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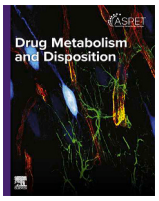

ARTICLE

# In vitro reversible enzyme inhibition assays to predict drug-drug interactions: Current state and industry perspective from the IQ Consortium Enzyme Inhibition Working Group

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## ABSTRACT

Inhibition of drug-metabolizing enzymes such as P450s and uridine 5′-diphosphoglucuronosyltransferase is routinely evaluated in drug discovery and development. Substantial efforts have been made over the years to standardize in vitro assay conditions and data interpretation. More recently, increasing importance has been given to the approaches for improving translation of in vitro data to predict in vivo outcomes. The International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) Enzyme Inhibition Working Group conducted a survey across IQ member companies to interrogate on the current state of inhibition assessment in the industry, including the strategies used as compounds progress through the pipeline, the kinetic endpoints utilized, and the accuracy of clinical drug-drug interaction predictions. Focus was also placed on how companies applied correction for unbound fraction of potential inhibitors as required by the various recent regulatory guidance documents. Results showed that most companies follow similar set-ups to identify strong inhibitors in discovery and to fully characterize drug candidates in development. Although there are some minor differences in evaluating and correcting for incubational binding, there is almost universal alignment regarding the use of kinetic endpoints with $K_i = IC_{50}/2$ widely accepted to determine the inhibition constant. A majority of predictions align with clinical drug-drug interaction results, although instances of over- or underprediction were reported, and examples of these are discussed here as case studies.

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*Significance Statement:* Practices utilized to assess reversible inhibition of drug-metabolizing enzymes were evaluated based on 25 survey responses from IQ member companies. Results show how assay conditions, endpoints, and modeling approaches evolve as compounds advance through discovery to development. The accuracy of drug-drug interaction prediction is discussed, and recommendations for best practices are provided.



## 1. Introduction

Drugs that substantially inhibit the enzyme(s) responsible for clearance of coadministered drugs are often referred to as precipitants (perpetrators) of a drug-drug interaction (DDI). The severity of the clinical DDI depends in part on the potency of the inhibitor, the therapeutic index of the victim drug, and the extent by which it is metabolized by the enzyme being inhibited (fraction metabolized, $f_m$). Cytochrome P450 (CYP, P450) and uridine 5′-diphosphoglucuronosyltransferase (UGT) enzymes are the major enzymes involved in metabolic drug clearance.[1] Drug candidates are routinely screened using in vitro assays for the potential to inhibit P450s, whereas UGT screening is often more limited. Assays for evaluating P450 inhibitors were initially developed in the 1990s, in the wake of adverse events including fatalities in individuals taking nonsedating antihistamines and strong P450 inhibitors.[2] Screening assays conducted in drug discovery are typically optimized for capacity and throughput. To facilitate those attributes, there can be concessions in the choice of probe substrate, enzyme source, protein and substrate concentrations, incubation duration, and inhibitor concentration range. As compounds progress to candidate selection, inhibition assays shift from high throughput to more definitive assays with higher degrees of translation and kinetic robustness. Regulatory authorities have issued multiple guidance documents pertaining to in vitro assays, and the first was issued in 1997. Guidance documents (from the Food and Drug Administration [FDA], European Medicines Agency [EMA], and International Council for Harmonization [ICH]) have become increasingly prescriptive over the years, incorporating key scientific advances and consensus-driven attributes, while still allowing flexibility in the design of in vitro assays. Such recommendations include use of highly selective P450-isoform probe substrates, correcting for fraction unbound, limiting organic solvent concentrations to below 1% volume/volume and preferably <0.5%, etc. As with all guidance documents, the overarching goal of recommendations is to ensure patient safety.

In vitro inhibition data sets with kinetic endpoints (eg, the half-maximal inhibitory concentration, $IC_{50}$, or the inhibition constant, $K_i$) are often used in early, predevelopment stages to assess the potential risks of a specific compound as a perpetrator, using static prediction models.[3] For more refined analysis, dynamic physiologically based pharmacokinetic (PBPK) models can be developed using the same in vitro data. With P450 isoforms, $IC_{50}$ values are apparent values, not intrinsic, because values can vary depending on assay conditions, such as choice of substrate, source of enzyme, total protein concentration, and incubation duration. Additionally, certain enzymes (eg, CYP3A4) are prone to substantial substrate-dependent inhibition responses.[4] $K_i$ values are usually obtained as mathematical derivations of the $IC_{50}$ value.[5] Since guidance documents permit flexibility, companies are free to develop individualized assay strategies. Nevertheless, all documents provide guidance to direct generation of robust and high-quality data needed to assess risk and form the basis of DDI predictions.

The Enzyme Inhibition Working Group (EIWG) representing 19 member companies of the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) has assessed the current state of the industry with regard to strategies across member companies to improve accuracy in translation of in vitro data to predictions of clinical DDI. The EIWG conducted a survey of all member companies in 2023 to assess the current approaches to identify P450 and UGT inhibitors and further evaluate the reliability of in vitro data in predicting clinical DDI. Although P450 inhibition assays and assay suites can interrogate time-dependent inhibition (TDI) concurrently, the focus of the EIWG was primarily on reversible inhibition. In this paper, the authors report the results of the survey and suggest recommendations for best practices for in vitro reversible inhibition assays and highlight case studies to provide examples.

