as steroid hormones, toxics, and carcinogens [3–5]. CYP enzymes consist of a superfamily of heme-thiolate proteins, and CYP enzymes responsible for drug metabolism are classified into three families, namely, CYP1, CYP2, and CYP3. The liver is the principal organ of drug metabolism and one or more of the various CYP enzymes expressed in the microsomal fraction are mainly involved in the metabolism. Shimada *et al*. [6] demonstrated that the most abundant CYP enzymes in the human liver are CYP3A subfamilies at 30% of the total spectrally determined CYP content, followed by the CYP2C subfamilies (20%), CYP1A2 (13%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (<2%), and CYP2B6 (<1%). Accordingly, metabolic screening has been performed using human liver microsomes and fresh or cryopreserved human hepatocytes. Furthermore, those materials have been utilized to evaluate CYP inhibition, and hepatocytes in primary culture have been an indispensable tool for the assessment of CYP induction.

There is a growing awareness of the importance of predicting the pharmacokinetic profile in humans based on preclinical *in vitro* and/or *in vivo* data. The estimated human clearance (CL) allows predicting an effective dose by taking the efficacious plasma concentration into consideration, and the estimated plasma concentration profile, including maximum plasma concentration (C_{max}) and half-life ($t_{1/2}$) in humans, are incorporated into the design of the dose regimen of the clinical study. Similarly, with regard to the inhibition and induction of CYP enzymes, the prediction of a change in CL by comedication provides an important position when selecting development candidates or creating a strategy for clinical DDI studies.

This chapter focuses on *in vitro* liver systems used to study metabolic stability, and CYP enzyme inhibition and induction both to optimize pharmacokinetics during early drug discovery and to characterize NMEs during late drug discovery or drug development. The predictive models to evaluate CL and/or plasma concentration profile are also reviewed, together with the evaluation of the influence of DDI on the pharmacokinetic profile in humans. Recently, it has been highlighted that first-pass metabolism in the small intestine has a great impact on bioavailability, and *in vitro* and *in vivo* studies have been directed toward the assessment of metabolism of CYP3A substrates in the small intestine [7]. However, this chapter focuses on such metabolic events in the human liver.

12.3 METABOLIC STABILITY

12.3.1 *In Vitro* System for Evaluating Metabolic Stability

In vitro systems of microsomes or hepatocytes are routinely used in drug metabolism studies to obtain an estimate of metabolic stability, usually expressed as intrinsic clearance (CL_{int}). In general, the CL_{int} can be estimated from the metabolite formation assay using the following equation:

$$CL_{int} = \frac{v}{S} = \frac{V_{max}}{K_m + S} \tag{12.1}$$

where S is the substrate concentration, v the metabolite formation rate, K_m the Michaelis constant, and V_{max} the maximum metabolite formation rate. In the drug discovery phase, identification of the main metabolites using *in vitro* or *in vivo* samples is difficult due to the labor-intensive and time-consuming processes. Hence, substrate depletion assay is widely used to estimate CL_{int} from the depletion or degradation rate constant (k_{dep}) of the substrate in microsome or hepatocyte incubation [8–10]. CL_{int} is estimated at a low substrate concentration such as 0.5–1 μM [11–15], assuming that K_m values are at least higher than 5–10 μM.

Drug metabolism is a major determinant factor to pharmacokinetics of NMEs. In prediction of the pharmacokinetic behavior, hepatic clearance (CL_H) can be estimated from the CL_{int} using physiological models: well-stirred model, parallel tube model, and dispersion model [16,17]. It is highly likely that the CL is calculated mainly based on the well-stirred model, simplified method among them, because the CL values estimated from the well-stirred model were reported to be within an acceptable range when compared with the other two models [16]. Assuming that drugs are cleared via hepatic metabolism from the body, the CL can be expressed by hepatic clearance (CL_H), which is calculated by the following equation based on the well-stirred model:

$$CL = CL_H = \frac{Q_H \times f_p \times CL_{int,\textit{in vitro}}}{Q_H + f_p \times CL_{int,\textit{in vitro}}} \tag{12.2}$$

where Q_H is hepatic blood flow, $CL_{int,\textit{in vitro}}$ the intrinsic clearance (CL_{int}) scaled up to whole body using a scaling factor (microsomal recovery from the entire liver), and f_p the unbound fraction of protein binding in blood. The intrinsic clearance ($CL_{int}/f_{u,inc}$), which is corrected by unbound fraction ($f_{u,inc}$) of microsomal or hepatocyte incubation, is utilized on a case-by-case basis. Both unbound fractions are determined by equilibrium dialysis and ultracentrifugation [18]. An equilibrium dialysis method using 96-well apparatus has been developed for efficiently determining plasma protein binding [19].

Although knowledge of the binding parameters would be an essential part in precisely predicting CL, the metabolic stability has been a major indicator to guide the direction of lead optimization. Increased throughput of the metabolic screening has been achieved by developing an advanced robotic system in miniaturized format, such as 96-well plates [20–24]. Currently, Xu *et al*. [25] proposed a streamlined and intelligent workflow for metabolic stability that allows for high throughput analyses based on a novel postincubation pooling strategy coupled with ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS). Establishment of a semiquantitative

relationship between CL_{int} and *in vivo* CL is a prerequisite for metabolic screening. However, some investigators pointed out a poor correlation between CL_{int} and CL in a relatively large data set, including various compounds [12,24,26]. Noticeably, Komura *et al*. [24] demonstrated that there was a certain relationship among derivatives with the same core structure, and even if a derivative showed a poor correlation, considering the f_p and/or $f_{u,inc}$ into the estimation of CL_{int} dramatically improved the relationship of $f_p \times CL_{int}$ versus CL or $f_p \times CL_{int}/f_{u,inc}$ versus CL. Moreover, Obach [27] and Riley *et al*. [28] demonstrated that the $CL_{int,\textit{in vitro}}$ was correlated with *in vivo* intrinsic clearance ($CL_{int,\textit{in vivo}}$) estimated from CL using physiological models incorporating both unbound fractions.

In the pharmacokinetic profiling for NMEs, linear pharmacokinetics would be qualitatively guaranteed on comparing the K_m value with the effective plasma concentration predicted in humans [8,29]. Obach and Reed-Hagen [8] applied the substrate depletion assay where the K_m value can be estimated from a concentration-dependent change in k_{dep} of unchanged compounds. For substrates metabolized possibly via a predominant pathway by one CYP enzyme, the estimated K_m values were comparable to those from metabolite formation assays using recombinant CYP enzyme. A similar result for K_m values was reported by Komura *et al*. [30]. Recently, a newly developed multiple depletion curve method has been reported to provide accurate estimates of K_m, in addition to CL_{int} [31].

Like liver microsomes, hepatocytes with integrated drug-metabolizing enzymes, including CYP enzymes, are an indispensable tool for metabolic studies. Cryopreserved human hepatocytes, which are preliminarily well characterized by metabolic activity of each CYP enzyme, are commercially available, and pooled or individual cryopreserved hepatocytes have been utilized. However, it is a question whether cryopreserved hepatocytes retain drug-metabolizing activities during storage. Griffin and Houston [32] comparatively evaluated CL_{int} for 20 biotransformations using fresh and cryopreserved rat hepatocytes. There was a tendency for both hepatocytes to provide a similar CL_{int} except for some highly cleared compounds that showed lower activity in cryopreserved hepatocytes. Additionally, comparable CL_{int} values in fresh and cryopreserved human hepatocytes were proven by Lau *et al*. [33] and Naritomi *et al*. [14].

In general, plasma protein binding, as well as metabolic stability, affect the *in vivo* pharmacokinetic behavior, and the dose-dependent CL of some drugs arises from concentration-dependent protein binding, particularly for drugs that are governed by drug metabolism [34,35]. A challenged approach, whereby test compounds were incubated with hepatocytes in the presence of serum, has been developed by Shibata *et al*. [13]. The approach, based on substrate depletion assay and termed the serum (or plasma) incubation method, provided CL_{int} values that included the influence of plasma protein binding, and the obtained value was regarded as the relevant parameter for predicting *in vivo* CL. Further investigation using the plasma incubation method with rat and human hepatocytes was performed to assess species differences in nonlinear pharmacokinetics of propafenone [10]. The nonlinear disposition in humans would be ascribed to the saturation of hepatic metabolism because of the low K_m values, which was in contrast to the linear pharmacokinetics in rats, which was associated with the offset of saturable metabolism by nonlinear plasma binding in rats. The plasma incubation approach was applied to investigate chiral-conversion-associated pharmacokinetics of ibuprofen in adjuvant-induced arthritis rats [36]. The developed substrate depletion

assay combined with plasma incubation can produce *in vivo* relevant parameter, including two different factors of metabolic stability and plasma protein binding, and must be a valuable tool in the drug discovery phase [17].

12.3.2 *In Vitro* System for Evaluating the Contribution of CYP Enzymes to Total Metabolism

The estimation of fraction (f_m) of drug metabolized by the target enzyme is required to precisely predict the effect of CYP inhibition and induction. Not only the identification of CYP enzyme involved in the major metabolic pathway of an NME but also the evaluation of contribution of the CYP enzyme to total metabolism has been conducted by investigating the effects of chemical inhibitors or an antibody against CYP enzymes on the metabolite formation rate in human liver microsomes after elucidation of the major metabolic pathways [37,38]. Further investigations have then been performed by comparing the metabolite formation rate with either recombinant human cytochrome P450 (rhCYP) enzymes or separate samples of human liver microsomes with a wide range of CYP activity [22,39]. The metabolic activity in each rhCYP enzyme needs to be extrapolated to the relevant data in human liver microsomes. Nakajima *et al*. [40] proposed the concept of relative activity factor (RAF) for the extrapolation by which ratios of marker activity specific to a certain CYP enzyme between rhCYP enzyme and human liver microsomes was used. The RAF method has been widely utilized to study the involvement of individual CYP enzyme in the formation of each metabolite [41–43].

There are practical difficulties involved in identifying the detailed chemical structures of the major metabolites during drug discovery and early drug development. The substrate depletion assays, which are a useful tool to estimate total metabolic intrinsic clearance [8,44], are also applied to evaluate the contributions of CYP enzymes to the metabolic elimination in human liver microsomes using chemical inhibitors specific to each CYP enzyme [9,45]. Furthermore, Emoto and Iwasaki [46] demonstrated the usefulness of a combination method of the RAF method and substrate depletion assay in early drug discovery.

Some of the drugs and NMEs are eliminated via multiple pathways, not only catalyzed by CYP enzymes but also conjugative enzymes. The usefulness of hepatocytes for evaluating the contribution of CYP enzymes in propafenone and propranolol metabolism, which are mediated by phase II conjugation alongside CYP2D6 [47], was demonstrated. The contribution of CYP2D6 to the metabolism of both drugs in human hepatocytes was consistent with corresponding *in vivo* data and was different from human liver microsomes that exhibited the overestimation of CYP2D6 involvement.

