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Review Article

Metabolism-mediated drug–drug interactions – Study design, data analysis, and implications for *in vitro* evaluations

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ABSTRACT

Therapeutics undergo metabolism as a major biotransformation pathway for their bioactivation or clearance from the body, which is a well-known factor for affecting drug pharmacokinetics, safety and efficacy. Drug metabolism primarily occurs in the liver and intestine, where expressing a wide variety of drug metabolizing enzymes, which are generally divided into Phase I enzymes like cytochrome P450s (CYP450s) and Phase II enzymes as UDP glucuronosyltransferases (UGTs). The chemical structures of therapeutic molecules are modified, which are called metabolites, as outcome of oxidation or conjugation by metabolism or biotransformation. Therefore, metabolism-mediated drug–drug interactions (MMDDI) may have more potential clinical significance than transporter-mediated drug–drug interactions (TMDDI), because both parent molecules and metabolites may contribute to the drug–drug interaction which may make the clinical significance more complicated. This article reviewed the challenges of MMDDI during therapeutic innovations, and industry solutions for supporting IND/NDA submissions according to the related guidances by the regulatory agencies as NMPA, FDA, PMDA and EMA.

Contents

Abbreviations: ADH, Alcohol dehydrogenase; AhR, Aryl hydrocarbon receptor; ALDH, Aldehyde dehydrogenase; AO, Aldehyde oxidase; AUC, Area under the plasma concentration-time curve; CAR, Constitutive androstane receptor; CES, Carboxylesterase; CL_{int}, Intrinsic clearance; C_{max}, Total maximal concentration in plasma; $C_{max,u}$, Unbound maximal plasma concentration; COC, Combined oral contraceptive; CYP450, Cytochrome P450; DDI, Drug–drug interaction; E_{max}, Maximum induction effect; EC_{50}, Concentration causing half-maximal effect; EMA, European Medicines Agency; F_2, *In vitro* test concentration leading to a 2-fold increase in baseline mRNA; FMO, Flavin monooxygenase; $f_{u,hep}$, Free fraction of the compound in hepatocytes; fu_{mic}, Microsomal unbound fraction; HDI, Herb-drug interaction; IC_{50}, Half-maximal inhibitory concentration; $[I]$, Maximal unbound plasma concentration of the NCE at steady state; $[I]_{gut}$, Intestinal luminal concentration of the NCE; $I_{max,u}$, Maximal unbound plasma concentration of the interacting drug at steady state; IND, Investigational new drug applications; ISEF, Intersystem extrapolation factors; k_{deg}, Apparent first-order degradation rate constant of the affected enzyme; K_i, Inhibition constant; K_I, Inhibitor concentration causing half-maximal inactivation; $K_{I,u}$, Unbound inhibitor concentration causing half-maximal inactivation; k_{inact}, Maximum inactivation rate constant; K_m, Michaelis-Menton constant; K_{obs}, Observed (apparent first order) inactivation rate of the affected enzyme; MAO, Monoamine oxidase; MBI, Mechanism based inhibition; MMDDI, Metabolism-mediated drug-drug interaction; NC, Negative control; NCE, New chemical entities; NDA, New drug applications; NMPA, National Medical Product Administration; PXR, Pregnane X receptor; P-gp, P-glycoprotein; PBPK, Physiologically-based pharmacokinetic; PC, Positive control; PCN, Pregnenolone-16a-carbonite; PK, Pharmacokinetic; PMDA, Pharmaceuticals and Medical Devices Agency; RAF, Relative activity factor; RIS, Relative induction scores; SULT, Sulfotransferase; TDI, Time-dependent inhibition; TP, Therapeutic protein; TMDDI, Transporter-mediated drug-drug interaction; UGT, UDP glucuronosyltransferase; FDA, U.S. Food and Drug Administration; V_{max}, Maximal rate; XO, Xanthine oxidase.

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1. Background

It is almost a routine clinical practice for treating disease by use of multiple therapeutics. As a consequence, the patients may take potential risks due to drug–drug interaction (DDI), resulting in the alteration of systemic exposure parameters as the area under curve (AUC) and/or plasma concentrations (C_{max}), and leading to either an increase of adverse reactions or decrease in efficacy [1]. The DDIs are generally mediated by drug metabolism enzymes (MMDDI) and/or drug transporters (TMDDI). The TMDDI evaluation using *in vitro* studies and the related regulatory guidelines were reviewed in our previous publication [2]. *In vitro* evaluation of MMDDI was reviewed in this article.

Many therapeutics undergo metabolism as a major mechanism of bioactivation or clearance from the body, which primarily occurs in the liver and intestine. These two organs express a wide variety of drug metabolizing enzymes [3], among which cytochrome P450s (CYP450s) enzymes are responsible for metabolizing chemical molecules of therapeutics or xenobiotics in humans. The CYP450s mediated DDIs have led to the withdrawn of several drugs, such as terfenadine, mibefradil, astemizole, cisapride and nefazodone, due to the increased risk of QTc prolongation, an adverse reaction [1]. Moreover, the attention should also be paid to metabolite caused DDI, such as inhibiting the enzyme activities of CYP3A4 and CYP2C8 by gemfibrozil 1-*O*-β-glucuronide, one of major circulating metabolites of gemfibrozil, resulting in increased exposure of cerivastatin with a higher incidence of fatal rhabdomyolysis, a severe adverse reaction [4]. Therefore, metabolites with high *in vivo* exposure or/and potential pharmacological activity should also be considered for MMDDI evaluation.

The MMDDI evaluation of the new chemical entities (NCE) generally starts with *in vitro* experiments for identifying potential DDI risks and elucidating related mechanisms, which are classified into three types of experiments [3,5-7]:

1) determining if the NCE is a substrate of metabolizing enzymes, and if so, identifying the responsible metabolism enzymes (phenotyping);
2) determining if the NCE is an inhibitor of metabolizing enzymes in mechanism of reversible and/or time-dependent;
3) determining if the NCE is an inducer of metabolizing enzymes.

As reported by a systematic analysis, two-thirds of the total 37 FDA approved drugs in 2017 were subject to the metabolism by CYP3A4 and followed by CYP2D6, CYP1A2 and CYP2C, suggesting CYP3A4 played a significant role in metabolizing drugs. Among the approved drugs, the largest number of drugs were identified as inhibitors of CYP3A, followed by CYP2C9, CYP1A2, CYP2C8, CYP2D6, CYP2C19 and CYP2B6, and some as inducers of CYP3A, CYP2B6 and CYP1A2 [8]. Out of 20 therapeutics approved by NMPA in 2020, 17 were labeled with DDI information, among which 11 were with CYP450 mediated DDI. Among 53 therapeutics approved by FDA in 2020, 20 were related to CYP450 mediated DDI [9]. The report indicated that MMDDI occurred primarily through the effect on the enzymes activity of CYP family. Accordingly, the *in vitro* substrate study and inhibition/induction study of NCEs mediated by CYP family, including CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A, were recommended for supporting IND/NDA submission by related regulatory guidances [3,5-7].

The regulatory DDI Guidance kept being updated according to the needs of therapeutic innovation and scientific research since 2005, taking FDA as an example, its DDI guidance had been modified for 4 times within 14 years from 2006 to 2020. As presented in Table 1, the endpoints and standard criteria of CYP450 induction were changed significantly by FDA [3,10-12].

Moreover, ICH M3 (R2) recommended that *in vitro* metabolic study for both animals and humans generally should be evaluated before ini-

tiating human clinical trials, and *in vitro* biochemical information relevant to potential drug interactions should be available before exposing large numbers of human subjects or treating for long duration (generally before Phase III) [13]. FDA Safety Testing of Drug Metabolites (MIST) Guidance also mentioned that *in vitro* studies of the metabolic profile of the NCE should generally be conducted before initiating clinical trials using liver microsomes, liver slices, or hepatocytes from animals and humans [14].