## 2. Materials and methods

The blinded survey was organized into 4 sections with the purpose of establishing industry practices regarding in vitro P450 and UGT inhibition assay strategies, and use of experimental or calculated data in predictive modeling (Supplemental Appendix 1). The survey also sought to garner information regarding the accuracy of predicting outcomes of clinical DDI studies. Questions were generally multiple choice with some questions permitting more than one response. Many questions included text boxes to allow for elaboration. In the first section, companies were asked for details on assay design and how that might be modified as candidates' progress in the pipeline. For example, design elements favoring efficiency and throughput may be replaced by those favoring robustness and closer alignment with regulatory agency guidance documents. Respondents were asked whether $IC_{50}$ or $K_i$ values are determined, whether TDI is evaluated, and whether inhibition from circulating metabolites is assessed. In the second section, companies were asked whether correction for unbound fraction in the incubation ($f_{u,inc}$) is applied to the inhibition parameters and whether $f_{u,inc}$ is determined experimentally or predicted using in silico approaches, with elaboration permitted. The third section inquired on practices for mathematical derivation of $K_i$ values, if these were not directly determined experimentally. In the fourth section, companies were asked about reversible enzyme inhibition modeling practices (eg, basic static, mechanistic static, or PBPK).[3] In addition, companies were asked about the accuracy of their predictions based on clinical DDI trial outcomes covering the period from 2010 to 2023. The survey sought to quantify incidents of poor predictions from in vitro data and potential explanations. Additionally, the survey inquired whether any compounds were progressed despite predictions of strong reversible inhibition. The survey concluded with questions regarding the incidence of observed clinical interactions that may have resulted from P450 inhibition in the intestine instead of, or in addition to, in the liver. Of 30 member companies, 25 provided responses (Supplemental Appendix 1 for aggregated responses to

**Table 1**
Enzyme inhibition assay format used in discovery and development

| | Discovery (%) | Development (%) |
|---|---|---|
| **Single Test Compound (Inhibitor) Concentration** | | |
| Cocktail of probes only | 24 | 4 |
| Individual probes only | 16 | 4 |
| Both approaches | 12 | 8 |
| **Total** | **52** | **16** |
| **Multiple Test Compound (Inhibitor) Concentrations** | | |
| Cocktail of probes only | 24 | 0 |
| Individual probes only | 56 | 92 |
| Both approaches | 8 | 8 |
| **Total** | **88** | **100** |

questions, Supplemental Appendix 2 for individual responses). We elected to provide numerical counts of responses as percentages of the 25 respondents; therefore, dividing percentages by 4 will equate to absolute number of companies responding in the affirmative. Each company provided responses to each question, unless specified otherwise.

## 3. Results

### 3.1. In vitro enzyme inhibition assay strategy

In this section, questions were aimed at discerning assay design strategies as well as the staging of these assays. All respondents indicated that they conduct assays in both discovery and development phases. In the discovery phase, some companies indicated that multiple assay formats are deployed (Table 1). Roughly half of the respondents (52%) employ single test article concentration assays, with 24% using a cocktail of probe substrates; 16% incubating individual probes; and 12% using both formats. These data suggest that many companies incorporate cost/time saving strategies in the discovery phase. However, the most common approach (88%), even in discovery, is incubating multiple test compound concentrations (thereby permitting $IC_{50}$ value determinations), with the majority (56%) incubating a single P450-specific probe substrate, 24% using a cocktail of probe substrates, and 8% reporting that both approaches can be used. In contrast, during the development phase, all laboratories use individual enzyme probe substrate concentrations close to the $K_m$ value, with multiple test article concentrations, indicative of the classic $IC_{50}$ approach.

Although a variety of enzyme sources can be used to assess inhibition risk, human liver microsomes (HLMs) are used almost universally, both in discovery and development phases (100% and 96% of the respondents, respectively, Fig. 1). However, 15%–30% of respondents appear to complement HLM data by generating additional data using human hepatocytes and/or recombinant enzymes, with a higher frequency at the development stage. Based on free text responses, the latter systems are more likely to be used for special circumstances, such as incorporating effects of a permeability barrier (hepatocytes) or to ensure specificity for the tested enzyme (eg, recombinant enzymes for UGT inhibition).

Respondents were asked which P450 isoforms among the agency-recommended list (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A) are studied at various stages (Fig. 2A). In the discovery phase, 44% of laboratories examine all isoforms listed, whereas 84% include at least CYP1A2, 2C9, 2D6, and 3A4. Fewer respondents tested CYP2B6 (48%), CYP2C8 (72%), and CYP2C19 (76%). For CYP3A, midazolam is the preferred probe substrate, but roughly half (52%) of laboratories use both midazolam and testosterone. In 4% of cases, neither midazolam nor testosterone is used, and are instead replaced by luminogenic substrates of CYP3A4. All

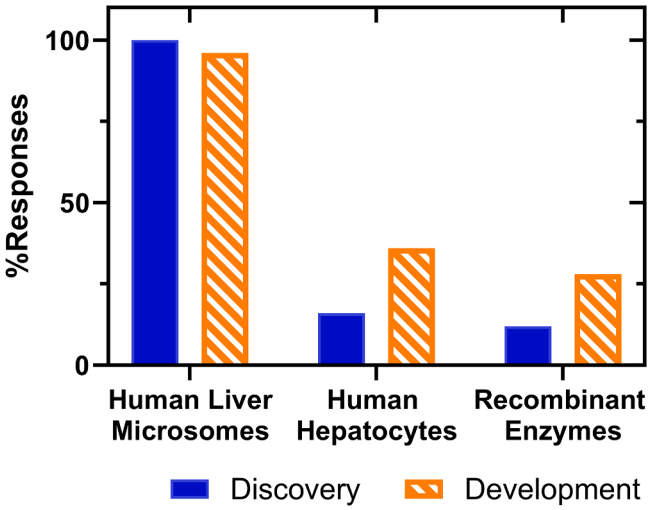


**Fig. 1.** In vitro enzyme systems used in the discovery and development phases for P450 inhibition assessment.

respondents assess CYP3A inhibition in the discovery stage, but few distinguish between CYP3A4 and CYP3A5 (only 12%). In the development phase, all laboratories surveyed assess inhibition for the major agency-recommended P450 isoforms. For CYP3A, both midazolam and testosterone are used as probes in almost all labs (96%) surveyed. Moreover, at this stage, 28% of the laboratories assess inhibition of CYP3A4 and CYP3A5 separately.