12.3.3 Prediction of *In Vivo* Pharmacokinetics in Humans

12.3.3.1 Physiological Approach (In Vitro–In Vivo Extrapolation). One of the major purposes of preclinical pharmacokinetic studies is to select NMEs and/or support a design of clinical studies including DDI, by the prediction of CL and plasma concentration profiles in humans [48]. The prediction of human CL is based on *in vitro–in vivo* extrapolation using the physiological models in which the $CL_{int,in\ vitro}$ obtained in *in vitro* systems is incorporated [16,49].

The first successful prediction of *in vivo* hepatic CL of YM796 from *in vitro* data with human liver microsomes and rhCYP enzymes was demonstrated by Iwatsubo *et al*. [50]. Moreover, a pronounced species difference in *in vivo* CL was kinetically explained by the K_m and V_{max} values in rat, dog, and human liver microsomes [51]. Retrospective prediction using marketed drugs and/or development candidates has been performed by some investigators. Obach *et al*. [52] evaluated the effect of plasma protein binding on CL prediction based on the well-stirred or parallel tube model. Impressively, not incorporating the f_p value in the physiological models yielded better predictability than taking the f_p value into consideration. On the other hand, investigation using a relatively large data set by Ito and Houston [53] demonstrated that a prediction model incorporating rather than ignoring the f_p value provided a better correlation between the predicted $CL_{int,in\ vitro}$ and the observed $CL_{int,in\ vivo}$. Furthermore, when both the f_p and $f_{u,inc}$ values were canceled out under the assumption that both binding parameters are equivalent, there was reportedly a poor relationship between the predicted $CL_{int,in\ vitro}$ and the observed $CL_{int,in\ vivo}$ values, particularly for acidic compounds [28]. This relied on the fact that the f_p values were lower than the $f_{u,inc}$ values, for acidic compounds unlike basic compounds.

The importance of nonspecific binding in *in vitro* system with microsomes and hepatocytes, on the CL prediction, was demonstrated by Riley *et al*. [28]. Compared with considering only plasma protein binding, incorporating both binding parameters in the well-stirred model produced a relatively better relationship regardless of the classification of acidic, neutral, and basic properties. Similar findings were reported in the prediction study of 29 drugs conducted by Obach [27], who concluded that the inclusion of both binding values gave the best agreement between the observed CL and the CL predicted from *in vitro* data. It should be noted that the unbound fractions in microsome and hepatocyte systems can be calculated using lipophilicity- or chemical structure-based *in silico* approaches [54–57].

It has been realized that there is a common issue of underestimation in various reported prediction studies. Some attempts have been made to clarify the underlying mechanism for the underestimation. Using benzodiazepine derivatives, which are subject to CYP-mediated elimination, kinetic profiles were compared between human liver microsomes and cryopreserved hepatocytes that possess the full spectrum of drug-metabolizing enzymes and transporters [58]. Although almost similar CL_{int} values were obtained with both evaluation systems, the retrospective analysis exhibited 5.6-fold underprediction in human hepatocytes as evidenced in liver microsomes.

A scaling factor has been regarded as a determining parameter of predictability. When scaling up the CL_{int} from microsome or hepatocyte studies to whole body basis, generally, a physiological scaling factor, which is obtained from average recovery of microsomal protein/number of cells per gram of liver multiplied by the average liver weight in humans, is utilized. However, the employed microsomal content and number of cells range widely from 29 to 77 mg protein/g liver and from 86 to 135×10^6 cells/g liver, respectively [59], and the most widely utilized values seem to be 45 mg protein/g liver and 120×10^6 cells/g liver, respectively. Ito and Houston [53] reported that the use of an empirical scaling factor, instead of the physiological scaling factor, dramatically improved predictability. In their analysis, the empirical factor was determined by regression analysis to obtain the best fit of $CL_{int,in\ vitro}$ and $CL_{int,in\ vivo}$ values for various drugs. Recently, Sohlenius-Sternebeck *et al*. [60] proposed a prediction method based on a regression line that adjusts for systematic underprediction. Namely,

the regression lines were drawn from the correlation between $CL_{int,in\ vitro}$ from rat liver microsome and hepatocyte studies and $CL_{int,in\ vivo}$ from intravenous studies with 52 drugs. The use of hepatocyte regression, in particular, yielded high predictability that CLs for 82% of the compounds were predicted within twofold error. An approach using the animal scaling factor was established by Naritomi *et al*. [61]. The animal scaling factor was determined by dividing $CL_{int,in\ vivo}$ by $CL_{int,in\ vitro}$ obtained from *in vitro* and *in vivo* studies of the individual compound in rats or dogs and was a drug-dependent intrinsic factor that possibly included unknown or unidentified mechanisms governing drug disposition. Correction of the scaling factor gave better prediction within twofold of the observed value. An impressive finding for the underprediction was reported by Huang *et al*. [62]. Their group highlighted that permeability and efflux status in P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP)-expressing cells are a key factor affecting predictability of CL from CL_{int} and would be associated with the underprediction. For compounds that displayed high passive permeability and that are not good substrates toward the efflux transporters, the CLs would be predicted reasonably from the CL_{int}. In contrast, for compounds that showed either poor permeability or good substrates toward the transporters, the CL_{int} was unlikely to be predictive of CLs possibly due to a significant role of the transporters on their eliminations from the body.

It is increasingly realized that transporter-mediated liver uptake is involved in pharmacokinetic behavior with evidence that plasma concentrations of cerivastatin were increased with the inhibition of uptake transporter by the coadministration of cyclosporine A [63,64]. On the basis of the difference in concentration-dependent depletion profile between rat liver microsomes and hepatocytes, Parker and Houston [65] revealed that the liver uptake was a rate-limiting step for hepatic metabolism of saquinavir, nelfinavir, and ritonavir. A media loss assay was developed with rat hepatocytes to evaluate the impact of hepatic uptake on CL_{int} [66]. In contrast to the conventional assay in which the initial depletion rate constant is estimated from whole incubate (medium plus cells), the new concept was based on estimation of the disappearance rate constant only from the medium, which significantly improved predictability for a data set of 36 AstraZeneca compounds. Hence, the media loss assay can evaluate the hepatic active uptake process that confounded the CL prediction. Furthermore, the disposition of atorvastatin, cerivastatin, and indomethacin, known as substrates of uptake transporters, was investigated based on the measurement of cell and media concentration–time data in rat hepatocytes [67]. The developed physiological pharmacokinetic model that describes the ratio of the intracellular to extracellular concentration successfully predicted the effect of active uptake on the hepatic CL of atorvastatin and cerivastatin. Such a model would be a useful tool to predict *in vivo* CL of compounds that are subject to active liver uptake. In fact, using the *in vitro* kinetic data derived from human hepatocytes, analysis with a complicated physiologically based pharmacokinetic (PBPK) model incorporating a hepatic uptake process can successfully predict the *in vivo* pharmacokinetic profile of pravastatin, which is a typical substrate to solute organic anion-transporting peptide (OATP)1B1/1B3 [68].

12.3.3.2 Empirical Approach (Allometric Scaling). Alongside the *in vitro–in vivo* scaling described above, one of the best described techniques for predicting CL is allometric scaling, in which many physiological processes (blood flow, creatinine CL, heart rate, glomerular filtration, etc.) and organ size are defined by a power–law relationship

with the body weight of the species [69,70]. Owing to its simplicity, allometric scaling has been widely used to obtain human CL by extrapolating CL in animal species. Several methods have been developed; that is, simple allometry (SA), maximum lifespan potential (MLP), brain weight (BrW), and rule of exponent (ROE) approaches associated with the choice of SA, MLP, or BrW based on the exponent of SA. Ward and Smith [71] developed a liver blood flow (LBF) method in which CL in humans is predicted based on the ratio of human and animal LBF rate. Monkey LBF method provided the best accuracy among the examined approaches, including allometric scaling [71,72]. Sinha *et al*. [73] reported that the ROE approach based on unbound oral clearance (unbound CL/F) gave the highest predictability among the examined allometric methods. Unbound fraction corrected intercept method (FCIM), by which both ratios of unbound fraction between rat and human plasma and a fixed power coefficient of 0.77 are considered, can be utilized for compounds that are not applicable to the ROE approach [73,74].

Interestingly, the concept that each property in the empirical and the physiological approach is integrated has been proposed by Lavé *et al*. [75,76]. The integrated approach was conducted using CLs in animal species that were normalized by a ratio of *in vitro* metabolic activities between hepatocytes of human and animal species and yielded 80% prediction within an error of twofold the observed CL.

Although the major disadvantage of allometric scaling is its empirical nature, this can, in some cases, be recognized as an advantage. Since it is different from *in vitro–in vivo* scaling, the application of allometric scaling does not need to clarify physiological mechanisms underlying elimination from the body and to understand species differences in the elimination mechanism [77]. In the early phase of drug development, characterizing other elimination mechanisms than the typical hepatic metabolism process, such as oxidation, is in many cases impossible. With regard to compounds that are eliminated from the body via unknown or multiple mechanisms, allometric scaling must be an indispensable predictor to human CLs.

12.3.3.3 Comparison of Empirical and Physiological Approaches. Comparative assessment between empirical and physiological approaches has been conducted by several investigators. Ito and Houston [53] demonstrated that the best method would be prediction from *in vitro* data using an empirical scaling factor. However, the use of a physiological scaling factor instead of the empirical scaling factor had similar accuracy as SA analysis. Obach *et al*. [52] evaluated the accuracy of human CLs predicted by an *in vitro* microsomal study without plasma protein binding (f_p) and by allometric scaling. The average error expressed by predicted/actual values was within a similar range (1.95 vs 1.91). Similar predictability between the empirical and the physiological approaches was also reported by Shiran *et al*. [78] and Tamaki *et al*. [79]. The latter group evaluated the comparative predictability of empirical and physiological approaches using data sets that were similarly characterized by a wide range of lipophilicity and CL. In contrast, using 16 marketed drugs, the ROE method was found to be more accurate than the *in vitro–in vivo* scaling method with *in vitro* microsomal data [80]. One issue of this kind of study is that each conclusion is derived from a different data set. To identify the best predictive method, predictability should be compared based on the same data set with a wide range of physicochemical properties.