Various well-developed modeling approaches, such as basic models, static mechanistic models, or dynamic mechanistic models, including physiologically-based pharmacokinetic (PBPK) models, were widely applied for translating data from *in vitro* experiments into *in vivo* predictons, or for the design of clinical DDI studies [3,5-7].

PBPK modeling is one approach that enables integration of physiological, chemical, and drug-dependent preclinical and clinical information to model an NCE's absorption, distribution, metabolism, and excretion and ultimately simulate untested clinical scenarios [15]. According to an overview of the application of PBPK modeling in the submissions between 2018 and 2019 received by FDA, DDI-related PBPK analyses accounted for 56% of the total 116 applications (40% and 16% for metabolism-mediated and transporter-mediated DDI, respectively) [16].

This article reviewed the challenges of MMDDI during therapeutic innovations, and industry solutions for supporting IND/NDA submissions according to the related guidances by the regulatory agencies. The decision tree for overall MMDDI studies was present in Fig. 1, which was referred from NMPA DDI guidance [7].

Table 1
The summary of endpoints and standard criteria of CYP450 induction changing from 2006 to 2020 DDI guidance by FDA.

Guidance Version	Endpoint	Standard Criteria
2006	Enzyme activities	A drug that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer *in vitro* and *in vivo* evaluation is warranted.
2012	mRNA level	If the *in vitro* induction results are positive according to predefined thresholds using basic models, the investigational drug is considered an enzyme inducer and therefore further *in vivo* evaluation may be warranted.
2017	mRNA level	A ≥ 2-fold increase in mRNA and a response ≥ 20% of the response of the positive control in the presence of an investigational drug are interpreted as a positive finding.
2020	mRNA level	A drug is interpreted as an inducer if: (1) it increased mRNA expression of a CYP enzyme in a concentration-dependent manner; and (2) the fold change of CYP mRNA expression relative to the vehicle control is ≥ 2-fold at the expected hepatic concentrations of the drug

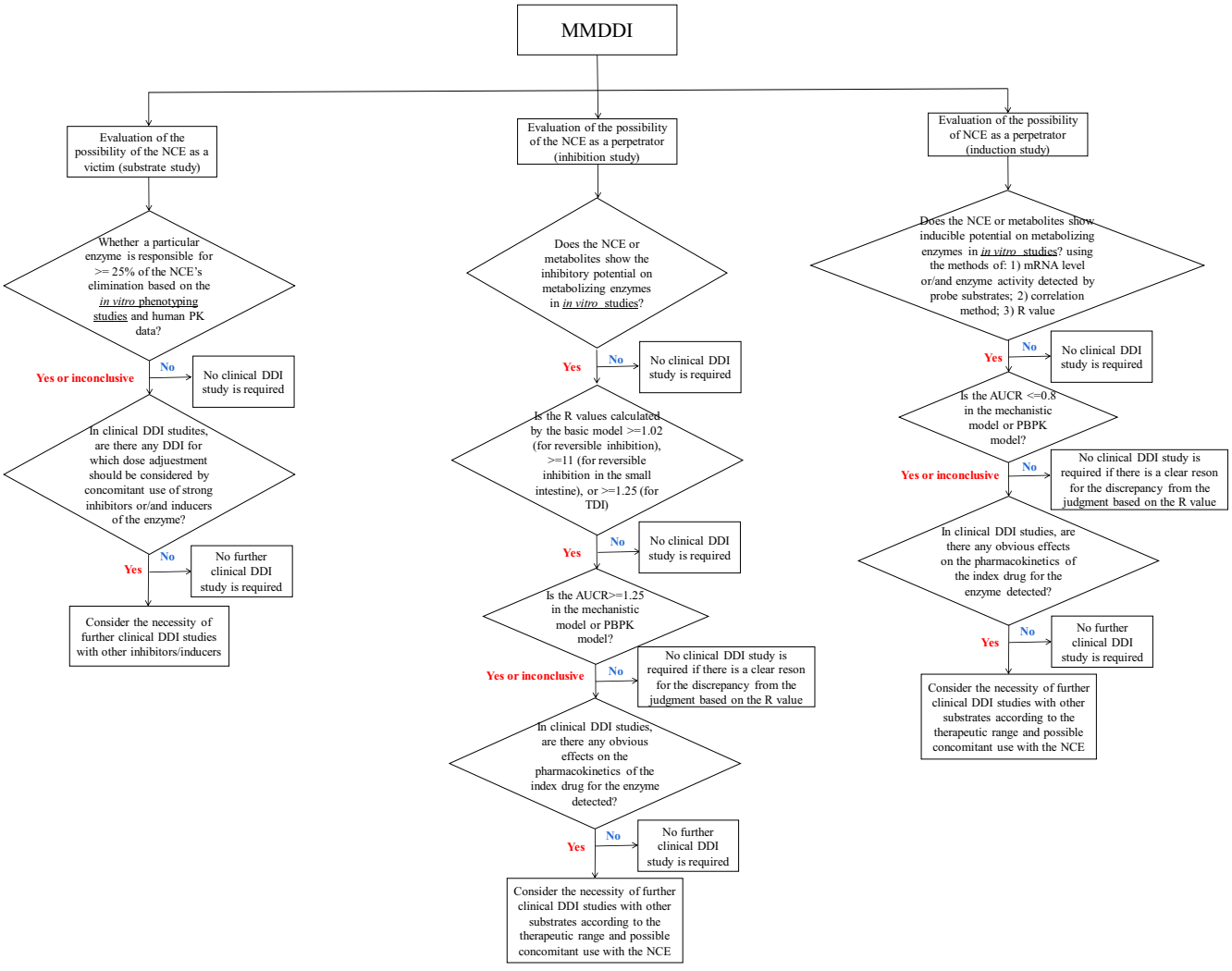

Fig. 1. The decision tree of MMDDI evaluation referred from NMPA guidance.

2. Substrate study

2.1. Background

The DDI guidelines recommend that *in vitro* substrate assay, also called reaction-phenotyping study, should be conducted to identify the responsible enzymes for metabolizing NCE, such as CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A, in case the NCE is subject to metabolism by CYP450s.

Additional metabolism enzymes should also be considered if NCE was not subject to the metabolism by the above CYP450s, and the followings (but not limited) should be taken into considerations:

1) other CYP450 metabolic enzymes such as CYP2A6, CYP2J2, CYP4F2 and CYP2E1;
2) other phase I metabolic enzymes such as monoamine oxidase (MAO), flavin monooxygenase (FMO), Xanthine oxidase (XO), Alcohol/Aldehyde dehydrogenase (ADH/ALDH), Aldehyde oxidase (AO) and Carboxylesterase (CES);
3) Phase II enzymes such as UDP glucuronosyl transferases (UGT) and sulfotransferases (SULTs).