In the ICH M12 guideline, UGTs 1A1, 1A4, 1A9, 2B7, and 2B15, as well as the UGT isoforms metabolizing the test compound, are suggested as enzymes to evaluate for potential reversible inhibition when glucuronidation is a major elimination pathway. However, the M12 guideline was finalized after the survey was completed and results reflect practices likely driven by the preceding EMA guidance,[6] which suggests that only UGT1A1 and UGT2B7 (as well as UGTs involved in drug glucuronidation) should be evaluated (Fig. 2B). During the development phase, over 72% of respondents evaluated inhibition of UGT1A1 and UGT2B7, followed by UGT1A4 (52%), UGT1A9 (48%), and UGT1A6 (44%). In some cases, bovine serum albumin (BSA) has been reported to recover activity of select UGTs, likely through sequestering of otherwise inhibitory fatty acids that are released during preparation of in vitro matrices.[7,8] Previous work has established that, for certain UGTs, characterization of reversible inhibitors in the absence of BSA may result in overestimation of inhibition kinetic parameters.[9] Regarding the use of BSA in UGT inhibition studies, 16% of respondents run assays both with and without BSA.

Companies were asked about the timing for generation of inhibition parameters (eg, either $IC_{50}$ or $K_i$). During a typical hit-to-lead stage, 28% of respondents generate these parameters, whereas 72% generate values during the lead optimization stage. Nearly all respondents (92%) generated values by candidate selection or prior to clinical development.

Although TDI was not the focus of the survey, one question was asked on timing of TDI and reversible inhibition assays. Most respondents conduct TDI assays in parallel with direct inhibition assays in discovery (72%) and in development (92%). However, the breadth of P450 coverage for TDI assays may differ depending on the stage of the test compound. Multiple respondents commented that in discovery, TDI assays are limited to CYP3A whereas other major P450 isoforms are not tested until the development stage.

Laboratories were surveyed regarding evaluation of DDI potential of metabolites. All respondents evaluated the inhibitory

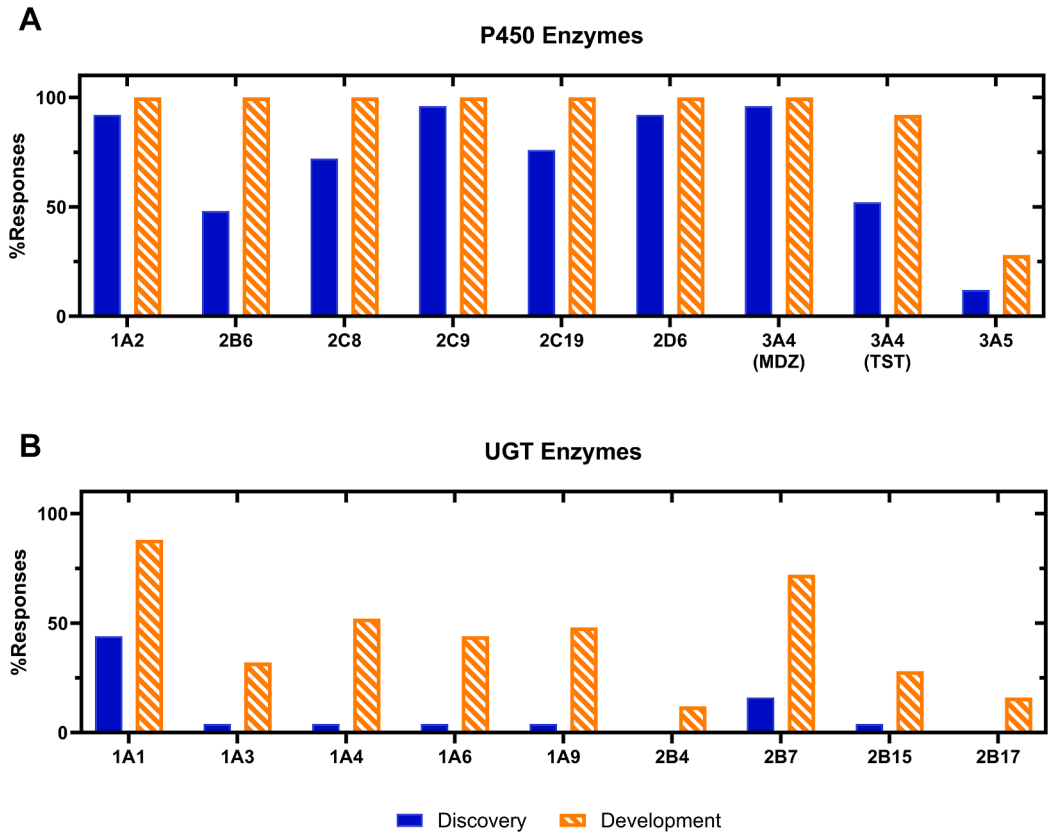


**Fig. 2.** P450 (A) and UGT (B) isoforms assessed in enzyme inhibition assays in discovery and development phases.

potential of circulating metabolites during the development phase. However, it was clear from the free text responses that the criterion for investigating metabolites is based on regulatory guidance, eg, inhibitory potential should be investigated for metabolites that have a total $AUC_{metabolite} \geq 25\%$ of $AUC_{parent}$ and account for at least 10% of drug-related material in circulation.[3] In the discovery phase, 32% of respondents evaluate the inhibitory potential of metabolites if deemed appropriate on a project-to-project basis but not routinely. Respondents mentioned that significant metabolism or the prediction of a specific metabolite circulating at appreciable levels were key factors for investigating metabolites in discovery.