12.4 CYP ENZYME INHIBITION

Comedication with two or more drugs is quite common in the treatment of diseases. One drug is often found to inhibit the drug-metabolizing enzymes of the second drug, mainly leading to an increased plasma concentration. As a consequence, such DDIs cause a risk particularly in patients given drugs with a narrow therapeutic index. In 1998, adverse drug reactions were reported as one of the most common causes of death of hospitalized patients in the United States, and most adverse effects involved drug metabolism [81]. Terfenadine [82], mibefradil [83], astemizole [84], cisapride [85], cerivastatin [86], and nefazodone have been withdrawn from the market due to toxicity or QT prolongation attributable to increased exposures by DDI [87]. Furthermore, DDIs have a huge impact on sales, as can be seen with cimetidine, a drug for the treatment of acid reflux, which lost sales because of DDIs that emerged in clinical and postmarketing approval studies of competing products [88]. To avoid costly failures of drug development, the assessment of DDIs has become an increasingly important task of the pharmacokinetic department.

The assessment of CYP inhibition is conducted both during the drug discovery and drug development phases. During drug discovery, the data of CYP inhibition are utilized to optimize the chemical structures and to identify NMEs with no or weak inhibitory activity by simplified moderate to high throughput methods. During drug development, detailed evaluation of CYP inhibition causes inhibitory kinetic data to both predict a change in plasma concentration profiles based on the mathematical models and to clarify the need for clinical DDI studies; for example, according to the FDA draft guidance [89] (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf), it was noted that CYP inhibition can be classified as either reversible or time-dependent inhibition (TDI) [90]. For reversible inhibition and TDI, our review focuses separately on assays that meet the requirements for the drug discovery and the development phases.

12.4.1 *In Vitro* Systems for Evaluating CYP Inhibition

12.4.1.1 Reversible Inhibition. Reversible inhibition involves rapid association and dissociation of drugs and enzymes and can be classified into competitive, noncompetitive, and uncompetitive modes based on the inhibitory mechanisms. The assay conditions of reversible inhibition have been described, together with the prediction criteria of the clinical relevance of competitive CYP inhibition, in the FDA's draft guidance and wave site [89]. Probe substrates and specific inhibitors of CYP enzymes are summarized in Table 12.1, together with time-dependent inhibitors. The guidance-defined method mainly using marketed drugs as practical probe substrates seems to be a gold standard for the assessment of CYP inhibition, in which the IC_{50} (inhibitor concentration causing a reduction of enzyme activity by 50%) or the K_i (inhibition constant) obtained from the dose response curve is estimated as a clinical relevant parameter to predict the possibility of a DDI in humans. However, the lead optimization process requires moderate to high throughput CYP inhibition screening to reduce the cost and shorten the time frame. The advantages and disadvantages of the various developed methods are shown in Table 12.2.

TABLE 12.1 Probe Substrates, Inhibitors, and/or Inducers in *In Vitro* CYP Inhibition or Induction Assessment

CYP Enzyme	Perspective of PhRMA (MBI)[a]		FDA Draft Guidance[b]		
	Substrate	Positive Control	Substrates	Inhibitors	Inducers
CYP1A2	Phenacetin Ethoxyresorufin	Furafylline	Caffeine Ethoxyresorufin Phenacetin[c] Tacrine Theophylline	Furafylline[c] α-Naphthoflavone	Lansoprazole Omeprazole
CYP2B6	Bupropion	Ticlopidine Thiotepa	Bupropion[c] Efavirenz[c] *S*-Mephenytoin Propofol	Clopidogrel Phencyclidine Ticlopidine Thiotepa Sertraline	Phenobaribital
CYP2C8	Paclitaxel Amodiaquine	Gemfibrozil-glucuronide Phenelzine	Amodiaquine Rosiglitazone Taxol[c]	Gemfibrozil Montelukast[c] Pioglitazone Quercetin[c] Rosiglitazone Trimethoprim	Rifampicin
CYP2C9	Diclofenac Tolbutamide *S*-Warfarin	Tienilic acid	Diclofenac[c] Flurbiprofen Phenytoin Tolbutamide[c] *S*-Warfarin[c]	Fluconazole Fluvoxamine Fluoxetine Sulfaphenazole[c]	Rifampicin

CYP2C19	*S*-Mephenytoin Omeprazole	Ticlopidine	Fluoxetine *S*-Mephenytoin[c] Omeprazole	Nootkatone Ticlopidine	Rifampicin
CYP2D6	Dextromethorphan Bufuralol	Paroxetine	Bufuralol[c] Debrisoquine Dextromethorphan[c]	Quinidine[c]	None identified
CYP3A4	Testosterone Midazolam	Mifepristone Verapamil Troleandomycin Erythromycin	Erythromycin Dextromethorphan Midazolam[c] Nifedipine Terfenadine Testosterone[c] Triazolam	Azamulin Ketoconzole[c] Itraconazole[c] Troleandomycin Verapamil	Rifampicin

[a]Grimm *et al*. [91]; the list of substrates and inhibitors for time-dependent inhibition is based on perspective of Pharmaceutical Research and Manufacturers of America.
[b]FDA's wave site, http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm#inVitro [89].
[c]Preferred substrates and inhibitors listed in the FDA's wave site [89].

TABLE 12.2 Advantages and Disadvantages on Various Methods for Assessment of CYP Inhibition

Methods	Advantages	Disadvantages
Fluorescence	• Very high throughput	• Low selectivity and only use of recombinant CYP enzymes • K_i or IC_{50} that shows relatively low correlation with the corresponding data derived from marketed drugs
Radioactivity	• High throughput • Use of marketed drug with high selectivity as probe substrate • Use of recombinant CYP enzymes, liver microsomes, and hepatocytes	• Limited availability of probe substrates • High possibility of nonspecific binding when microsome concentration is high
LC/MS/MS	• Moderate–high throughput • Use of marketed drug with high selectivity as probe substrate • Use of recombinant CYP enzymes, liver microsomes, and hepatocytes • Inhibition on well-defined metabolic pathway • Cassette dosing (cocktailed approach) • Results applicable to new drug application (NDA)	• Ion suppression • High possibility of nonspecific binding when microsome concentration is high

Fluometric-based assays are regarded as a high throughput screening method by which substrates converted to fluorescent metabolites by CYP enzymes are utilized. Since the substrates exhibit low specificity toward each CYP enzyme, recombinant CYP enzymes but not human liver microsomes need to be used. Crespsi *et al*. [92] developed high throughput screening using 3-cyano-7-ethoxycoumarin as the substrate of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 and resorufin benzyl ether as the substrate of CYP3A4. The obtained IC_{50} values showed high correlation to those of the traditional method using phenacetin, diclofenac, *S*-mephenytoin, bufuralol, and testosterone as the respective substrates. A similar microtiter plate assay with recombinant CYP enzymes and fluorescent probes was reported by Yamamoto *et al*. [93], who demonstrated comparable K_i values for nine CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) to the reported values of the conventional assay using human liver microsomes. Such a good relationship between the two assays was also reported by Bapiro *et al*. [94]. On the other hand, different results were found due to low sensitivity when Vivid®Red as the fluorescent substrate was utilized for CYP3A4 inhibition [95]. Furthermore, relatively lower IC_{50} values of CYP2C9, CYP2D6, and CYP3A4 in the fluometric assay than the conventional assay were found and were possibly associated with nonspecific binding

due to high microsome concentrations in the latter assay [96]. The reduction of microsomal concentration from 0.5 to 0.1 mg/mL led to an increase in the relationship. To increase the throughput, as well as to reduce the used amount of test compound, the fluometric assay was miniaturized to a 384-well plate format [97], and an integrated robotic system that consisted of liquid handlers, a plate hotel, and an incubator was applied to the fluometric assay [98].

Importantly, Kenworthy *et al*. [99] have recommended that multiple CYP3A4 probes, representing individual substrate groups, are used for *in vitro* assessment of CYP3A4-mediated DDI. Their group evaluated the inhibitory effects of 34 CYP3A4 substrates in the metabolism of 10 CYP3A4 probes in rhCYP3A4. As a consequence, erythromycin, cyclosporine, and testosterone were categorized as the large molecular group and dextromethorphan, diazepam, and benzodiazepine derivatives as the second group. Dihyropyridine derivatives such as felodipine and nifedipine showed a different inhibition profile from the other two groups. It was thus recommended that different CYP3A substrates (two or more substrates) chosen from each group are applied to the CYP3A inhibition assay. The sensitivity of various fluorescent substrates to CYP3A4 such as benzoxyresorufin (BzRes), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-benzyloxyquinoline (BQ), and dibenzylfluorescein (DBF) was comparatively evaluated by Stresser *et al*. [100]. BFC and BQ were the most and the least sensitive substrates, respectively, for evaluation of CYP3A4 inhibition. Additionally, IC_{50} values from DBF correlated to those from testosterone 6β-hydroxylation. Chauret *et al*. [101] also showed evidence for a similar correlation between DFB (3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-furan-2-one) and testosterone turnover. On the basis of the aspects of high sensitivity and/or clinical relevant substrate, BFC, DBF, and DFB are regarded to be suitable fluorescent CYP3A4 substrates for the high throughput assay.

Amelioration of conventional assay with marketed drug substrates was advanced with a short running time per sample analysis by developing the technologies of analytical equipment such as LC/MS/MS. Establishing simultaneous determination methods for metabolic activities of seven or nine CYP enzymes using LC/MS/MS allowed increasing the throughput in the assay [102,103]. Given that the use of two different CYP3A substrates was required, further development of simultaneous analysis, including two CYP3A activities (midazolam and dextromethorphan), was conducted by Li *et al*. [104]. Interestingly, as a cassette probe dosing/cocktail approach, a simultaneous incubation method using several probe substrates was developed [97,105–107]. In a validation study of the cocktail approach by Zientek *et al*. [97], the estimated IC_{50} values of furafylline to CYP1A2, sulfaphenazole to CYP2C9, ticlopidine to CYP2C19, quinidine to CYP2D6, and ketoconazole to CYP3A4 were almost consistent between the cassette dosing and single dosing approaches.

In general, the IC_{50} value is estimated based on the remaining CYP activity at multiple inhibitor concentrations such as 5–10 points. To increase the efficiency, various methodologies for the estimation of IC_{50} have been developed. Moody *et al*. [108] developed two-point IC_{50} determination in automated radiometric inhibition assay. The IC_{50} estimated from 5 and 50 μM inhibitor concentrations showed a good relationship with full IC_{50} determination regardless of CYP enzymes. Moreover, a great challenge by Lin *et al*. [109] employed one-point IC_{50} determination to maintain screening throughput instead of cassette incubation and pooled sample analysis. On the basis of the experiences of their group, there were high possibilities that the cassette dosing

generated additional interactions and that pooled sample analysis could potentially compromise MS/MS detection in case of strong inhibitors. The IC_{50} values calculated based on the one-point assay was in good agreement to those obtained by the eight-point assay, with slop of 0.95–1.02 for CYP enzyme activities. Additionally, Nakamura *et al*. [110] pointed out the possibility of ion suppression in LC/MS/MS analysis, which probably misleads the inhibitory effect of test compounds; that is, stable isotopes as internal standards of the quantification of metabolites of probe substrates are, in many cases, not commercially available. Use of [$^{13}C_4$–,^{15}N]-midazolam and 1′-hydroxymidazolam as the substrate and internal standard, respectively, successfully improved the effect of ion suppression.