2.2. Methodology

As recommended by the regulatory guidances, the routine methodology with experimental design is summarized as below:

1) Prior to phenotyping, the metabolic stability of the NCE using human liver microsomes or/and hepatocytes should be evaluated with two purposes (1) briefly estimating that the responsible enzyme belongs to CYP450s or non-CYP450s by comparing metabolic differences between incubations using liver microsomes and hepatocytes (Table 2); (2) supporting design of phenotyping study such as incubation time and test concentrations.
2) Due to the difficulty for synthesis of standard metabolites, the parent depletion rate is used as the parameter of phenotyping study during non-clinical phase, and referred to as metabolic rate. Both parent depletion rate of pro-drug and formation rate of active metabolite as well as phenotyping should be evaluated if NCE is a pro-drug.
3) By careful consideration of different test systems, both human liver microsomes with chemical inhibitors and human recombinant enzymes are recommended for phenotyping of NCE by the regulatory guidances. The liver microsomes test system for evaluating the absolute contribution of each responsible enzyme will be suitable to high metabolic NCE with better *in vitro* and *in vivo* correlation. On the contrary, the recombinant enzymes test system is more suitable for NCE with lower metabolic rate, e.g. the parent remaining is over 50% at the end of incubation compared to that before incubation, and has better specificity for identifying the responsible metabolism enzyme than liver microsomes system.
4) For supporting IND filing and helping design of potential clinical DDI study, as discussed above, in the stage of IND filing, *in vitro* phenotyping study should be considered preliminarily, and followed by the relative contribution of each enzyme to the metabolism. The relative contribution could be estimated using recombinant enzyme abundance method, relative activity factor (RAF) method, and intersystem extrapolation factors (ISEF) method. The RAF method is the most recognized and commonly used because it generates the RAF (CL_{int}) algorithm based on the inherent clearance rate from the experiment design of multiple incubation time points, and provides more accurate parameters [17]. In principle, a correlation between activities from experiment using recombinant enzymes and those from experiment using pooled human liver microsomes will be established by the parameter of RAF, be initially measured using a CYP-specific probe substrate, and be extended to the data set acquired for the NCE [18,19].

Table 2
Some data examples useful to determine the non-CYP450s metabolic pathways .

Data example	Enzymes likely involved in the metabolism	Specific *in vitro* test system
A large amount of Phase II metabolites such as glucuronic acid conjugates was detected *in vivo* or *in vitro* hepatocytes test system	UDP glucuronosyl transferases (UGT)	Further verification by using liver microsomes +/− UDPGA, a cofactor of UGT; Identification of UGT isoform responsible for the metabolism using recombinant UGT enzymes.
The NCE showed good chemical stability in incubation buffer, while it was metabolized apparently in liver microsomes without any cofactor	Carboxylesterase 1 (CES1), carboxylesterase 2 (CES2), Arylacetamide deacetylase (AADAC)	Further determination based on the plasma stability of the NCE and its species difference; Evaluate whether the NCE metabolism is suppressed by the addition of CES inhibitors (*e.g.* PMSF) using liver microsomes
The NCE was not metabolized in liver microsmes but metabolized apparently in hepatocytes	Some non-CYP450 enzymes, such as ADH/ALHD, MAO and AO/XO	According to the different location of the target metabolizing enzymes, S9, mitochondria, cytosol and hepatocytes are chosen for further determination.

2.3. Data analysis and interpretation

If a specific metabolizing enzyme responsible ≥ 25% elimination of the NCE is identified by *in vitro* phenotyping study, the clinical DDI study using strong index inhibitor or inducer of such an enzyme should be considered and conducted [3,7]. Considering the complexity of substrate studies including metabolic stability, phenotying and contribution of enzymes, focuses during different stages of drug development are summarized as below:

Screening phase: the metabolic stability experiment using human liver microsomes and/or hepatocytes is widely applied for screening and identifying the lead compounds, such as molecule optimization and prediction of oral bioavailability, clearance and *in vivo* half time and exposure [20]. The metabolic pathway identification (phenotyping) might not be considered in this phase unless co-medications with inhibitors or inducers of the metabolizing enzyme are concerned.

Non-Clinical Phase: *in vitro* substrate study should be conducted using human liver microsomes +/− NADPH, a co-factor of CYP450, since over 90% of the drugs were substrates of CYP450s [8,21]. If NCE is subject to metabolism by CYP450s, the phenotyping study should be conducted using chemical inhibitors and recombinant enzymes for identifying the major CYP450 isoforms responsible for metabolizing NCE, and respective contributions if multiple isoforms involved. If non-CYP450 enzymes are concerned, *in vitro* metabolic stability study is recommended to be considered using primary hepatocytes [7].

Clinical Trial Phase: the relative contribution of each enzyme responsible for metabolizing NCE should be evaluated in this phase. The clinical significance should be concerned if the relative contribution is over 25%, suggesting that the exposure of NCE may be vulnerable to enzyme inhibitors or inducers. The relative contribution is a valuable parameter for calculating the value of f_m (fraction of metabolism) for clinical significance evaluation [22], which is one of the

major factors governing the magnitude of a drug's DDI and the impact of polymorphism on total drug clearance [23]. Additionally, the pathways of metabolites formation with significant plasma exposure levels or pharmacological activities should be considered and further evaluated [24].

3. Inhibition study

Inhibition of CYP450s can be classified into reversible or time-dependent inhibition (TDI). Reversible inhibition is defined as the inhibition potency that is time-invariant, and enzyme activity that recovers once the inhibitor is no longer there [25]. TDI sometimes is also called mechanism based inhibition (MBI). The mechanism of reversible inhibition involves rapid association and dissociation of drugs and enzymes, while TDI results from irreversible covalent binding or quasi-irreversible noncovalent tight binding of a chemically reactive intermediate to the enzyme. Therefore, the consequences of TDI are considered to be more serious than reversible inhibition because the inactivated enzyme won't be responsible for drug metabolism.

3.1. Reversible inhibition study

3.1.1. Background

According to the recently updated NMPA [7] and FDA guidances [3], the inhibition of NCE on CYP450s including CYP1A2, CYP2B, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A should be conducted using *in vitro* test systems. As recommended by EMA [5] and PMDA guidance [6], *in vitro* inhibition study should include UGT1A1 and UGT2B7 if glucuronidation is involved in metabolism of NCE.

As recommended by the guidance of EMA [5], the major metabolizing enzymes (*e.g.* CYP2E1, MAO) for NCEs should be included in *in vitro* inhibitory study. By considering a potential logical relationship between reaction-phenotyping and inhibition effect, in case that NCE is subject to metabolism by one enzyme, the inhibition of NCE on this enzyme should be evaluated.

The inhibition constant K_i should be estimated if NCE shows inhibitory potency on metabolism enzyme(s), and could be used for elucidating inhibitory mechanism as competitive, non-competitive, uncompetitive and mixed, respectively. As one of the key parameters, K_i helps to calculate the value of R_1 (or R_{1gut}) by basic model, and the value of AUCR by static mechanistic model. The clinical significance of MMDDI could be evaluated by using the values of K_i, R_1, and AUCR (Fig. 1).

3.1.2. Methodology

The most applicable methods for evaluating reversible inhibition of NCE on drug metabolizing enzymes are presented in Table 3. The single point assay is suitable for preliminary large-scale screening. The methods of IC_{50} and K_i determination are suitable for mechanism research and clinical significance evaluation to support IND and NDA filing [26]. The study design is outlined as below:

1) Test concentrations: for the single point assay, it is recommended to set a higher test concentration, often above 10 μM, to prevent "false negatives"; For the IC_{50} and K_i assay, in order to obtain a relatively accurate IC_{50} or K_i value, it is recommended to design 4–8 concentrations, with a general span of 2–3 LOG, on the basis of the compound information, such as cytotoxicity, maximum solubility and plasma concentration. The highest test concentration is preferred to cover the estimated liver or intestinal lumen concentration (*e.g.* 50 times of the unbound C_{max} or 0.1 times of the dose/250 mL), and the lowest test concentration is preferred to reach or be lower than the blood concentration at the effective dosage.
2) Probe substrates: because CYP3A4 has at least two active binding sites, it is recommended to use midazolam and testosterone to evaluate the binding (inhibition) effect of the NCE on two different sites; However, a single specific probe substrate is enough for the activity evaluation of other isoforms of CYP450s. In addition, in order to be able to accurately predict the K_i value, the final concentration of probe substrates in the incubation system cannot exceed its K_m value. However, when substrates have a low K_m, the exhaustion of substrates should be considered. It should be noted that reversible inhibition assay is to evaluate the parent inhibitory effect on enzymes, thus for the NCE that is rapidly metabolized in the test system like liver microsomes or hepatocytes, to avoid the decrease in test concentration caused by rapid metabolism, it is recommended to use substrates metabolized faster than the NCE.
3) Test systems: although the guidance recommends that liver microsomes and hepatocytes pooled from more than 10 donors, and recombinant enzymes can be used for the inhibition assay, liver microsomes is considered to be the first choice with the following reasons: one is, compared with hepatocytes, liver microsomes are more suitable for the mechanism study because the interferences of cell membrane permeability and drug transporters are eliminated; the other is, compared with recombinant enzymes over-expressing CYP450 protein, liver microsomes has a higher correlation with *in vivo*.
4) Microsomal protein concentration: in order to prevent non-specific binding, it is recommended that the protein concentration should be lower than 1 mg/mL, usually 0.3–0.5 mg/mL. Additionally, if the nonspecific binding is expected to influence the analysis of kinetic data, the microsomal protein binding of the NCE should be measured by using equilibrium dialysis or ultra-filtration, which is indicated as microsomal unbound fraction (fu_{mic}), and used for subsequent correction of IC_{50} and K_i values by using following equation [27-29]:

$$\text{Unbound } IC_{50} = IC_{50} \times fu_{mic}$$

$$\text{Unbound } K_i = K_i \times fu_{mic}$$

3.1.3. Data analysis and interpretation

According to the guidances by NMPA and FDA [3,7], the R_1 value is calculated by using the basic model firstly for evaluating the potential

Table 3
The 3 methods commonly used in reversible inhibition study.

Methods	Objections	Endpoints	Evaluation standard
Single-point	Single test concentration used for predicting IC_{50} for screening	Relative activity (% of NC)	$IC_{50} = x \times \left(\frac{100-(percent\ control\ at\ x)}{percent\ control\ at\ x}\right)^{1/h}$ Where x is test concentration, the Hill parameter h is set to -1.
IC_{50} determination	Gradient test concentrations for IC_{50} determination under single concentration of probe substrates	IC_{50}	Combined with unbound C_{max} for evaluating clinical significance
K_i determination	Gradient test concentrations for K_i determination under multiple concentration of probe substrates	K_i Inhibition type	Combined with clinical PK data to calculate R_1 and R_{1gut} values for further evaluation of clinical significance

clinical significance of the inhibitory effect. Because CYP3A4 is expressed relatively high in the intestinal tract, R_{1gut} is recommended for estimating the inhibitory effect of oral drugs on CYP3A4 expressed in intestinal cavity. If either $R_1 \geq 1.02$ or $R_{1gut} \geq 11$, the clinical potential of MMDDI should be further investigated by using mechanistic models.

K_i and human PK data are usually not available in pre-clinical phase, and the potential risk of MMDDI could be alternatively estimated as below:

1) The category of inhibitory potency: according to the IC_{50} value, it can be typically divided into 3 categories: strong inhibition ($IC_{50} < 1$ μM), moderate inhibition (1 μM ≤ IC_{50} ≤ 10 μM) and weak or no inhibition ($IC_{50} > 10$ μM) [30]. When the NCE is classified as strong inhibitor, it is recommended to further determine K_i for identifying the inhibition mechanism.
2) Verification by hepatocyte: human liver microsomes is a feasible and reliable test system for MMDDI study, however, this system is not an intact one including membrane permeability by drug transporters and metabolism by non-CYP450s enzymes, which may result in false-positive finding. Therefore, human primary hepatocytes are recommended for verifying the positive finding of CYP450 inhibition by liver microsomes.
3) Prediction of K_i through IC_{50}: The relationship between K_i and values of IC_{50} can be determined when the final concentration of probe substrate is near its value of K_m ([S] = K_m). Brief judgement could be reached as: $K_i = IC_{50}$ suggesting noncompetitive inhibition, $K_i = IC_{50}/2$ competitive and uncompetitive inhibition, and K_i values ranging from IC_{50} to $IC_{50}/2$ mixed inhibition [31,32]. As suggested by FDA guidance (2012 version) [11], K_i values are often estimated by using $IC_{50}/2$ and following the conservative assumption that the mechanism of reversible inhibition is competitive in nature. K_i being much higher than maximal blood concentration of the NCE suggests potential MMDDI's risk be not significant.
4) Clinical co-medications: The analysis of possible co-medications would also be helpful to estimate MMDDI's clinical significance [33]. For example, if the NCE is an inhibitor of CYP2C8, the clinical risk of MMDDI might be considered in-significant if the co-medicated drugs are not subject to metabolism by this enzyme. Therefore, the NCE could be labeled using *in vitro* data directly.

3.2. Time-dependent inhibition study

3.2.1. Background

TDI is defined as an interaction from pre-incubation with test system prior to addition of the probe substrate of tested CYP450, which specifically refers to time-dependent inhibition by chemically reactive metabolite [34,35].

TDI should be conducted to cover seven CYP450s isoforms, including CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 same as reversible inhibition as recommend by NMPA and FDA.

The evaluation route of TDI study is similar to reversible inhibition. Generally, IC_{50} shift fold should be calculated by comparing the values of IC_{50} from the incubation in the presence or absence of NADPH, to start or do not start the CYP450-mediated metabolism. The maximum inactivation rate constant (k_{inact}) and the inhibitor concentration causing half-maximal inactivation (K_I) should be further estimated for evaluating the clinical significance in case that IC_{50} shift fold of NCE is concerned.

3.2.2. Methodology

As presented by Table 4, the single-point assay is suitable for screening and optimizing leads, and the evaluations of IC_{50} shift and K_{inact}/K_I suitable for estimating mechanism of MMDDI and evaluating clinical significance. The followings may help the study design:

1) Test concentrations: the test concentrations of NCE for evaluating reversible inhibition (Section 3.1.2) could be considered as a reference to design TDI study. A pilot dosing range finding assay, e.g. single-point experiment, may benefit a rational design of test concentrations for accurately calculating IC_{50} shift fold.
2) The TDI-specific inhibitors: the known inhibitors as positive control for reversible inhibition study can't be directly used for TDI study. The compounds with time-dependent inhibitory effect could be served as positive controls in TDI assay.
3) Pre-incubation time: in the IC_{50} shift assay, the pre-incubation time is often set as 30 min, which can be appropriately changed according to the metabolic rate of the NCE in liver microsomes. Sometimes, the 0-min pre-incubation is set to estimate the potential inhibitory effect on metabolism by non-NADPH dependent enzymes. In the 30-min pre-incubation without NADPH, almost no IC_{50} shift was observed including in the positive control, suggesting that CYP450 enzymes be bound by the metabolite dependent on CYP450s [36].
4) The incubation system: compared to reversible inhibition study, there is a "1 in 10" dilution step between the pre-incubation and incubation followed by the addition of increased substrate concentration of 5–10 × K_m in TDI assay with two considerations (1) for minimizing the effect of reversible inhibition on quantification of enzyme activity; and (2) relatively increasing microsomal protein concentrations and minimizing the effect of "free drug" on quantification of enzyme activity [36].

Table 4
The 3 methods commonly used in time-dependent inhibition study.