### 3.2. Incubational binding

This section was aimed at understanding the current practices used for the determination or prediction of incubational binding of the test compound ($f_{u,inc}$) as this parameter is used to correct for the free fraction of compound in the assay. Questions probed whether $f_{u,inc}$ values were determined experimentally or predicted using in silico equations.[10,11] If determined experimentally, companies were then asked whether the assay is performed at the same protein concentration as the in vitro inhibitory $IC_{50}$ or $K_i$ assay. Since different enzyme matrices can be utilized to assess inhibitory potential (eg, liver microsomes, recombinant proteins, or hepatocytes), questions were designed to understand the methodology used for each matrix.

Responses received from the IQ survey regarding whether $f_{u,inc}$ values are measured or predicted are summarized in Table 2. Overall, 96% of the respondents indicated that some form of experimental or predictive $f_{u,inc}$ value is determined either at the discovery or development stages. Microsomes are the matrix of choice to use whether $f_{u,inc}$ is measured or predicted. At the discovery stage, $f_{u,inc}$ values are determined by 80% of the respondents and as compounds progress into development, this number increases to 92%. In discovery, of the 80% that actively determine $f_{u,inc}$, 24% report only measuring values experimentally, 16% use predictive tools only, and 40% indicated that both measurements and predictions can be employed. As compounds progress into development, 60% of the respondents experimentally measure $f_{u,inc}$, 12% using predictive tools only, and 20% report using both measurements and predictions.

Although microsomes are the preferred matrix, 56% of the respondents indicated that $f_{u,inc}$ values are also determined in hepatocytes, either experimentally (52%) or as predictions (24%), in discovery or development. Conversely, the use of recombinant enzymes as a matrix for $f_{u,inc}$ calculations appears to be selected only in a small minority of cases (as reported by 8% of respondents in discovery and 12% in development).

Regarding whether $f_{u,inc}$ values are measured under the same protein concentrations as the inhibition assays, of the 96% of companies reporting some measurement, the majority (56%) maintain consistent protein concentrations between incubation binding assays and inhibition assays when liver microsomes are used (Fig. 3). HLMs are the predominant test system, though recombinant enzymes are also utilized by 28% of the respondents. Of these, 16% keep consistent protein concentration in both binding and inhibition assays, whereas 12% of the companies use different protein concentrations. In addition, where hepatocytes are utilized to assess the potential for P450 inhibition (52% of overall respondents), 48% determine incubational binding using the same hepatocyte density, whereas 4% do not. Since microsomes are the

**Table 2**
Determination of fraction unbound in the incubation ($f_{u,inc}$) of enzyme inhibition assays, whether measured experimentally or predicted via in silico tools

| Overall | Microsomes | | | | Hepatocytes | | | | Recombinant Enzymes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 96% | | | | 56% | | | | 12% | | | |
| Discovery | Yes | 80% | Measured | 24% | Yes | 52% | Measured | 28% | Yes | 8% | Measured | 4% |
| | | | Predicted | 16% | | | Predicted | 16% | | | Predicted | 4% |
| | | | Both | 40% | | | Both | 8% | | | Both | 0% |
| | No | 20% | | | No | 48% | | | No | 92% | | |
| Development | Yes | 92% | Measured | 60% | Yes | 48% | Measured | 32% | Yes | 12% | Measured | 8% |
| | | | Predicted | 12% | | | Predicted | 8% | | | Predicted | 4% |
| | | | Both | 20% | | | Both | 8% | | | Both | 0% |
| | No | 8% | | | No | 52% | | | No | 88% | | |

matrix of choice, most respondents (84%) reported that equilibrium dialysis is the methodology typically used, although 20% reported that in some instances either ultracentrifugation or ultrafiltration can also be used. It was also surveyed whether companies assess nonspecific binding to plastics (eg, adsorption to plates or tubes) in drug discovery, and 100% of the respondents indicated that this was not assessed ($n = 23$ responses).

### 3.3. In vitro enzyme kinetics

Inhibition kinetic endpoints can be determined experimentally as $IC_{50}$ or as $K_i$ values. The latter is generally a more robust endpoint but is typically not determined until after prior determination of an $IC_{50}$ value and requires assessing inhibition by the test compound at multiple substrate and inhibitor concentrations. Companies were asked which enzyme inhibition endpoint is used for DDI risk assessment. The responses ($n = 23$) showed substantial overlap, indicating that companies may use multiple approaches. This is summarized in the Venn diagrams in Fig. 4. In the discovery phase, only 4% of the responding companies obtain $K_i$ values experimentally, but 56% respond that they use $K_i$ as the inhibition endpoint, indicating that 52% also calculate it from the $IC_{50}$ value. The $IC_{50}$ value is used as the endpoint by 72% of the companies, which also implies that several companies (36%) report using both $IC_{50}$ and $K_i$ values as endpoints. In the development phase, the number of companies that determine $K_i$ values experimentally increases to 36%. However, 88% of the companies use $K_i$ values (experimentally or calculated from $IC_{50}$) as inhibition endpoints, whereas $IC_{50}$ values are used by 48% of the companies.