Pharmacokinetics of some drugs involves various mechanisms such as uptake transporters, intracellular protein binding, and lysosomal uptake in addition to CYP-mediated metabolism. Hepatocytes are considered to retain those functions, and the usefulness of cryopreserved human hepatocytes in the assessment of DDI, alongside metabolic stability, was demonstrated by Li *et al*. [111]. *In vitro* DDI study for four hepatic uptake substrates, including atorvastatin and pitavastatin, was implemented using rat and human hepatocyte systems [112]. Those substrates were concentrated up to 1000-fold in the intracellular space of rat hepatocytes; however, only a fivefold difference in the IC_{50} values was pronounced. This was due to intracellular binding rather than extensive hepatic uptake. The importance of intracellular binding was also revealed by Brown *et al*. [113], who employed miconazole, fluconazole, ketoconazole, quinine, fluoxetine, and fluvoxamine with a high cell-to-medium ratio ranging from 4.2 to 6000. The cell-to-medium ratio was not correlated with the ratio of the K_i values between rat hepatocytes and liver microsomes; instead, there was a relationship between the unbound K_i values of the two assays. Accordingly, the high cell-to-medium ratio was not ascribed to hepatic uptake but to intracellular nonspecific binding. Intracellular accumulation associated with the binding must be considered as a key factor in *in vitro–in vivo* extrapolation. Importantly, McGinnity *et al*. [114] reported the usefulness of inhibition data of hepatocytes rather than recombinant CYP enzymes in the assessment of inhibition of quinidine and propafenone, CYP2D6 inhibitors. A significant difference in unbound IC_{50} ($IC_{50,u}$) toward the metabolism of bufuralol values between rhCYP2D6 and human hepatocytes was found with $IC_{50,u}$ of 0.02 versus 0.5 μM for quinidine and 0.02 versus 4.1 μM for propafenone, which was in contrast to similar $IC_{50,u}$ values for other examined CYP2D6 inhibitors.

As reported in the metabolic stability study, the plasma incubation method with human hepatocytes was applied to evaluate CYP inhibition by ketoconazole and fluconazole, a strong and a moderate CYP3A inhibitor, respectively [115–117]. On the basis of the concept that the examined inhibitor concentration in the plasma incubation is clinically relevant, *in vivo* inhibition potential was successfully derived from a relationship between the inhibitor concentrations and the remaining metabolic activity of the probe substrates in the plasma incubation, taking the f_m value into consideration. Moreover, the plasma incubation approach with human hepatocytes was applied to develop a single preincubation time method, which provided reliable prediction of DDI, regardless of CYP3A inhibitory mechanism [118].

For a drug that is biotransformed via multiple pathways mediated by CYP enzymes, in the general approach, the K_i value obtained in the individual metabolite formation need to be converted to the K_i value of whole metabolism by the weighted method with

the contribution fraction (f_m) of the inhibited CYP enzyme to CL for each pathway [114]. The substrate depletion assay has also been used to evaluate *in vitro* DDI [119] and can easily provide the K_i value of the inhibitor to whole metabolism. The estimated K_i values were similar to those of the metabolite formation assay in case of inhibition of omeprazole to diazepam metabolism. Hence, substrate depletion assay would enable to easily generate a K_i value for drugs that are eliminated via unidentified metabolic pathways.

12.4.1.2 Time-Dependent Inhibition. TDI, in general, results from irreversible covalent binding or quasi-irreversible noncovalent tight binding of a chemically reactive intermediate to enzymes that catalyze its formation. Grimm *et al*. [91] has distinguishably defined TDI and mechanism-based inactivation (MBI). TDI only indicates a kinetic definition and can be defined as a compound that time dependently increases an inhibitory effect during incubation with enzymes before the addition of substrates. MBI can be referred to as a *subset of TDI* demonstrating that enzymes act on the substrate to form a chemically reactive metabolite that subsequently inactivates the enzyme [120]. Accordingly, TDI, in some cases, includes reversible inhibition of the product (metabolite) formed during the incubation with enzymes. On the basis of the results of biochemical experiments, MBI can be classified into two groups: quasi-irreversible and irreversible inhibition.

Assessment of TDI has generally been performed by a shift in IC_{50} after preincubation with human liver microsomes or recombinant CYP enzymes in the presence of NADPH before incubation with CYP enzyme-selective probe substrates (Table 12.1). The shift in the IC_{50} value depending on the preincubation time cannot distinguish MBI and inhibition of the formed metabolite. In a general approach, after preincubation with human liver microsomes at high concentrations of 1–2 mg/mL, at least 10- or 20-fold dilution is necessary to quench further inhibition of probe substrate metabolism by the test compound or its metabolite. Use of a probe substrate concentration under saturation conditions (>four- or fivefold K_m) is recommended to minimize further inhibition of probe substrate metabolism by the test compound and its formed metabolite.

The rate of CYP enzyme inactivation is proportionally dependent on the inhibition concentration as described by the following equation [121]:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \tag{12.3}$$

where k_{obs} is the pseudo first-order rate constant of inhibition at the inactivator concentration [I], k_{inact} the maximum inactivation rate, and K_I the inactivator concentration when the rate of inactivation reaches half that of k_{inact}. The k_{obs} can be determined from percentage remaining activity in multiple time- and concentration-dependent TDI assay. The logarithm of percentage remaining activity is plotted against the preincubation time for each concentration of test compounds, and the k_{obs} can be obtained by a slope from the linear regression analysis, and the k_{obs} and the inactivator concentration are fitted into Equation 12.3. Hence, to determine K_I and k_{inact}, five to six preincubation time points and multiple inactivator concentrations are required, and the TDI study is a time-consuming and labor-intensive work. Tables 12.1 and 12.3 indicate probe substrates and inactivators, and K_I and k_{inact} for CYP3A-mediated TDI using human liver microsomes, rhCYP3A4 or human hepatocytes, respectively.

TABLE 12.3 *In Vitro* Parameters for Time-Dependent Inactivators in Humans

	Enzyme Sources	Substrates	K_I (μM)	k_{inact} (min^{-1})	References
Amprenavir	HLM	Testosterone	0.37	0.59	[122]
Azamulin	HLM	Midazolam	0.17	0.68	[123]
Clarithromycin	HLM	Midazolam	15.5	0.0192	[121]
	HLM	Nifedipine	15.9	0.0244	[121]
	HLM	Testosterone	12.9	0.0324	[121]
	HLM	Midazolam	5.49	0.072	[124]
Diltiazem	HLM	Midazolam	8.7	0.0050	[123]
	HLM	Testosterone	4.7	0.0230	[123]
	rhCYP3A4	Midazolam	0.49	0.010	[125]
Erthinylestradiol	rhCYP3A4	Midazolam	23	0.09	[125]
Erythromycin	HLM	Midazolam	12.1	0.0215	[121]
	HLM	Nifedipine	11.3	0.0295	[121]
	HLM	Testosterone	10.9	0.0352	[121]
	rhCYP3A4	Midazolam	8.82	0.12	[126]
	rhCYP3A5	Midazolam	No TDI	—	[126]
	Hepatocyte	Midazolam	11	0.07	[127]
Fluoxetine	HLM	Midazolam	5.26	0.017	[124]
	Hepatocyte	Midazolam	1	0.01	[127]
Lopinavir	HLM	Testosterone	1.0	0.11	[122]
Mifepristone	rhCYP3A4	Midazolam	0.61	0.080	[126]
	rhCYP3A4	Midazolam	No TDI	—	[126]
Nelfinavir	HLM	Testosterone	1.0	0.22	[122]
Ritonavir	HLM	Testosterone	0.17	0.40	[122]
Saquinavir	HLM	Testosterone	0.65	0.26	[122]
Troleandomycin	HLM	Midazolam	2	0.03	[127]
	rhCYP3A4	Midazolam	0.26	0.12	[126]
	rhCYP3A5	Midazolam	No TDI	—	[126]
	Hepatocyte	Midazolam	0.4	0.05	[127]
Verapamil	HLM	Midazolam	2.55	0.0277	[121]
	HLM	Nifedipine	3.12	0.0486	[121]
	HLM	Testosterone	5.79	0.0591	[121]
	HLM	Testosterone	2.6	0.038	[123]
	rhCYP3A4	Midazolam	0.74	0.041	[125]

Abbreviation: HLM, human liver microsomes.

As a simplified screening system for TDI with moderate to high throughput, IC_{50} shift assay using fluometric substrates (3-cyano-7-ethoxycoumarin for CYP1A2, CYP2C9, CYP2C19, and CYP2D6; BzRes and BFC for CYP3A4) has been developed [128]. In this assay, IC_{50} was continuously measured without terminating the enzyme reactions. Time-dependent reduction in IC_{50} values was found for quasi-irreversible inhibitors (isosafrole, erythromycin, troleandomycin, and diltiazem) and irreversible inhibitors (furafylline, propranolol, and mifepristone), which was in contrast to the constant IC_{50} for typical reversible inhibitors such as sulfaphenazole, tranylcypromine, quinidine, and ketoconazole. Similarly, Sekiguchi *et al*. [129] also conducted TDI assay of CYP3A4 using fluorescent substrate (BFC). In contrast, a disadvantage of

those fluometric assays is that metabolite inhibition and MBI cannot be distinguishably evaluated.

Single time- and concentration-dependent assay has been proposed as the first screening for TDI [121,125]. Watanabe *et al*. [121] reported automated single time- and concentration-dependent inhibition assay using midazolam as the CYP3A probe substrate, combined with a 10-fold dilution process of preincubation to avoid the effect of product inhibition. Using 171 marketed drugs, a diagram to evaluate the possibility of MBI-associated *in vivo* DDI was successfully described from the remaining activity of midazolam 1′-hydroxylation in the MBI assay and *in vivo* DDI information that included therapeutic blood or plasma concentration. Their group also developed a screening method to distinguish quasi-irreversible and irreversible inhibitors by the addition of ferricyanide into the preincubation solution. Noticeably, the assessment of MBI does not depend on the substrates (midazolam, nifedipine, and testosterone), while reversible inhibition requires the use of different types of substrates. A two-time point shift assay for TDI with a 5- or 10-fold dilution of preincubation solution was applied to the assessment of five major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) [123]. Moreover, to increase the throughput in the TDI assay, a cassette dosing approach was applied to the estimation of K_I and k_{inact} of prototypic inhibitors in cultured human hepatocytes [127].