Methods	Objections	Endpoints	Evaluation standard
Single-point	Single test concentration used for predicting TDI for screening and the design of following study	Remaining activity (%) = (% of $NC_{(+NADPH)}$/% of $NC_{(-NADPH)}$) × 100	A compound under the 80% remaining threshold is classified as having potential TDI risk If the remaining activity is lower than 80%, an IC_{50} shift assay is recommended to be conducted.
IC_{50} shift	Gradient test concentrations for IC_{50} determination under pre-incubation with/without NADPH for one time point (usually for 30 min)	IC_{50} shift = IC_{50} (30 min pre-incubation without NADPH)/IC_{50} (30 min pre-incubation with NADPH)	A compound which exhibits an IC_{50} shift of ≥1.5 with a pre-incubation is classified as a TDI inhibitor If an IC_{50} shift is observed, a k_{inact}/K_I assay is recommended to be performed
k_{inact}/K_I assay	Gradient test concentrations for Gradient test concentrations of the compound under pre-incubation with/without NADPH for different time point s	The values of k_{inact} and K_I	Combined with clinical PK data to calculate R_2 values for further evaluation of clinical significance

3.2.3. Data analysis and interpretation

1) Judgement of the inhibitory mechanism by IC_{50} shift: Solely reversible inhibitors do not make IC_{50} shift after a 30-min pre-incubation; Solely time-dependent inhibitors will inhibit CYP450s with IC_{50} in 30-min pre-incubation with NADPH. Compounds with both reversible and time-dependent inhibitory effects will show stronger inhibition with lower IC_{50} in the pre-incuabtion in the presence of NADPH.
2) R_2: the value of R_2 can be calculated by k_{inact} and K_I based on the basic model as following formula:

$$R_2 = (k_{obs} + k_{deg})/k_{deg}$$

Where $k_{obs} = k_{inact} \times 50 \times [I]/(K_{I,u} + 50 \times [I])$

[I] is the maximal unbound plasma concentration of the NCE at steady state.
$K_{I,u}$ is the unbound inhibitor concentration causing half-maximal inactivation.
K_{obs} is the observed (apparent first order) inactivation rate of the affected enzyme.
If $R_2 \geq 1.25$, the TDI potential should be further evaluated by using mechanistic models for considering if clinical DDI study is necessary.

3) The apparent first-order degradation rate constant (k_{deg}): k_{deg} of the inhibited enzyme could be obtained from the literature, for example, the k_{deg} of CYP3A4 expressed in liver and small intestine were reported as 0.000321 and 0.000481, respectively [37]. However, be very careful to cite literature reported k_{deg} values, which varied with different authors, especially for the NCE with both TDI and induction effects on CYP450s. The literature summarized the k_{deg} values of each CYP450s isoforms by different methods [38].
4) k_{obs}: it should also be mentioned that besides liver, CYP3A4 is also highly expressed in intestine, the following formula can be used to estimate the value of k_{obs} of CYP3A in intestine [6]:

$$k_{obs} = k_{inact} \times 0.1 \times [I]_{gut}/(K_I + 0.1 \times [I]_{gut})$$

where $[I]_{gut}$ is the intestinal luminal concentration of the NCE calculated as the dose/250 mL.

4. Induction study

4.1. Background

The clinical significance of metabolizing enzyme induction could (1) increase the clearance of drugs, resulting in decreased therapeutic effect; (2) increase the activation of pro-drug, causing an alteration in efficacy and pharmacokinetics; (3) increase the metabolism of drugs that contribute to toxicity via reactive intermediates [39].

The mechanisms for inducing enzyme activity of CYP450s are as below:

1) Stabilization of the enzymes mRNA or protein. As the case of troleandomycin, it induced CYP3A activity by decreasing the rate of CYP3A protein degradation but increasing the rate of protein synthesis [40]. As the case of isoniazid, it induced CYP2E1 activity by stabilizing the enzyme itself but not expediting protein synthesis [39].
2) Nuclear receptor-mediation. The most common mechanism of CYP450s induction is transcriptional gene activation. In general, a drug activates a nuclear pathway, which then causes upregulation of transcription of the receptors' target genes. These nuclear receptors commonly involved in regulation of drug metabolizing enzymes are aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR), which are responsible for the induction of CYP1A2, CYP2B6 and CYP3A4, respectively [41]. However, there are apparent species differences in nuclear receptor activation. For instance, omeprazole is CYP1A inducer in humans while it has little effect on CYP1A isoforms in the rat, mouse and rabbit [42]. Rat CYP3A enzymes are readily induced by pregnenolone-16a-carbonite (PCN), whereas neither rabbit nor human CYP3A enzymes are induced by PCN [43].

Human-derived cell tissue, such as human primary hepatocytes, is one of the best *in vitro* systems for estimating the induction effect of the NCE on CYP450s. *In vitro* induction study should be considered if decrease of drug exposure as AUC and C_{max} was observed in *in vivo* multiple-dose pharmacokinetics in animals and humans.

Not all CYP450s isoforms are subject to induction by chemicals based on published research articles, such as CYP2D6 and CYP2J2 [21].

In terms of enzyme induction studies, CYP1A2, CYP2B6, CYP2C8, CYP2C9 and CYP3A4 should be evaluated according to the regulatory guidances issued by NMPA, FDA, and EMA. CYP2C enzymes should be considered if the induction of CYP3A4 enzyme is observed, otherwise, induction study for CYP2Cs are not necessary, because both CYP3A4 and CYP2C enzymes are induced via activation of the same nuclear receptor - PXR.

4.2. Methodology

1) Number of donors: by taking into account the large inter-individual variation in the response to inducers, the human hepatocytes should be prepared from at least three individual donors by following considerations [44]:
   - Apparent individual differences in the expression levels of efflux and uptake transporters on hepatocytes, such as P-gp (MDR1) and OATP;
   - Individual difference of the clearance of inducers;
   - Genetic variations of nuclear receptors;
   - Physiological and environmental factors, such as hormonal and disease status, age, gender, are different.

Therefore, if the positive finding, e.g. NCE induced CYP450s, is detected in hepatocytes incubation from one donor, the NCE should be considered as a potential inducer of the tested CYP450s isoform (s). The donor with a lower basal level of enzyme activity, which is more inducible, is preferred to be selected for induction assay.

2) NCE exposure time: NCE should be incubated with hepatocytes for 48–72 h for ensuring the enough exposure time and avoiding false negative results because the half-life of enzyme protein is about 20–40 h [45]. However, reducing exposure time should be considered if cytotoxicity of hepatocytes by NCE is observed. Usually for anti-tumor drugs, 24-hour exposure time is considered for estimating mRNA expression. A pilot cytotoxicity assay prior to induction study is strongly suggested for avoiding the interference of drug-induced hepatotoxicity on the induction potential evaluation.
3) Test concentration: plasma therapeutic exposures as the minimal therapeutic concentration C_{max}, and liver concentration for NCE highly distributed and/or accumulated in liver should be considered, when designing the test concentration range. Generally, 4–8 concentrations should be designed for accurately evaluating dose-dependent induction including the parameters as E_{max} (the maximum induction effect) and EC_{50} (the concentration causing half-maximal effect), which are essential for further evaluating the potential clinical significance.