The $K_i$ value can be obtained from an $IC_{50}$ value based on the principles of the Cheng-Prusoff equation, where $K_i = IC_{50}/(1+[S]/K_m)$ for competitive inhibitors.[5] Almost all (92%) companies calculate $K_i$ value by simply dividing $IC_{50}$ values by 2, indicating assay substrate concentrations are at or near the $K_m$ (Fig. 5). Some (12%) of the companies invoke the Cheng-Prusoff equation to incorporate the assay substrate concentration (eg, not assumed to be identical to the $K_m$ value) to derive the $K_i$ values. A smaller subset (8%) uses inhibition assays in which the probe substrate concentrations are much lower than the $K_m$ value ($[S] << K_m$) and calculate $K_i$ values as $K_i = IC_{50}$. No company uses an experimental setup in which the probe substrate concentrations are much higher than their $K_m$ values ($[S] >> K_m$) which would elevate $IC_{50}$ values and make the corresponding $K_i$ values calculated from the $IC_{50}/2$ rule too high.

### 3.4. Translation DDI risk prediction

#### 3.4.1. Hepatic reversible P450 inhibition

Drug developers utilize a range of models across discovery and development to predict the probability and magnitude of clinical DDIs. These range from the basic method (ie, $K_{i,u} > 50 \times C_{max,u}$), where compound data are limited, to mechanistic static models, and finally to dynamic PBPK modeling when a more comprehensive understanding of the compound properties is available. The reader is referred to ICH M12 section 7.5 for more context and other frequently used equations in this regard.

To further understand the assay formats employed, the survey was designed to query timing and use of various predictive models as compounds advance toward clinical development. In early discovery and before selection of a candidate molecule, most companies rely on basic static (84%) or mechanistic static models (48%), whereas only 36% of companies make use of PBPK modeling at this stage (Fig. 6). As expected, the survey highlighted that as a compound progresses through development, companies transition more to mechanistic static and PBPK modeling. After entering the clinical phase, 92% of companies apply PBPK modeling.

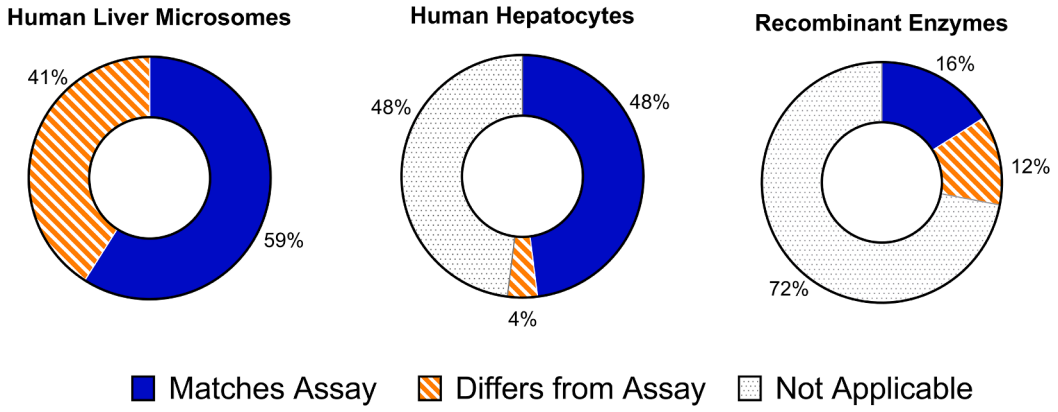


**Fig. 3.** Concentration of protein or cells utilized to assess incubational binding for enzyme inhibition assays.

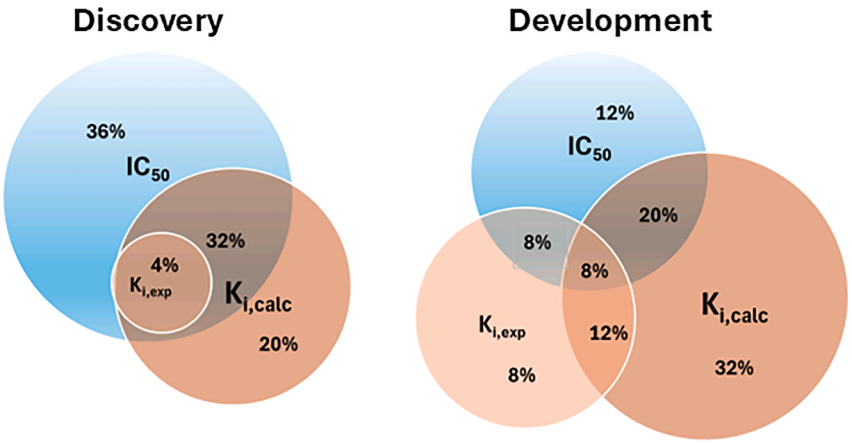


**Fig. 4.** Enzyme inhibition parameter endpoint utilized for DDI-risk assessment in drug discovery and development; overlap of circles indicate multiple approaches being used by same respondents.

Respondents noted that the use of PBPK was initially used to increase their understanding of perpetrator potential and later to support clinical DDI study designs or assist in justifying waivers for a clinical DDI study. Models were also used by some respondents to support absorption modeling or address special population considerations (Fig. 7).