A different strategy to evaluate TDI of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 was proposed by Lim *et al*. [130]. In their strategy, test compounds are first evaluated in apparent partition ratio (APR) screening, followed by detailed study with time- and concentration-dependent inhibition, and differentiation of quasi-irreversible inhibition and irreversible inactivation by oxidation of potassium ferricyanide and/or dialysis. APR was estimated by plotting percentage remaining activity as the function of the molar ratio of the test compound to CYP enzyme. Among 19 mechanism-based inhibitors and 4 quasi-irreversible inhibitors, the assay identified ritonavir, mibefradil, and azamulin as highly effective mechanism-based inhibitors of CYP3A, with an APR of 1.4–2.1, and isoniazid as a less effective inhibitor of CYP2C19 with an APR of 13,491. Such a widely dynamic range is regarded as an advantage in APR screening.

CYP3A5, along with CYP3A4, one of the major CYP enzymes, is expressed in adult human liver and small intestine [131], although the expression is genetically polymorphic [132]. The time-dependent inhibitors such as verapamil and its metabolites reportedly showed different inhibitory kinetics for CYP3A4 and CYP3A5 [133]. In addition, some of the compounds investigated by Soars *et al*. [126] did not show TDI to rhCYP3A5 but rhCYP3A4. Therefore, there is a possibility that a variable expression ratio of CYP3A4/3A5 in human liver microsomes contributes to interindividual variations in CYP3A-mediated DDI [134].

12.4.2 Prediction of *In Vivo* CYP Inhibition

12.4.2.1 Static Prediction Model. Assessment of the possibility of DDI and/or risk based on the predicted data has been included in the framework of pharmacokinetic studies. The prediction methods can be divided into two groups; namely, the static model describing the fraction of remaining CYP activity at a fixed inhibitor concentration and a PBPK model describing the time-dependent fraction of the remaining CYP activity associated with the plasma concentration–time curve of the inhibitor after administration.

In the static model, the most widely utilized method is the FDA-recommended approach based on [I]/K_i ratio, in which [I] indicates the mean steady-state C_{max} value for total drug (bound plus unbound) after administration of the highest proposed clinical dose [89]. An estimated [I]/K_i ratio exceeding 0.1 is considered positive and follow-up *in vivo* assessment and/or detailed evaluation using mechanistic models are recommended.

The critical point of the static is which inhibitor concentration to use preferentially as the surrogate of the inhibitor concentration in the surroundings of the target enzymes in the liver because direct measurement of the inhibitor concentration is impossible. Some surrogate inhibitor concentrations have been investigated comparatively to evaluate the possibility of *in vivo* DDI. Komatsu *et al*. [135] challenged DDI prediction based on reversible inhibition between tolbutamide and various sulfonamides using K_i values estimated from an *in vitro* experiment in human liver microsomes. Irrespective of competitive or noncompetitive inhibition, the ratio of CL_{int} in the presence ($CL_{int,+I}$) and absence of inhibitor ($CL_{int,-I}$) can be described as the following equation when the substrate concentration is much lower than the K_m value:

$$CL_{int,+I} = \frac{CL_{int,-I}}{1 + \left(\frac{C_{hep,inlet,u}}{K_i}\right)} \tag{12.4}$$

where $C_{hep,inlet,u}$ is the unbound concentration of inhibitor. To avoid the false negative predictions, $C_{hep,inlet,u}$, which presents unbound maximum hepatic inlet concentration, was calculated as the sum of maximum unbound plasma concentration in the systemic vein ($C_{max} \times f_p$) and the portal vein ($I_{max} \times f_p$) from gastrointestinal absorption after oral administration, under the assumption that unbound inhibitor concentration in the liver equals that in the plasma. The blood concentration in the portal vein can be estimated as follows:

$$I_{max} = \frac{D \times k_a \times F_a}{Q_h} \tag{12.5}$$

where k_a is the absorption rate constant, F_a the fraction of absorption, D the dose of administered inhibitor, and Q_h the hepatic blood flow. Approximately 4.8- and 1.8-fold increases in area under the plasma concentration curve (AUC) of tolbutamide, which were predicted by the coadministration of sulfaphenazole and sulfamethizole, were within a similar range as the reported *in vivo* data.

A comprehensive study on the prediction methods for reversible inhibition using Monte Carlo simulation [136] indicated that the use of maximum unbound hepatic inlet concentration did not provide false negative prediction, often accompanied with a produced overestimation, whereas an attempt using maximum unbound blood concentration in the systemic vein produced a false negative prediction. Furthermore, Ito and Houston [137] intensively investigated the effect of various estimates of inhibitor concentrations on the predictability of DDI based on the results of 193 DDI studies involving CYP2C9, CYP2D6, and CYP3A4. Excluding mechanism-based inhibitors, the use of total hepatic inlet concentration did not result in false negative prediction. This method may be a preferential approach in the selection of NMEs, which need to avoid false negative predictions, although the incidence of false positive predictions is controversial.

Importance of f_m and k_a values was highlighted by several investigators. The use of f_m values and the free hepatic inlet C_{max} successfully identified drugs that showed at least twofold increase in the AUC of the CYP-probe substrates, excluding known mechanism-based inhibitors from among 40 examined drugs [138]. In addition, Brown *et al*. [139] focused on the impact of f_m and k_a values on the prediction accuracy based on 115 clinical DDI studies involving CYP2C9, CYP2D6, and CYP3A4. The incorporation of f_m values into *in vivo* predictions using hepatic input plasma concentration resulted in 84% of studies within twofold of the *in vivo* data. In addition, the refined k_a value calculated from pharmacokinetic analysis reduced the number of overpredictions of CYP2D6 and CYP3A4. Ohno *et al*. [140] developed a robust and practical model, in which the time-averaged apparent inhibition ratio of CYP3A4 (IR_{CYP3A4}) calculated primarily based on AUC increases in DDI studies with 18 CYP3A inhibitors and midazolam was incorporated. The proposed method with f_m and IR_{CYP3A4} values is a versatile tool that enables the easy prediction of AUC increase by CYP3A4-mediated unknown interaction.

Regarding TDI, retrospective investigation by Ito *et al*. [141] focused on what inhibitor concentration was appropriate to predict midazolam and macrolide interaction. Application of maximum unbound hepatic inlet concentration gave a more accurate prediction, whereas underprediction tended to be observed in case of maximum unbound systemic concentration. Galetin *et al*. [142] evaluated 37 *in vivo* cases of irreversible inhibition of macrolides and diltiazem on the metabolism of CYP3A4 substrates, considering the inhibitory effect on the intestinal first-pass metabolism, using the following equation:

$$\frac{AUC_I}{AUC} = \frac{F'_G}{F_G} \times \frac{1}{\frac{f_{m,CYP3A4}}{1+\sum_{i=1}^{n} \frac{k_{inact,i} \times I_{u,i}}{k_{deg,CYP3A4} \times (K_{I,u}+I_u)_i}} + (1 - f_{m,CYP3A4})} \tag{12.6}$$

where $f_{m,CYP3A4}$ is the fraction of probe substrate metabolized by CYP3A4, $k_{deg,CYP3A4}$ is the *in vivo* degradation rate constant of CYP3A4 in the liver, I_u is either the average unbound systemic plasma concentration after repeated administration or the maximum unbound hepatic inlet concentration, and F'_G and F_G are the intestinal availability in the presence and absence of inhibitor, respectively. The use of unbound systemic plasma concentration of the inhibitor and f_m value resulted in 89% of studies predicted within twofold of the *in vivo* data.

The prediction accuracy of TDI is very sensitive to the CYP enzyme degradation rate. However, regarding CYP3A4 enzyme, the reported turnover ranged widely from 26 to 79 or 36 to 140 h, estimated from the *in vitro* and *in vivo* data, respectively [91]. Obach *et al*. [143] reported a prediction method for CYP3A-related MBI incorporating an *in vivo* CYP enzyme degradation rate constant estimated from clinical pharmacokinetic data, which was a similar model as to that by Galetin *et al*. [142], additionally with intestinal inhibitory kinetics. When using unbound systemic C_{max} as the *in vivo* inhibitor concentration, the most accurate predictions of DDI was obtained with a mean error of 1.64-fold. Additionally, using IC_{50}, IC_{50} fold-shift, K_I, and K_{inact} obtained in irreversible inhibition analysis, the relationship between each parameter was investigated. Surprisingly, fold-shift of IC_{50} before and after preincubation, which is often used as an indicator in the first screening, showed no correlation with K_{inact}/K_I, one of the indicators for potential irreversible inhibition. A good relationship was noted

between the shifted IC_{50} determined after 30-min preincubation and the K_{inact}/K_I or K_I value. Burt *et al*. [144] also reported that K_{inact}/K_I was correlated with $1/IC_{50}$ value after either a 10- and 30-min preincubation.

12.4.2.2 Physiologically Based Pharmacokinetic Model. In addition to K_i or K_I generally used as an index of hepatic enzyme inhibition, a key factor to the predictability is which inhibitor concentration is used. Those retrospective assessments using fixed inhibitor concentration would have given an important indication that the use of unbound hepatic inlet inhibitor concentration and unbound systemic inhibitor concentration appears to yield better prediction for reversible and irreversible inhibition, respectively. Not only to increase the predictability of DDI but also to describe the plasma concentration profile of substrates altered by a concomitantly given drug, the use of a plasma concentration–time curve of the inhibitor simulated from a PBPK model is essential instead of the fixed inhibitor concentrations.

With regard to reversible inhibition, estimation of *in vivo* relevant K_i value allowed quantitative prediction of CYP-mediated DDI [145]. The *in vivo* K_i values were determined by fitting the plasma concentration–time profiles of a substrate and an inhibitor to a simple PBPK model. Increased ratios of AUCs predicted using the *in vivo* K_i value estimated for each CYP inhibitor were in agreement with *in vivo* corresponding data and were more accurate relative to the prediction by the static model. Importantly, the discrepancy between *in vivo* K_i and *in vitro* K_i values was pronounced, possibly ascribed to nonspecific binding to liver microsomes that was well defined by lipophilicity.

For irreversible inhibition, prediction using a PBPK model has been challenged for DDI between 5-fluorouracil and sorivudine [146], triazolam and erythromycin [147], and midazolam and macrolides [141]. As already pointed out, CYP turnover rate ($k_{deg,CYP}$) is a key parameter that has an impact on the predictability. There has been difficulty to estimate the $k_{deg,CYP}$ of each CYP enzyme in the human liver in biological experiments, and hence, rat CYP data of 0.0005/ min was employed as human $k_{deg,CYP}$ in those studies.