4) Endpoint: mRNA levels and/or enzyme activity are used as routine endpoints in *in vitro* induction study. However, one of the major challenges of enzyme activity assay is that the outcome could be mixed with CYP450's induction and inhibition if NCE is both inducer and inhibitor, which might result in a false-negative judgement. Therefore, the regulatory authorities recommend that mRNA expression assay should be conducted by using fold-change method or correlation method, and enzyme activity assay be considered if induction with the mechanism of protein stabilization is suspected.
5) Other indicators: in order to accurately evaluate the induction effect and potential clinical significance, the following tests can be carried out with the induction assay:
   - Cytotoxicity assessment in primary human hepatocytes
   - Assessment of non-specific binding to hepatocytes
   - Measurement of parent drug in the medium at the last day of dosing

The cytotoxicity assessment can help to understand non-linear induction especially at the higher test concentrations due to cytotoxicity or other mechanisms of CYP downregulation. The free fraction of NCE in hepatocytes incubation ($f_{u,hep}$) can be estimated by the experiment of non-specific binding to hepatocytes using equilibrium dialysis method, and be used for calculating unbound EC_{50} by using following equation [46-48]:

$$\text{Unbound } EC_{50} = EC_{50} \times f_{u,hep}$$

4.3. Data analysis and interpretation

1) Fold-change method: This method is commonly used to evaluate the fold change of mRNA expression by comparison of NCE treated-hepatocytes with non-NCE treated one (vehicle control, VC). A NCE should be considered as an inducer if the mRNA expression of a CYP enzyme increases ≥ 2 folds of VC in a concentration-dependent manner; or the mRNA expression ≥ 20% of the positive control (PC) treated with known inducers of CYP450s. Then, the induction parameters of E_{max} and EC_{50} could be calculated by non-linear regression analysis with plotting the *in vitro* fold induction data against the nominal *in vitro* concentration [49-51].
2) Correlation methods: The relative induction scores (RIS) and $I_{max,u}/EC_{50}$ could be calculated by the following formula for predicting the magnitude of a clinical induction effect of NCE.
   - Correlation method 1: $RIS = (E_{max} \times I_{max,u})/(EC_{50} + I_{max,u})$
   - Correlation method 2: $I_{max,u}/EC_{50}$

Where E_{max} is the maximum induction effect determined *in vitro*. EC_{50} is the concentration causing half-maximal effect determined *in vitro*. $I_{max,u}$ is the maximal unbound plasma concentration of the interacting drug at steady state.

If the predicted magnitude, such as AUC ratio of index substrate in the presence and absence of a NCE (inducer), is ≤0.8, the NCE should be considered potential inducer *in vivo*.

Sometimes, accurate and reliable E_{max} or EC_{50} won't be available due to an incomplete *in vitro* induction profile (*e.g.* limited by solubility or cytotoxicity). The following methods could be used for estimating the induction potential alternatively:

- Method 1: AUC/F_2
- Method 2: $C_{max,u}/EC_{50}$
- Method 3: AUC/EC_{50}

where AUC is the area under the curve of NCE from clinical pharmacokinetic study; F_2 is the *in vitro* test concentration leading to a 2-fold increase of mRNA; $C_{max,u}$ is the unbound maximal plasma concentration; EC_{50} is the concentration causing half-maximal induction effect *in vitro*.

If $AUC/EC_{50} \leq 0.005$, NCE will be considered with no *in vivo* induction potential. If ≥0.1, strong inducer was considered. For $C_{max,u}/EC_{50}$ values of ≤0.001, >0.001–≤0.01 and >0.01 would predict a NCE to be a weak, moderate and strong inducer, respectively [41,51].

3) Basic kinetic model: the value of R_3 could be calculated using this model, and ≤0.8 suggests potential induction *in vivo*.

If the above methods suggest NCE has the potential induction of metabolizing enzymes, follow-up evaluation is recommended to use mechanistic models including static mechanistic models and dynamic mechanistic models. A clinical DDI study with a sensitive index substrate will be the final one.

4) Prediction of the induction potential on other non-CYP enzymes: it has been reported that certain non-CYP450 enzymes such as UGT1A1/1A6 are induced through a mechanism similar to those of CYP450 enzymes. Therefore, the positive result of CYP induction assay can help to predict the potential induction risk of other enzymes [41].

5. The DDI potential of metabolites

There have been several clinical reports indicating that the metabolites of NCE also cause MMDDI, such as gemfibrozil metabolite gemfibrozil 1-O-β-glucuronide described before, finally resulted in the worldwide withdrawn of cerivastatin [4].

Therefore, the metabolites with significant plasma exposure or pharmacological activities was recommended to be evaluated for its MMDDI potential as a substrate or as a perpetrator of enzymes [3,7].

5.1. Metabolite as a substrate

When a pharmacologically active metabolite that contributes to ≥ 50% of the overall activity, its MMDDI potential as s substrate was recommended to be evaluated through *in vitro* or *in vivo* methods that are the same as those for the parent drugs [3,7].

5.2. Metabolite as an inhibitor

In vitro CYP450s inhibition study was recommended to be conducted if 1) the metabolite is less polar than the parent drug and the metabolite AUC ≥ 25% of the parent AUC, or 2) the metabolite is more polar than the parent drug and the metabolite AUC ≥ the parent AUC. It should be mentioned that if the metabolites with structural alerts for potential TDI, a lower cut-off value for the metabolite-to-parent AUC ratio may also be considered, and the AUC ratio was recommended to be determined by human mass balance study [3,7].

6. Case study

Ibrutinib is an irreversible Bruton's tyrosine kinase (BTK) inhibitor, which has been approved for the treatment of patients with mantle cell lymphorma (MCL) and chronic lymphocytic leukemia (CLL). The recommended dose of ibrutinib is 560 mg orally once daily in MCL and 420 mg once daily in CLL. The metabolism- and transporter-mediated DDI risks of both ibrutinib and its metabolites were evaluated according to the DDI Guidance. The MMDDI studies in different development stages are summarized in Fig. 2 [52-56].