The survey showed that 76% of respondents evaluated their candidates as reversible P450 inhibitors in the clinic during the period from 2010 to 2023. Of these, 60% evaluated up to 10 drug candidates clinically during this time frame, and 16% exceeded this number. The survey collated the total number of clinical DDI studies for enzyme inhibition completed by all respondents (173 studies), of which 153 (88%) had been correctly classified from predictions based on in vitro data. For the 20 studies which were not correctly predicted, there was a tendency to overestimate the DDI risk for predictions from basic static (67% of compounds) and static mechanistic models (50% of compounds, observed data was not available for 40% of compounds), whereas PBPK models underpredicted the DDI risk (100% of compounds) (Fig. 8). In 4 cases with incorrectly predicted outcomes, the respondent did not share the actual clinical outcome (R10-C1-4, Fig. 8). However, respondents shared that the maximum concentration levels ($C_{max}$) utilized in the static models were conservative, suggesting that these were additional examples of overpredictions. For 2 of the underpredictions, additional DDI mechanisms of TDI or formation of potent inhibitory metabolites had been observed after the model build. Another confounding aspect reported was down-regulation of enzyme activity and the specific challenges to describe the baseline activity of CYP2D6, where large variability in the exposure of dextromethorphan was not clearly related to poor or extensive metabolizers. Interestingly, half of the underpredictions observed with PBPK models occurred at the beginning of the survey window, between 2010 and 2012. Although we did not interrogate the quality of the PBPK model applied at the time of prediction, the commentaries that were included by the respondents indicated that PBPK modelling in the later period of the survey likely benefitted from continuous improvement of the models and wider familiarity of PBPK modeling across the industry. The fact that basic and mechanistic static models tended to overpredict clinical DDI risk in this survey is consistent with their application to flag risks early in the process while data gathering is not yet complete. Since 2010, respondents have noted a shift from utilizing basic static models to predict clinical DDIs by focusing on PBPK models.

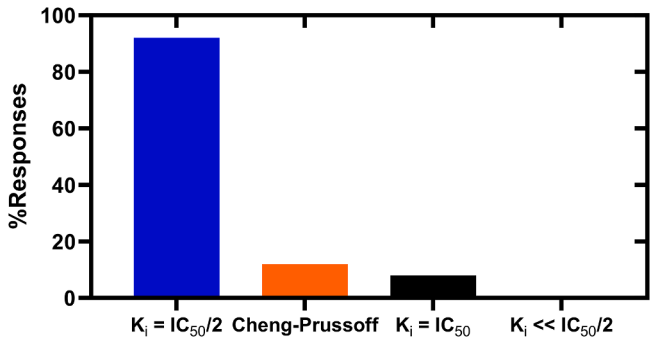


**Fig. 5.** Method utilized to calculate $K_i$ from $IC_{50}$.

Interestingly, 44% of companies progressed compounds from discovery into development despite findings of potent reversible P450 (all CYP3A) inhibition. Justification for progression was primarily reported as opportunities to address unmet medical needs (58%). Other reasons given were low confidence in DDI prediction at this stage (27%) or compounds being first or best in class (14%). It is important to note that the respondents highlighted that the DDI risks were reassessed in development.

### 3.4.2. *Intestinal reversible P450 inhibition*

All companies indicated they evaluate intestinal P450 inhibition, albeit indirectly. Although not assessed in the survey, we believe companies likely utilize the same P450 inhibition parameters generated in liver matrices as described above and do not measure inhibition in in vitro intestinal systems. The majority (92%) assess potential enzyme inhibition and DDI at the intestinal level incorporating inhibitor concentrations calculated as the dose/250 mL equation (ie, dose/250 mL/$K_{i,u}$ < 10), whereas 8% evaluated inhibition at concentrations up to the limit of solubility. A question was asked about observed clinical DDI interactions due to P450 inhibition in the intestine, instead of or in addition to the liver. Whereas the differentiation was unclear to 40% of the respondents, 32% observed an intestinal inhibitory interaction and were asked to elaborate. One respondent shared that they compared the oral and intravenous administration of probe substrate midazolam[12]; another cited a study with grapefruit juice versus ritonavir to differentiate between intestinal and total CYP3A inhibition. In another example, the systemic exposures with the tested doses were found to be too low to account for the interaction observed. In absence of these studies, respondents reported that they use mechanistic static or PBPK modeling and