DDI prediction has, in general, been performed under the following assumptions [141,147]: (i) substrate and inhibitor are orally administered according to the first-order rate constant and only undergo hepatic metabolism, (ii) unbound concentration in the hepatic vein and liver are equal due to a rapid equilibrium according to the well-stirred model, (iii) only unbound drug in the liver is subject to elimination, and (iv) disposition and metabolism of inhibitor are not altered by the inhibition of CYP3A4. In the prediction of triazolam and erythromycin interaction, when the K_I and k_{inact} values estimated in human liver microsomes or recombinant CYP3A4 were incorporated into a PBPK model, repeated administration of erythromycin at 333 mg three times a day for three days resulted in simulated reduction in hepatic CYP3A4 enzyme to 50–60% of the initial level [147]. Subsequently, the predicted 2.0- and 2.6-fold increase in triazolam AUC was derived from human liver microsomes and recombinant CYP3A4, respectively, which was similar to *in vivo* clinical data with a 2.1-fold increase. Similarly, for midazolam and macrolide interaction, simulation using the K_I and k_{inact} of macrolides estimated in human liver microsomes yielded a 2.9- to 3.0-fold increase in midazolam AUC after pretreatment with erythromycin, a 2.1- to 2.5-fold increase after pretreatment with clarithromycin, but little effect for azithromycin [141]. It should be noted that the

assessment of DDI in first-pass metabolism in the small intestine after oral administration is important to increase predictability, because the intestinal metabolism has reportedly had a great influence on the bioavailability of CYP3A4 substrates [7,145].

An advantage of the PBPK model beyond the static model in the DDI prediction is to estimate a change in the plasma concentration–time curves, including C_{max} and $t_{1/2}$, which would be associated with pharmacodynamics, in single and multiple ascending dose studies. Recently, advanced modeling and simulation tools combining *in vitro* data have increasingly been utilized to predict *in vivo* profile to optimize the dose regimen in a clinical trial to ensure that any interaction is appropriately measured. One such program is SIMCYP® (Simcyp Ltd., Shefield, UK), which would give confidence to CL and DDI prediction [148,149].

12.5 CYP ENZYME INDUCTION

In vivo induction of CYP enzymes can result in significant clinical consequences with suboptimal pharmacological efficacy through the reduction in plasma concentration of comedicated drugs, and in the worst case, the increased formation of reactive metabolites. Clinical DDIs have been reported, with the therapeutic failures that comedication with CYP3A inducers lead to reduced ethinylestradiol levels from contraceptives, resulting in breakthrough pregnancies [150], and reduced cyclosporine level with organ rejection in transplant patients [151,152]. Induction activity on CYP enzymes has become one of the criteria for the selection of NMEs.

Studies on the induction have mainly been focused on CYP enzymes that are involved in the metabolism of a large number of drugs. CYP induction can occur as a result of either an increase in *de novo* synthesis of enzymes or a decrease in degradation associated with stabilization of the protein [153]. Regarding all CYP enzymes, excluding CYP2E1, which is subject to the stabilization [154], increased synthesis is common. Induction of CYP enzymes by drugs and other xenobiotics typically occurs via activation of receptors that regulate transcription. Induction of CYP1A1 and 1A2 is mediated through the aryl hydrocarbon receptor (AhR) [155]. CYP3A4 is induced through the pregnane X receptor (PXR), which is also involved in the induction of CYP2C9 and organic anion transporters [156]. Therefore, it is considered that CYP2C9 induction consistently tracts with the expression level of CYP3A4. PXR is reported to have a synergistic effect with the glucocorticoid receptor (GR), although the mechanism is not fully understood [157]. The constitutive androstane receptor (CAR) is a member of the gene superfamily of nuclear hormone receptors and a key transcription factor in hepatic CYP induction [158,159]. PXR and CAR are demonstrated to be responsible for coordinating the regulation of a large number of drug-metabolizing enzymes, including CYP isoforms (CYP2B6, CYP2C9, and CYP3A4) and transporter genes [160,161].

Since almost all *in vivo* hepatic events, including drug metabolism and induction, can be evaluated by the hepatocyte system, human hepatocytes in primary culture are currently utilized in the assessment of enzyme induction as the gold standard in preclinical DDI studies. However, due to the limited availability of high quality human hepatocytes and interindividual variability in response to inducers, alternative assay systems with at least moderate throughput have been developed for the lead optimization under pressure from project organizations: reporter gene assay for PXR and AhR, and other cell lines such as nontumorigenic immortalized human hepatocyte line, Fa2N-4 cells, and a new human hepatoma cell line, HepaRG. The current status

TABLE 12.4 Advantages and Disadvantages on Various Methods for Assessment of CYP Induction

Methods	Advantages	Disadvantages
Reporter gene assay	• Very high throughput • Cost effective	• No consideration of metabolic elimination of inducer • Poor correlation of E_{max} with hepatocyte • Assessment of transcription activation only
Hepatocyte	• Gold standard method • Possible existence of functions regarding induction mechanism, nuclear transporters, uptake transporter, efflux transporter, and drug metabolism • Determination of both mRNA and activity level • Results applicable to NDA • Evaluation of all process involved in induction	• Low throughput • High cost and labor intensive • Limited availability of high quality fresh hepatocytes • High interindividual variation
HepaRG	• Moderate throughput • Determination of mRNA and activity level • High expression of nuclear factors and transporters • A certain level of drug-metabolizing activities • EC_{50} and/or E_{max} correlated with hepatocytes	• Alternation of drug-metabolizing activities by culture condition with DMSO • A relatively large lot-to-lot variation in fold induction reported
Fa2N-4	• Moderate throughput • Similar response over the passage • EC_{50} correlated with hepatocytes	• Extremely low expression of CAR • Low drug-metabolizing activities • Low expression of hepatic uptake transporters

of CYP induction assay and its prediction is separately reviewed. The advantages and disadvantages of those assay methods and the *in vitro* inducers recommended by the FDA draft guidance are listed in Tables 12.1 and 12.4, respectively. In addition, *in vitro* parameters for CYP induction are summarized in Table 12.5.

12.5.1 *In Vitro* System for Evaluating CYP Enzyme Induction

12.5.1.1 Reporter Gene Assay. PXR was first identified as an orphan nuclear receptor responsive to pregnanes by Kliewer *et al*. [169], who found that PXR induces the CYP3A family. Furthermore, their group [170] demonstrated a differential activation profile in human pregnane X receptor (hPXR) and mouse pregnane X receptor (mPXR)

TABLE 12.5 ***In Vitro*** **Parameters for CYP3A4 mRNA in Various Assay Systems of CYP Induction**

	PXR Reporter Gene Assay		Hepatocytes		HepaRG Cells		Fa2N-4 Cells	
	EC_{50} (μM)	E_{max}	EC_{50} (μM)	E_{max}	EC_{50} (μM)	E_{max}	EC_{50} (μM)	E_{max}
Carbamazepine	51[a]	8	60, 55.8[b]	10.2, 34.3	50[c]	58	62[d]	2.5
	0.9[e,f]	14.1	42[a]	23	—	—	11[g]	4
Clotrimazole	1.2[a]	11	—	—	—	—	0.4[g]	4
	1.1[e,f]	15.2	—	—	—	—	—	—
Dexamethasone	100[a]	12	43, 39[a]	71, 13	—	—	5[g]	3
	2.6[e,f]	52.3	—	—	—	—	—	—
Nifedipine	2[a]	14	16.6, 18[b]	104, 34.3	16[c]	12	17[d]	8.3
	—	—	—	—	—	—	8[g]	13
Phenobarital	100[a]	9	153, 159[b]	14.4, 49	139[c]	22	222[d]	5.5
	9.7[e,f]	41.3	142[a]	17	—	—	205[g]	12
Phenytoin	0.3[a]	3	3.7, 24[b]	8.8, 15.8	21[c]	30	44[d]	5.2
	25.1[e,f]	27.5	18, 12[a]	15, 12	—	—	74[g]	10
	—	—	5.1[h]	16.9	—	—	10[h]	9.2
Rifampicin	0.9[a]	19	2.6, 0.57[b]	14.9, 33	4.3[i,j]	42.5	1.9[d]	13
	1.7[e,f]	54.4	0.1, 0.4[a]	76, 33	0.8[c]	83	4[g]	36
	—	—	0.4[h]	52.5	—	—	8[h]	59.5
Ritonavir	1.4[a]	18	—	—	—	—	0.2[g]	8
Rosiglitazone	—	—	14.3, 13.6[b]	5.4, 23.9	14[c]	15	14.5[d]	2.9
Troglitazone	NA[a,k]	3	2.0, 8.5[b]	9.8, 23	—	—	3[d]	2.4
	0.2[e,f]	17.1	0.8[a]	8.7	—	—	—	

[a] McGinnity *et al*. [162].
[b] Fahmi *et al*. [164].
[c] Grime *et al*. [204].
[d] Ripp *et al*. [166].
[e] El-Sankary *et al*. [163].
[f] hGR/hPXR cotransfected cells.
[g] Kenny *et al*. [167].
[h] Hariparsad *et al*. [168].
[i] Kaneko *et al*. [165].
[j] Midazolam 1′-hydroxylation activity.
[k] Not appropriate to define.

in the reporter gene assay. That is, hPXR was activated by clotrimazole, rifampicin, and dexamethasone-*t*-butylacetate, which was in contrast to low activation of mPXR by clotrimazole and rifampicin together with high activation by pregnenolone 16α-carbonitrile (PCN).

The reporter gene assay for PXR has been used as a convenient high throughput tool to identify compounds with the potential to induce CYP3A4. Successful results to classify inducers and noninducers based on PXR reporter gene assay were reported by Persson *et al*. [171]. Seven *in vivo* inducers, one weak *in vivo* inducer, and noninducers were evaluated by the luciferase reporter gene assay for PXR with xenobiotic responsive enhancer module (−7836/−7208) and the −362/+53 proximal promoter from CYP3A4 in the pGL3 basic vector. When unbound *in vivo* AUC was taken into consideration, the assay identified inducers and noninducers separately. For phenobarbital, phenytoin, and troglitazone, which are prototypic inducers, E_{max} value, expressed as percentage of maximum rifampicin induction, was 71.7%, 20.6%, and 45.0%, respectively; however, lovastatin showed no response in the assay. Similar results for the rank order of induction responses (rifampicin > phenobaribital > phenytoin) in PXR activation assay was reported by Luo *et al*. [172].

Since hPXR and human glucocorticoid receptor (hGR) would reportedly contribute to the transcriptional regulation of CYP3A4 gene by a large number of xenobiotics [173], *in vitro* reporter gene assay, in which plasmid of hPXR and hGR was transfected into human hepatocyte carcinoma cell line, HepG2 cells, was developed [163]. This system utilized CYP3A4 proximal promoter (−1087 to −57) linked to a secretory alkaline phosphatase (SEAP) reporter gene. To increase the efficiency of estimating EC_{50} (concentration at half the maximum induction ratio) values in the assay, it was attempted to decrease to four- from eight-point concentrations on the dose–response curve. The four-point assay with respect to well-known CYP3A4 inducers produced a similar dose–response curve to the eight-point assay, and the highest fold induction based on E_{max} in lovastatin, followed by simvastatin ≈ troglitazone > rifampicin > phenobarbital > phenytoin.