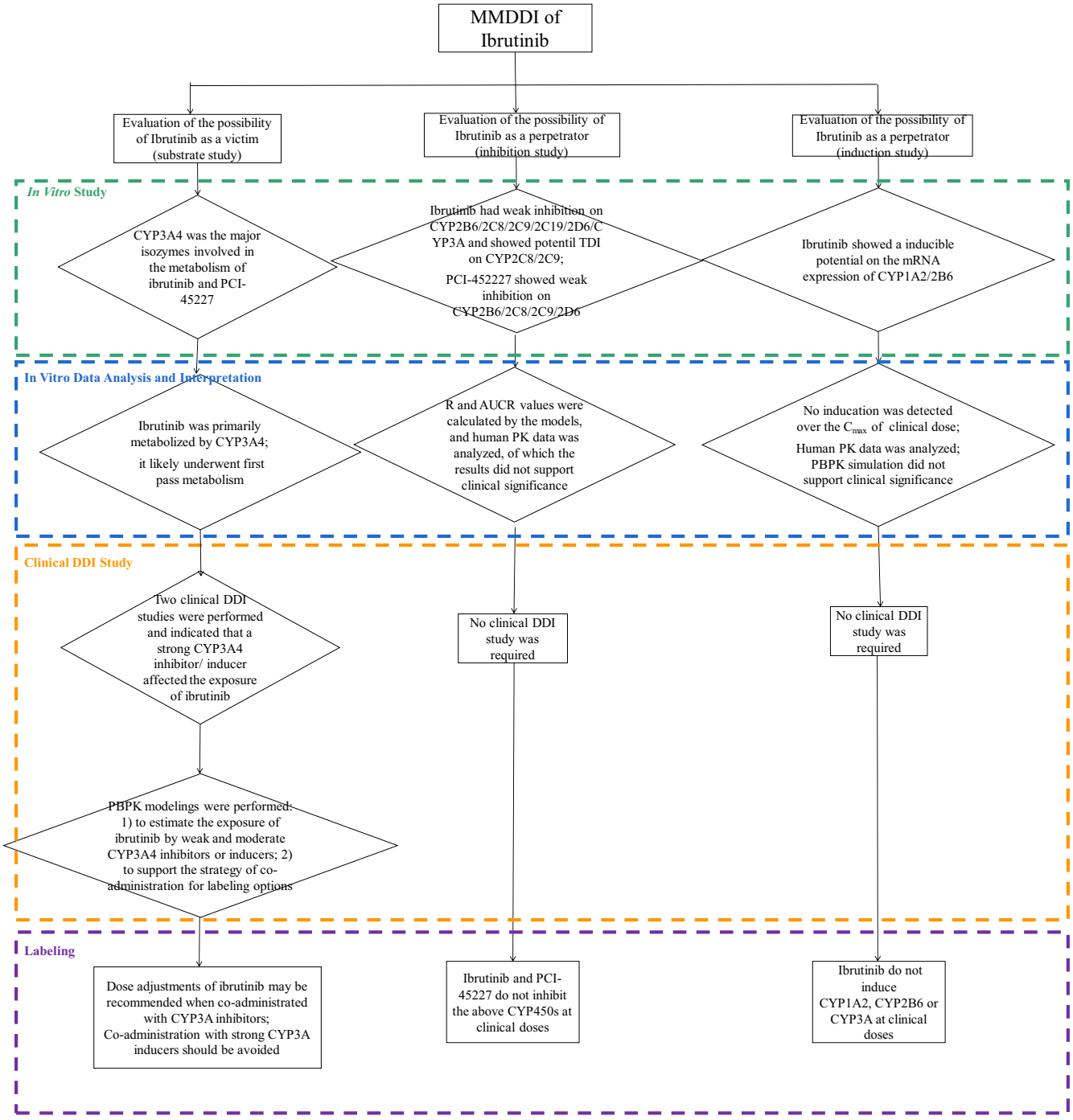

Fig. 2. The DDI evaluation of ibrutinib in different development stages.

6.1. Results of in vitro DDI study

Substrate study: *In vitro* substrate study indicated that CYP3A4 were the major isozymes involved in the metabolism of ibrutinib and its active metabolite PCI-45227 (M37) [53,54].

Inhibition study: The inhibitory effect of ibrutinib and PCI-45227 on the activity of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5 were investigated, of which the results showed that ibrutinib had no inhibitory effects on CYP1A2 and CYP2E1 while exerted weak inhibition on the rest of the CYP450s isozymes described above; PCI-45227 had no inhibition on the activity of CYP1A2, CYP2C19, CYP2E1 and CYP3A4 while it showed weak inhibition on the rest of the CYP450s isozymes tested [53,54].

TDI study: *In vitro* study suggested that ibrutinib had no TDI on either CYP1A2 or CYP3A4, while might have a relatively TDI on CYP1B6, CYP2C19 and CYP2D6 with the fold of shifted IC_{50} ranging from 1.11 to 1.40, and it showed a moderate TDI on both CYP2C8 and CYP2C9 with a 1.62- and 1.97-fold shifted IC_{50}, respectively; The TDI effect of PCI-45227 was not reported in submission reviews [53,54].

Induction study: A wide dosing range with high dosages (0.05, 0.2, 1 and 2 μM), which was > 50 folds of free C_{max} with repeated oral

doses, and twice daily dosing procedure was applied for ensuring effective concentrations because ibrutinib was subject to the significant first pass metabolism. The dosing concentration of ibrutinib was monitored at the final dosing for this purpose. The results showed that after being treated by ibrutinib, the mRNA expression of CYP1A2 in hepatocytes from one of three donors was increased to 140% of that of vehicle control (VC) at the concentrations over 0.5 μM, and the mRNA expression of CYP2B6 was increased to more than 2-fold of that of VC and 20% of that of positive controls (treated by known inducers) at the concentrations of 1 and 2 μM [54].

6.2. In vitro data analysis and interpretation

The *in vitro* data of inhibitory and inducible effect of ibrutinib were further analyzed by using the model introduced before and combining with the human PK data.

Substrate study: Ibrutinib was primarily metabolized by CYP3A4, and it likely underwent significant first pass metabolism with unchanged drug as less than 1% of the administered dose in feces, and human microsomal stability study showed that it was rapid with 66% metabolized within 10 min. Therefore, two clinical DDI studies coadministated with a strong CYP3A4 inducer and a CYP3A4 strong inhibitor, respectively, were performed [53,54].

Inhibition study: Considering that ibrutinib was identified as both a substrate and a weak inihibitor of CYP3A4, its potential risk of CYP3A4 inhibition was further evaluated using PBPK model. The PBPK simulation suggested that the C_{max} and AUC of midazolam (a probe substrate of CYP3A4), be increased to 1.15 folds by the co-administration of 560 mg ibrutinib, which is <1.25 folds, a cutoff value regulated by FDA DDI Guidance. Therefore, no significant clinical significance of CYP3A4 inhibition related DDI could be concluded [54]. Additionally, it should be mentioned that the inhibition constant K_i used for simulation, was reassessed based on ibrutinib levels measured at the end of the incubation because of its rapid metabolism in human microsomes [54].

TDI study: Based on the fold of shifted IC_{50}, ibrutinib might have a relatively significant TDI on CYP2C9 compared to the other isoforms, therefore, its TDI potential risk on CYP2C9 was further evaluated using different models. The details of the evaluation were as below:

Firstly, the R_2 value was determined as 3.5 using a basic model described in DDI guidances [3,7], which is morn than 1.25 folds, a cutoff value recommended by guidances, suggesing a clinical potential on CYP2C9. Here, it should be noted that the K_I and k_{inact} value used to predict the R_3 value was an adjusted value obtaiined by detecting the remaining parent concentrations after incubation rather than the designed dosing concentration.

Then, a static mechanistic model was used to further predict the exposure ratio (AUCR) of S-warfarin (a substrate of CYP2C9) in the presence or absence of ibrutinib. The AUCR value was determined as 1.7, which means the exposure of S-warfarin would be increased by 70% with the co-administration of ibrutinib, suggesting a potential clinical risk.

Finally, PBPK models were used to simulation the variety of C_{max} and AUC of S-warfarin in the presence or absence of ibrutinib. The simulation result showed that the C_{max} and AUC were increased by 1.02- and 1.04-fold, with the coadministration of ibrutinib, respectively, suggesting that ibrutinib had no serious effect on the clinical exposure of S-warfarin because the increased ratio was <1.25 folds, a cutoff value according to DDI guidances [3,7,54].

After all, because the PBPK simulation result indicated that ibrutinib had no clinical risk of TDI on CYP2C9, of which the fold of shifted IC_{50} was highest, it is could be considered that its TDI on the other isoforms including CYP2B6, CYP2C8, CYP2C19 and CYP2D6 might have no serious clinical risk [54].

On the other hand, although previous *in vitro* study showed that ibrutinib did not have TDI on CYP3A4, of which the data should be further interpreted by considering that rapid metabolism of the parent ibrutinib might cause false-negative result. According to the human PK data, there is no significant different between the exposure under repeated and single administration, which helps to confirm that ibrutinib does have no potential risk of TDI on CYP3A4 [54].

Induction study: The increase of CYP1A2 mRNA expression was only detected at the concentrations over 0.5 μM, which were over the C_{max} of clinical repeated dose, therefore, it was considered that ibrutinib had no apparent induction risk on CYP1A2 at clinical dose; The increase of CYP2B6 mRNA levels by ibrutinib was detected at the concentrations of 1 and 2 μM, which were over 50-fold of the free C_{max}. However, PBPK modeling simulation did not support clinical significance because a decreased AUC of bupropion (a substrate of CYP2B6) was not simulated by co-administration of ibrutinib with 560 mg daily dose [54].

Moreover, it should be mentioned that although previous *in vitro* study indicated that ibrutinib did not have inducible potential on CYP3A4, of which the data should be further interpreted by considering that rapid metabolism of the parent ibrutinib might cause false-negative result. The human PK data, which indicated that there was no apparent variety on the exposure between repeated and single dose, was helpful for considering that ibrutinib had no induction effect on CYP3A4 at clinical dose [54].