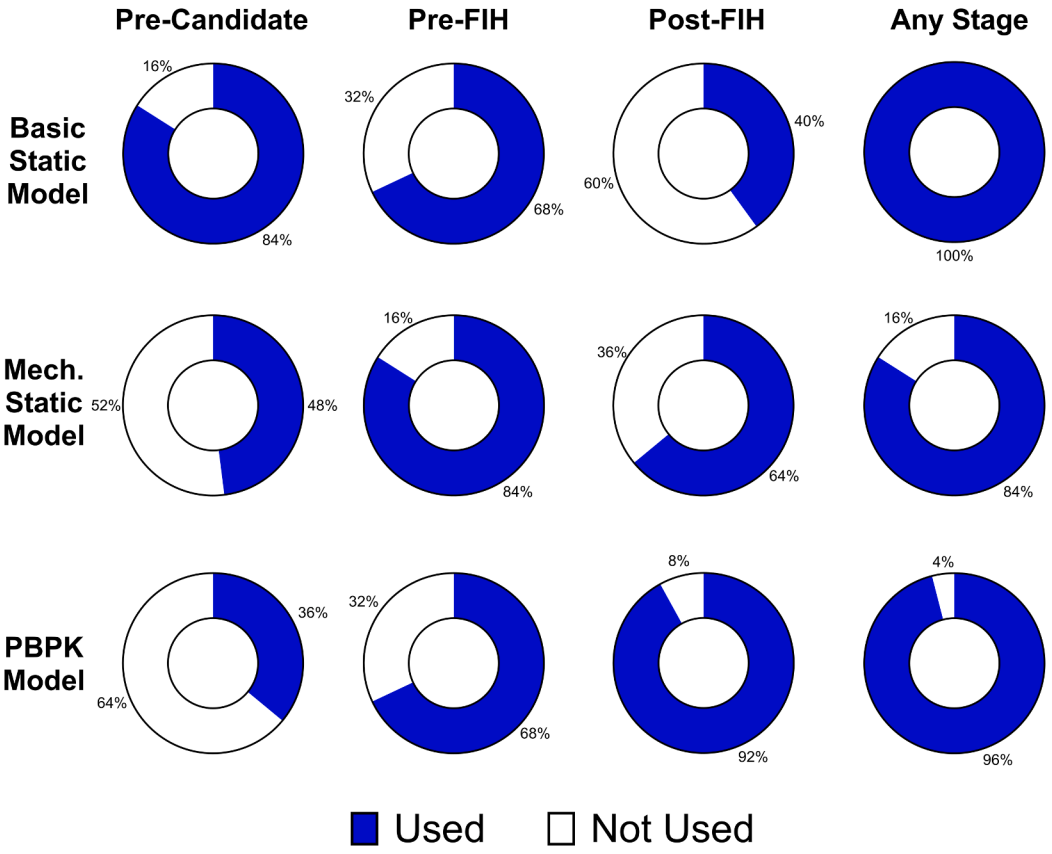


**Fig. 6.** DDI prediction models utilized throughout stages in drug discovery and development. (Multiple responses per respondent allowed. Pre-candidate, before candidate drug selection; Pre-FIH, before first in human study, Post-FIH, after first in human study).

evaluate the PK profiles to understand whether $C_{max}$ is impacted more than the elimination rate or half-life, suggesting that the interaction was mostly occurring at the intestinal level.

## 4. Discussion

### *4.1. In vitro enzyme inhibition assay strategy*

Assessing inhibition of P450 and UGT enzymes by drug candidates during the discovery and development stages is often conducted to determine the potential risk of DDIs that can affect the exposure of concomitant medications. Indeed, testing has been routine for most pharmaceutical companies for more than 2 decades and regulatory authorities have progressively fine-tuned the expectations of how pharmaceutical companies should present and interpret in vitro P450 inhibition data. It was not surprising therefore to observe consistency in the strategies adopted by the respondents to our survey.

The most common format reported at both discovery and development phases was determination of $IC_{50}$ values, using individual enzyme probe substrates with HLMs as an enzyme source. Seven P450 isoforms (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) are typically studied especially at the development phase with 2 substrates for CYP3A4 (usually midazolam and testosterone), to address potential substrate-dependent responses.[4] About half of the companies expand their panel routinely or “on a case-by-case" basis with additional isoforms (eg, CYP3A5, 2J2, 2A6, 2E1). Although rationale was not given, potential explanations for