To evaluate CYP1A1 induction, a cell-based high throughput screening system is also available. Yueh *et al*. [174] and Chao *et al*. [175] established stable cell lines harboring a luciferase reporter gene integrated in artificial multiple xenobiotic response elements. Moreover, the reporter gene assay was developed using HepG2 cells stably transfected pTX.DIR and pSV_2-Neo [171]. On the basis of the luciferase activity associated with AhR activation, the EC_{50} value of lansoprazole, omeprazole, and indole-3-carbinol was estimated to be 5.9, 18.1, and 55.8 μM, respectively, whereas activation was not pronounced for phenytoin, primaquine, rifampicin, and troglitazone. Noticeably, the liver slice system with the treatment of primaquine, however, produced 17-fold induction of CYP1A1 mRNA, and phenytoin, rifampicin, and troglitazone appeared to show the induction of CYP1A1 mRNA, although to a lesser extent.

The molecular mechanism of AhR-mediated activation has been extensively studied for CYP1A1; however, the mechanism for CYP1A2 remains unclear. Recently, Sato *et al*. [176] have set up a dual reporter gene assay that enables simultaneous and rapid determination of transcriptional activation of CYP1A1 and 1A2 genes using indicators of luciferase and SEAP activity, respectively. Given that transcriptional activation of CYP1A1 and 1A2 genes is reportedly regulated simultaneously through a common regulatory element existing between the genes that act bidirectionally [177], the stable cell lines contain 23-kb intergenic spacer regions alongside the reporter gene. The

reporter gene assay detected the induction of CYP1A2 by omeprazole, lansoprazole, and albendazole, although the extent of induction of CYP1A2 was lower than that of CYP1A1. Therefore, the assay with the stable cell line may be a useful assessment tool for induction of CYP1A2 in addition to CYP1A1.

On the basis of the survey of Pharmaceutical Research Manufactures of America (PhRMA) to its member companies [178], PXR reporter gene assay is used by a majority of PhRMA companies surveyed, but only 23% of the companies assesses CYP1A induction potential of compounds in the AhR reporter gene assay.

12.5.1.2 Human Hepatocytes. Human hepatocytes have molecular mechanisms underlying CYP induction from signal transductions via nuclear receptors, in addition to drug-metabolizing enzymes and transporters involved in liver uptake and efflux, and those features are a benefit over other assessment systems. Accordingly, human hepatocytes have been regarded as a reliable *in vitro* system and the gold standard for evaluating enzyme induction of NMEs [179,180].

FDA draft guidance for DDI studies has recommended the evaluation of CYP induction, before clinical study, by a change in CYP enzyme activities (and CYP mRNA) in the primary culture of hepatocytes treated with test compounds and positive controls [89]. Fresh and cryopreserved human hepatocytes are commercially available. Comparative assessment of induction response between both hepatocytes demonstrated that there is not a significant difference in fold induction of CYP1A2 and CYP3A4 by lansoprazole and rifampicin, respectively [181]. The fresh hepatocytes exhibited higher activities of CYP1A2 and CYP3A4 enzymes than the cryopreserved hepatocytes; however, the use of fresh hepatocytes in a timely manner is difficult. Furthermore, there is large interindividual variability in response to inducers in fresh hepatocytes as demonstrated by Kostrubsky *et al*. [179].

Large interindividual variation in the basal activities of testosterone 6β-hydroxylation and ethoxyresorfin deethylation was reported by some investigators [179,182,183]. Interestingly, Kostrubsky *et al*. [179] made it clear that there was an inverse relationship between fold induction and basal CYP3A4 activity when either taxol or rifampicin was exposed in hepatocytes; that is, hepatocytes with a low basal CYP3A4 activity tended to provide a large extent of induction and vice versa. As a consequence, the variation was decreased for the level of CYP3A4 activity enhanced after the treatment of the inducer, being possibly associated with a feedback regulation. A small lot-to-lot difference in the induced CYP3A4 activity was suggested by Chang *et al*. [184]. Moreover, a weak relationship was pronounced for the E_{max} values between different lots of hepatocytes, but the EC_{50} values were well correlated [162]. To dissolve these issues in the fresh hepatocytes, cryopreserved hepatocytes precharacterized as high quality lot in terms of induction and drug-metabolizing activities would be preferentially applicable to the CYP induction study.

A comprehensive work using a large number of human hepatocyte lots (62 lots) was conducted by Madan *et al*. [182], who evaluated the extent of induction of individual CYP activity after incubation with either β-naphthoflavone, phenobarbital, or refampicin. The highest extent of induction in the treatment of rifampicin was noted for CYP2C19 activity (37-fold, $n = 10$), followed by CYP2B6 activity (13-fold, $n = 14$), CYP3A4 activity (10-fold, $n = 61$), CYP2C8 activity (5.2-fold, $n = 4$), CYP2C9 activity (3.5-fold, $n = 10$), and CYP2E1 activity (2.2-fold, $n = 5$). CYP1A2 activity was induced by 13-fold after treatment of β-naphthoflavone ($n = 28$).

Ritonavir is not only an inducer but also an irreversible inhibitor of CYP3A4, and the induction activity of ritonavir was evaluated in both the PXR reporter gene assay and the hepatocyte assay [172]. Unlike mRNA level, testosterone 6β-hydroxylation activity in hepatocytes was reduced below the basal lever after the treatment of ritonavir, whereas ritonavir displayed a strong induction in the PXR reporter gene assay. Similar dual activities as CYP inducer and inhibitor were also reported for troleandomycin. Induction parameters derived from the PXR reporter gene and human hepatocyte assay were comparatively evaluated using 24 compounds, including 14 time-dependent inhibitors [162]. The EC_{50} values of CYP3A4 mRNA in hepatocytes were correlated with those in the PXR reporter gene assay. On the contrary, the E_{max} values of midazolam 1′-hydroxylation activity in hepatocytes showed poor correlation with the corresponding data for mRNA, which was attributed to metabolic activity reduced by TDI in hepatocytes. As expected, the exclusion of time-dependent inhibitors from the compounds examined led to dramatic improvement of the relationship. Fahmi *et al*. [185] comparatively investigated usefulness of CYP3A activity and mRNA as the reliable marker in the assessment of CYP3A induction. It was pointed out that cutoff value based on fold increase in CYP3A mRNA is the best classification on induction potential relative to that in CYP3A activity. This suggests that the measurement of CYP mRNAs, in addition to CYP activities, is important to characterize the induction profile of compounds when detailed information on TDI is not available. Interestingly, quantitative protein determination method for CYP induction by LC/MS/MS was reported [186]. A high throughput LC/MS/MS approach quantified CYP protein itself by measuring the isoform-specific peptides released by enzymatic digestion of hepatocyte incubation, with high reproducibility. This approach detected small fold change in CYP isoforms, and the induction of CYP3A5 by phenobarbital in human hepatocytes was detectable. The protein determination may be an alternative approach in the assessment of CYP induction.

CYP induction study using hepatocytes is a labor-intensive and time-consuming process. The use of a cassette dosing approach in the measurement of CYP enzyme activity would lead to a decrease in the workload of the induction study. Mohutsky *et al*. [187] employed cocktail probe substrates that were composed of phenacetin (CYP1A2), diclofenac (CYP2C), and midazolam (CYP3A4) to evaluate alternation of CYP enzyme activities. The extent of induction of CYP activities was comparable between the single and cassette dosing approaches regardless of CYP inducer or hepatocyte lot. In current study on profiling CYP induction of statins in human hepatocytes, Feidt *et al*. [188] demonstrated applicability of cocktail assay with metabolic activities of seven CYP enzymes: CYP1A2 (phenacetin), CYP2B6 (bupropion), CYP2C8 (amodiaquine), CYP2C9 (tolbutamide), CYP2C19 (*S*-mephenytoin), CYP2D6 (propafenone), and CYP3A4 (atorvastatin).

12.5.1.3 HepaRG Cells. A human hepatoma cell line, HepaRG, derived from a hepatoma cellular carcinoma has been utilized to evaluate CYP induction activity. In differentiated HepaRG cells after seeded at low density, mRNA levels of CYP3A4 and activity of testosterone 6β-hydroxylation were low in the absence of 2% dimethyl sulfoxide (DMSO), compared with the corresponding data in the presence of 2% DMSO [189]. The low activity in the absence of DMSO was remarkably induced by rifampicin treatment. Profiling of the mRNA and activity level of drug-metabolizing enzymes in

differentiated HepaRG cells was conducted by Kanebratt and Andersson [190]. The differentiated HepaRG cells at one day after removal of DMSO from the medium showed comparable mRNA levels to human hepatocytes. Relative to human hepatocytes, the metabolic activities toward midazolam, naloxone, and clozapine in HepaRG cells were similar, whereas the HepaRG cells exhibited lower activity for 7-ethoxycoumarin and dextromethorphan associated with low levels of CYP2E1 and CYP2D6. Various nuclear receptors such as AhR, CAR, and PXR were retained at a high level, regardless of the presence or absence of DMSO [189]. HepaRG cells that exhibited the expression of nuclear receptors alongside most of all drug-metabolizing activities would be suitable for CYP induction screening, although the activity levels may depend on the culture condition.

Evaluation of EC_{50} needs a widely ranged dose–response curve. To avoid problems with cellular toxicity and solubility in the higher inducer concentration range, a new score to evaluate induction activity at lower concentrations was introduced. The score was defined as an F_2 value, which was obtained from the concentration at a twofold increase from baseline for mRNA and enzyme activity levels, instead of EC_{50} values [191]. The obtained F_2 values were correlated with decreases in AUC of CYP3A4 probe substrate drugs after treatment with inducer.

Lot-to-lot variability in CYP3A induction assays was reported in HepaRG cells [165]. The coefficients of variation (CV%) of the E_{max} of rifampicin, phenobarbital, and carbamazepine were 61.0%, 138.4%, and 198.8% and of the EC_{50} were 95.8%, 88.6%, and 198.3%, respectively, in four different lots of HepaRG. Since the variation tended to be smaller at lower rather than higher concentrations in the dose–response curve, the use of rifampicin- or phenobarbital-normalized fold induction at lower concentrations successfully minimized the variation.