6.3. Clinical DDI study

6.3.1. The effect of strong inducers and inhibitors

In vitro studies in microsomes and recombinant CYP450s identified CYP3A as the major enzyme responsible for the metabolism of ibrutinib, which indicated that the exposure of ibrutinib might be affected when co-adminstrated with CYP3A4 inhibitors or inducers. Therefore, two clinical DDI studies were performed to assess the effect of ketoconazole (a strong CYP3A4 inhibitor) and rifampicin (a strong CYP3A4 inducer) on the pharmacokinetics of ibrutinib, respectively. The results of clinical DDI studies indicated that the co-administration of multiple doses of ketoconazole (400 mg QD) increased the C_{max} of single dose ibrutinib by 29 folds and AUC by 24 folds, and the coadministration of rifampicin (600 mg QD) decreased the C_{max} of ibrutinib by more than 13 folds and AUC by approximately 10 folds [52,53].

6.3.2. The effect of weak/moderate inducers and inhibitors

Considering the DDI risk detected in two clinical DDI studies, the sponsor used the PBPK modeling to estimate the exposure of ibrutinib by weak and moderate CYP3A4 inhibitors or inducers. The modeling followed three steps [53]:

Step 1-Model building: the model was built based on *in vitro* data and human PK studies.

Step 2-Model verification: the model was verified using data from two clinical DDI studies described in 6.3.1.

Step 3-Model predictions: The model was used to predict the effect of moderate CYP3A4 inhibitors like diltiazem and erythromycin, a weak CYP3A4 inhibitor fluvoxamine, and a moderate CYP3A4 inducer efavirenz, and CYP3A4 time-dependent inhibitor ritonavir on the exposure of ibrutinib.

Simulations of PBPK model suggested that diltiazem, erythromycin and ritonavir might increase the C_{max} of 560 mg ibrutinib by 4.1-, 6.4- and 24-fold, respectively, and AUC by 4.2-, 7.0- and 40-fold. Simulations of PBPK model suggested that efavirenz might decrease the C_{max} and AUC of 560 mg ibrutinib by 1.6 folds.

6.4. Labeling

Followed by the determination that the exposure of ibrutinib would be affected by the co-administration of CYP3A4 inhibitors or inducers, the sponsor used PBPK model to support the strategy of co-administration for labeling options: (1) to predict the effect of dosing

staggering and/or dose reduction on ibrutinib exposure co-administrated with strong and moderate CYP3A4 inhibitors; (2) to predict the effect of dose doubling or no dose adjustment on ibrutinib exposure co-administrated with strong or moderate CYP3A4 inducers, respectively [53]. The results of simulation were presented in the part of Dose Modifications in the Label [52].

The results of DDI studies including *in vitro* and clinical studies and PBPK modeling simulation were used to support the labeling, for Ibrutinib as a victim, which describes that dose adjustments of ibrutinib may be recommended when co-administrated with CYP3A inhibitors, and the co-administration with strong CYP3A inducers should be avoided; for ibrutinib as a perpetrator, which describes that ibrutinib and PCI-45227 are unlikely to inhibit CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 or 3A at clinical doses, and ibrutinib are unlikely to induce CYP1A2, CYP2B6 or CYP3A at clinical doses [52].

7. Special concerns

7.1. For therapeutic proteins

Because therapeutic proteins (TPs) are not eliminated by general drug metabolizing enzymes (such as CYP450s) and drug transporters, the risk of pharmacokinetic DDIs is lower for them. However, TPs have been reported to affect the disposition of drugs that are substrates of CYP450s by altering the expression CYP450s. In general, TPs that increase proinflammatory cytokine levels and proinflammatory cytokines can down-regulate the CYP450s expression, and may further reduce the metabolism of drugs. If the co-administered drugs are substrates of CYP450s, their exposure levels might be increased by TPs. Conversely, TPs that reduce cytokine levels might reduce the exposure of co-administered drugs that are substrates of CYP450s. Therefore, if necessary, the potential risk of MMDDI between a TP with proinflammatory cytokine-related mechanism and small molecular drugs that are substrates of CYP450s should be evaluated [57-59]. It also should be mentioned that because of the mechanism of TPs on the CYP450s expression are generally related to proinflammatory cytokine, the *in vitro* study methods that are applicable for small molecules introduced before, are generally not applicable to TPs. Compared with *in vitro* studies, *in vivo* studies are likely to be more suitable for TPs.

For example, risankizumab (ABBV-066) is an anti-IL-23 monoclonal antibody drug, is used to treat a variety of inflammatory diseases. Studies have shown that the inhibition of IL-23 will directly or indirectly affect the levels of its downstream inflammatory factors IL-17, IL-6, IL10, INF-γ and TNF-α, which may affect the expression of CYP450s. Taking into account the limitations of *in vitro* and animal experiments for the prediction, a clinical DDI trial was conducted to evaluate the potential effect of risankizumab on the activity of CYP1A2 (caffeine), CYP2C9 (warfarin), CYP2C19 (omeprazole), CYP2D6 (metoprolol) and CYP3A4 (midazolam) using a cocktail approach. The results showed that risankizumab had no clinically meaningful effect on the activities of these CYP450s [60,61].

7.2. For antibody–drug conjugates

Antibody–drug conjugates (ADC) is a unique class of therapeutic agent that consists of both a small molecule and a antibody component [62]. The DDI evaluation strategy of antibody is recommended to refer the DDI Guidance for TP [57], while the DDI potential consideration of small molecule component can refer the DDI guidances introduced in this review [3,5-7].

For instance, Fam-trastuzumab deruxtecan (DS8201a) is a HER2-directed antibody and topoisomerase I inhibitor conjugate, for the treatment of petients with HER2-positive breast cancer. As present in the submission reports, series of *in vitro* DDI studies of its small molecule drug, MAAA-1181a, were designed and performed completely [63].

7.3. Combined oral contraceptives

There are some special issues on MMDDI. For example, combined oral contraceptives (COCs) usually contain two synthetic steroid hormones, a progestin and an estrogen, both of which are substrates of CYP3A4. Therefore, the human pharmacokinetics of COCs would be affected if CYP3A4 was inhibited and/or induced by NCE. As required by FDA, clinical DDI study should be seriously considered in childbearing potential women if COCs will be co-administered with a NCE inhibiting or inducing CYP450s and having teratogenic potential for long term usage [64-66].

7.4. For herb medicines or ingredients

Because some herb medicines or their ingredients have been confirmed as the substrates, inhibitors, and/or inducers of CYP450s [67-70], metabolism mediated herb drug interaction (MMHDI) should also be evaluated carefully if necessary [7]. *In vitro* methods discussed in this review can be used for evaluating MMHDI and their clinical significance [7].

8. Summary

Co-medications plays more and more important role for preparing solutions for more serious challenges from human diseases. Therefore, more and more serious scientific and regulatory attentions have been paid to the evaluations and reviews of DDI, including TMDDI and MMDDI, by FDA, NMPA, PMDA, and EMA [3,5-7].

As consequence of DDI, the exposure of victim drug as C_{max} and AUC will be elevated by enzyme inhibition, and those reduced by enzyme induction, which will affect efficacy or toxicity of victim drugs, which clearly indicated that DDI do have potential effects on drug efficacy and safety.

This review article shared the authors' understanding and experiences on designing, conducting, and interpreting MMDDI studies for supporting discovery, IND/NDA registrations according to the related Guidance. Evaluation of MMDDI has become one of routine procedures for NCE's discovery and development, including both *in vitro* experiments and clinical studies, and will contribute greatly to the drug labeling, especially for co-medication therapies.

CRediT authorship contribution statement

Shujun Fu: Original draft writing, editing. **Feifei Yu:** Original draft writing, editing. **Zhuohan Hu:** Conceptualization, review. **Tao Sun:** Conceptualization, review.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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