12.5.1.4 Fa2N-4 Cells. Fa2N-4 cells are nontumorgenic and originated from human hepatocytes immortalized by transfection with the SV40 large T-antigen [192]. Hariparsad *et al*. [168] extensively characterized basal mRNA expression profiles with respect to 64 drug disposition genes that included drug-metabolizing enzymes, nuclear receptors, and transporters in Fa2N-4 cells, compared with four batches of human hepatocytes. Fa2N-4 cells showed a higher expression in almost all transcription factors and coactivators/corepressors associated with PXR-mediated enzyme induction. CYP3A4 expression level in hepatocytes was variable between the lots and ranged from 0.3- to 18-fold of Fa2N-4 cells. It was pointed out that midazolam 1′-hydroxylation activity in Fa2N-4 cells was ~100-fold lower than that in hepatocytes [167]. Compared with hepatocytes, the expression level of PXR, AhR, and GR was higher in Fa2N-4 cells, but significantly, the expression level of CAR was two orders of magnitude lower in Fa2N-4 cells [168]. Fa2N-4 cells also tended to show a significantly lower basal expression of several major hepatic uptake transporters, including sodium/bile acid cotransporter 10A1 (NTCP), solute carrier organic cation transporter 22A1 (OCT1), OATP1B1, and OATP1B3. In particular, the expression level of OATP1B1/1B3 in Fa2N-4 cells was 50-fold lower than that in hepatocytes, indicating that there is high possibility that substrates of those transporters apparently show low induction potency in Fa2N-4 cells because of low intracellular concentrations. This has been evidenced by the relatively higher EC_{50} values of rifampicin, which is substrate to OATP1B1/3, in Fa2N-4 cells (1.9–8 μM) than hepatocytes (0.1–2.6 μM), as shown in Table 12.5.

Rifampicin was regarded as an outlier in the obtained good relationship when EC_{50} values for various CYP3A inducers were plotted between Fa2N-4 cells and hepatocytes [162]. Noticeably, 6-(4-chlorophenyl) imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime, a CYP2B6 inducer, produced no induction in Fa2N-4 cells, which was in contrast to hepatocyte data with E_{max} of 6.8-fold. This difference was consistent to extremely low expression of CAR in Fa2N-4 cells.

The usefulness of Fa2N-4 cells to identify CYP3A4 inducer was evaluated using 24 compounds selected from induction profiles in human hepatocytes [166]. Fa2N-4 cells showed CYP3A4 induction exceeding twofold for all 18 positive controls and less than 1.5-fold for all 6 negative controls. In addition to CYP3A4, induction of CYP1A2 and CYP2C9 was evaluated in Fa2N-4 cells [193]. Treatment of β-naphthoflavone, rifampicin, or phenobarbital yielded similar fold induction to CYP1A2, CYP2C9, and CYP3A4 mRNA levels, relative to human hepatocytes. Furthermore, a cassette dosing approach with tacrine for CYP1A2, diclofenac for CYP2C9, *S*-mephenytoin for CYP2C19, and midazolam for CYP3A4 was employed to assess induction based on changes in CYP activities in Fa2N-4 cells [194]. A comparable dose–response curve was obtained in both the cassette dosing and single dosing approaches. Similarly, successful measurement of induction activities in the cassette dosing approach was conducted by Kenny *et al*. [167].

12.5.2 Prediction of *In Vivo* CYP Induction

The first investigation of quantitatively predicting effects of CYP1A and CYP3A induction on *in vivo* exposure of substrates to those CYP enzymes was conducted by Kato *et al*. [195], who used *in vitro* and *in vivo* data of various inducers such as rifampicin, carbamazepine, phenytoin, dexamethasone, phenobarbital, and omeprazole. *In vitro* EC_{50} and E_{max} values were calculated from an E_{max} model using an averaged unbound concentration of inducers in the incubation medium with hepatocytes. The magnitude of AUC increase in comedicated CYP1A2 or CYP3A substrates was predicted based on the difference in CL_{int} values before and after treatment of inducers using the E_{max} model in which unbound steady-state plasma concentration of inducers after repeated administration was incorporated. With respect to CYP3A, predicted and observed induction ratios were correlated, whereas the prediction of CYP1A induction by omeprazole was overestimated. This discrepancy in CYP1A induction would be partly because of the underestimation of omeprazole exposure in the incubation medium with hepatocytes.

Ohno *et al*. [196] constructed a robust and accurate prediction method based on the equation of $1/(1 + CR_{CYP3A4} \times IC_{CYP3A4})$, in which CR_{CYP3A4} is the ratio of the apparent contribution of CYP3A4 to oral CL of a substrate (i.e., f_m) and IC_{CYP3A4} represents the apparent increase in CL of substrate by coadministration of prototypical inducers to CYP3A4. IC_{CYP3A4} was calculated for seven inducers based on a reduction in AUC of coadministered simvastatin, a standard substrate of CYP3A4. Rifampicin was found to be the most potent inducer among the tested compounds, with an IC_{CYP3A4} of 7.7, followed by phenytoin (4.7) and carbamazepine (3.0).

Relative induction ratio (RIS) was proposed as an indicator to predict percentage decrease in AUC of CYP3A4 substrate by comedication of CYP3A4 inducers [166]. RIS can be estimated from *in vitro* induction data (E_{max} and EC_{50} values) in Fa2N-4 cells and human hepatocytes with efficacious free plasma concentration using the

following equation:

$$\mathrm{RIS} = \frac{E_{\max} \times C_{\mathrm{eff,free}}}{\mathrm{EC}_{50} + C_{\mathrm{eff,free}}} \tag{12.7}$$

where $C_{\mathrm{eff,free}}$ is the unbound plasma concentration of inducers after a standard therapeutic dose. In case of CYP3A4 inducers (rifampicin, phenobarbital, carbamazepine, troglitazone, etc.), the RIS score estimated from *in vitro* parameters in Fa2N-4 and human hepatocytes was in good agreement with the decrease in AUC of coadministered CYP3A4 substrates such as midazolam or ethinylestradiol.

The dose–response curve using widely ranged inducer concentrations can be described using *in vitro* parameters such as $E_{\max}$ and EC_{50}. In some cases, those parameters cannot be estimated as a result of cellular toxicity or a solubility problem in the high concentration of inducers. As an alternative indicator, Kanebratt and Andersson [191] have proposed to use F_2 parameter, which is an inducer concentration giving twofold induction of CYP3A4 mRNA from baseline. Relationship between the AUC/F_2 (ratio of *in vivo* AUC of inducer to F_2) and percentage decrease in *in vivo* AUC of CYP3A substrate was investigated. Use of either total (bound + unbound) or unbound AUC showed a high relationship with the *in vivo* data, accompanied with a correlation coefficient (R^2) of 0.859–0.863. Kaneko *et al*. [165] demonstrated a large lot-to-lot variation in the $E_{\max}$ and EC_{50} of CYP3A induction in HepaRG cells. The variation in induction response appeared smaller at the low exposure of inducers or positive control than at the high exposure in the dose–response curve. To minimize the variation, an index value termed the *relative factor* (RF) was introduced and is defined by the ratio of the minimum concentration to cause induction response of the inducers tested to the positive control such as rifampicin or phenobarbital. It was revealed that the ratio of the *in vivo* unbound plasma concentration of inducers toward the RF values was a good indicator to classify into three categories: low, medium, and high potency.

In contrast to qualitative assessment using constant inducer concentration or AUC, quantitative prediction of the extent of induction of CYP3A4 was performed based on time-dependent mass balance of CYP3A4 protein calculated by the $E_{\max}$ model incorporating the data in reporter gene assay and plasma concentration profile of inducers simulated by the one-compartment model [197]. The predicted induction ratio was related to urinary metabolite ratio of 6β-hydroxycortisol/cortisol before and after treatment of CYP3A4 inducers.

Prediction approaches have been developed for each mechanism of DDI: reversible, irreversible inhibition, and induction. The method that focuses on a single interaction mechanism is likely to, in some cases, produce misleading predictions of compounds with multiple mechanisms such as ritonavir characterized as an irreversible inhibitor and inducer of CYP3A4. Fahmi *et al*. [198] has developed a more comprehensive mathematical model for simultaneously evaluating the effect of multiple mechanisms of CYP3A4-related DDI. Utilizing this model, DDI arising via inactivation (e.g., erythromycin), competitive inhibition (e.g., ketoconazole), or mixed mechanism (e.g., ritonavir) was well predicted within the ranges reported in clinical studies. The geometric mean fold errors were somewhat decreased by the combined DDI model. The integrated prediction model produced a success rate of 88%, which was comparable to that predicted by DDI functions in the computational PBPK model such as SIMCYP® [199].

12.6 FUTURE PERSPECTIVE

A great need for efficient identification of NMEs with desirable pharmacokinetic profiles has enhanced the efficiency of lead optimization processes based on metabolic stability, CYP inhibition, and induction, coupled with a paradigm shift from throughput to quality. By this trend, the development of high quality screening systems that can be more relevant to *in vivo* humans is needed. In the fine tuning of chemical structures that have an important position in the optimization process to create NMEs, the utilization of human hepatocytes has enhanced the quality of the data in the study of metabolic stability, CYP inhibition, and induction, but the demand for high quality hepatocytes applicable to those studies heavily outweighs the availability. Si-Tayeb *et al*. [200] imply the possibility that human induced pluripotent stem (iPS) cells will be used as a source of hepatocytes for toxicological and drug metabolism studies. The use of iPS cells has required the establishment of culturing conditions using microengineering approaches that support expression of a full panel of phase I and II enzymes and transporters. More recently, an advanced technology with transient overexpression of hemeobox gene using adenovirus vector allowed for differentiation of human iPS cells to hepatocytes, which successfully displayed CYP3A4 induction activity by rifampicin. Efficient generation of the hepatocytes from iPS cells would show a high possibility of giving an evolutional change in the CYP induction study [201].

Furthermore, chromosome engineering techniques, which are based on transfer of a large size of chromosome with a defined region, have enabled to produce humanized model animals. The human chromosome 7 region around CYP3A locus was cloned into the SC20 vector. It is interesting to note that transcript of CYP3A gene was also detected specifically in the liver and small intestine of SC20-transferred chimeric mice, which is coincident with the tissue specificity of CYP3A expression in humans [202,203]. There has been possibility that HAC-mediated gene expressions of human drug metabolism enzymes in transgenic animals produce animal models to study drug metabolism and predict DDI-associated events in humans. Those *in vitro* and *in vivo* assessment tools will be a breakthrough in pharmacokinetic studies in drug discovery and development.

It has been revealed that multiple mechanisms such as transporter-mediated liver uptake and efflux and drug metabolism influence CYP inhibition and induction. Accordingly, using those human-relevant *in vitro* data, the selection of NMEs with high developability among the candidates will be routinely conducted based on the predicted pharmacokinetic profiles and the evaluated possibility of DDI by comedication in humans through utilizing a whole-body PBPK model incorporating those multiple mechanisms.

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