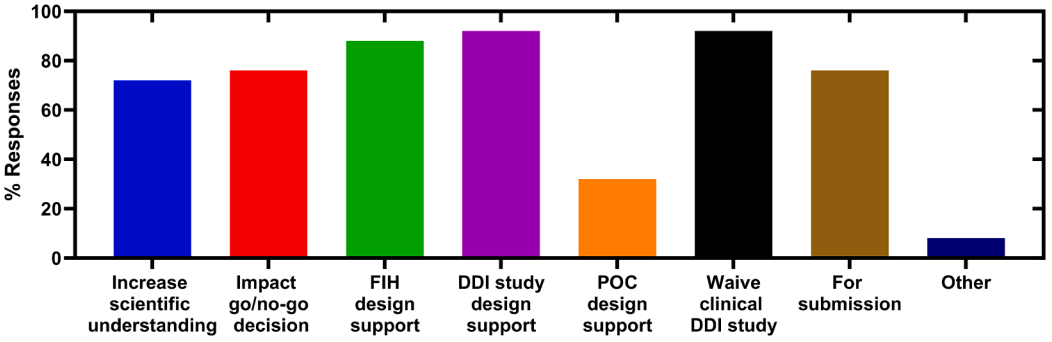


**Fig. 7.** Rationale for performing PBPK modeling.

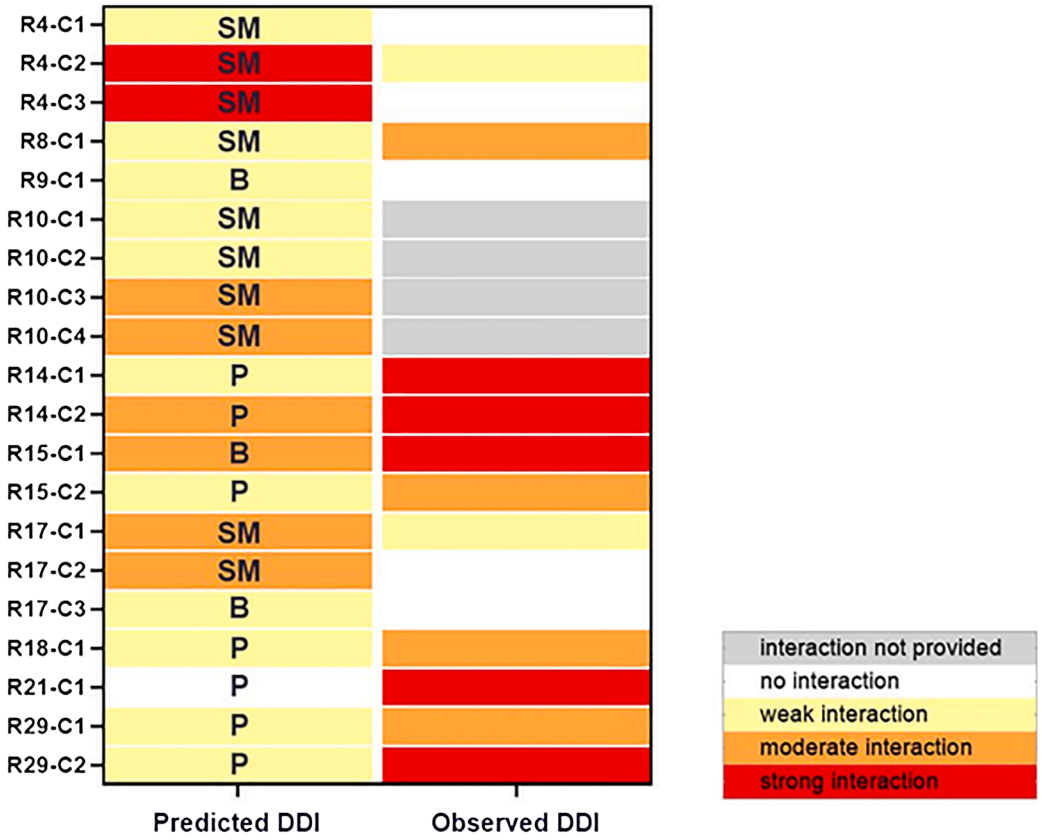


**Fig. 8.** Examples of mismatch between predicted DDI (left column, abbreviations indicate DDI prediction model utilized: B, basic model; SM, static mechanistic; P, PBPK) and observed DDI outcome (right column; depth of shading corresponds to the severity; cross-barring indicates that no information was provided by the respondent). R indicates respondent number; C indicates compound number – anonymized data preclude further interpretation.

testing these additional enzymes might include (1) a conservative strategy by the company, (2) test compounds were found to be substrates of those enzymes, or (3) test compounds with unusually low molecular weights (eg, <250), prompting potential interactions with CYP2A6 and CYP2E1 which are known to metabolize small molecular weight compounds. Three companies also tested nifedipine, either as a third CYP3A4 substrate in the panel or as an alternate to testosterone, likely because it may respond distinctly from midazolam and testosterone.[12–14]

Regarding UGT inhibition, following earlier EMA guidelines in cases when glucuronidation is a metabolic pathway, many respondents also tested for UGT1A1 and 2B7 inhibition, with fewer companies also assessing UGT1A4, 1A9, and 1A6. Whether or not UGT inhibition was tested also depended on chemotype, the extent of glucuronidation as a metabolic pathway, the contribution of specific UGTs in glucuronidation of drug candidates, and/or potential comedications. As the recently finalized M12 guideline indicates that testing for inhibition of UGT1A1, 1A4, 1A9, 2B7, and 2B15 should also be considered when investigational drugs exhibit significant glucuronidation, evaluation of these 5 UGTs is likely to increase. Although there is limited clinical data on UGT-mediated DDIs, this is an emerging area where further research would be valuable, as highlighted by recent reviews.[15,16]

Use of in silico models[17] to predict enzyme inhibition was prevalent, with 40% of respondents indicating that various technologies and platforms (TensorFlow, Python, SVM fingerprint or other in-house QSAR models) are used, primarily in early prediction and often prior to compound synthesis. Technological advancement in this area is obviously rapid and can be expected to substantially influence testing paradigms in the coming years. Emerging in silico models for DDI prediction are highlighted in the recent review by Handa et al.[18]

### 4.2. Incubational binding

According to the free drug hypothesis, only unbound drug is available for pharmacological activities including target binding, metabolism, and inhibitory effects on drug-metabolizing enzyme.[19] Although not without apparent contradictions,[20] the free drug hypothesis has become a well accepted concept, and regulatory and advisory agencies (including FDA, EMA, Pharmaceuticals and Medical Devices Agency, and ICH, based on guidance documents) are increasingly recognizing its utility in DDI risk assessment using in vitro data. For example, recent guidance documents from the FDA and the ICH now require the use of unbound plasma or tissue concentration data along with determination of incubational free drug concentration to estimate unbound inhibitory potency and assess DDI potential for drug candidates.[3] In turn, this has spurred the development of strategies to assess incubational binding and represented an area we wanted to investigate. Specifically, we sought to interrogate methods and potential for discrepancies in the estimation of incubational binding due to factors such as protein concentration variability, extrapolation from other matrices, predicted versus experimentally determined $f_{u,inc}$, etc. Most respondents apply $f_{u,inc}$ in microsomal and hepatocytes system for DDI assessment either by experimental measurement or in silico prediction of $f_{u,inc}$ at discovery and development phases while leaning more toward experimental data in the development phase (Table 2). Gardner et al[21] evaluated the prediction accuracy of $f_{u,inc}$ in liver

microsomes and hepatocytes using several commonly used computational prediction methods (such as Austin, Hallifax, and Houston, Turner & Poulin methods).[21] The authors described $f_{u,inc}$ data variability as a big challenge for the development of in silico prediction methods, since data are collected by different techniques and/or at different protein concentrations. Therefore, in silico methods were not fully predictable of experimental $f_{u,inc}$ values for entire chemical spaces. Based on the analysis, the authors recommended to use prediction methods early in discovery, but when more definitive data are needed the authors recommended experimental $f_{u,inc}$ data which is in good agreement with the responses from this survey for $f_{u,inc}$ determination and application for DDI estimation.

To the question as to whether $f_{u,inc}$ values are measured under the same protein concentrations as the inhibition assays, of the 96% of companies indicating some form of measurement, the majority (56%) reported using the same protein concentration in incubation binding assays when liver microsomes are used. Among the remaining companies, 24% of the respondents measure incubational binding at different concentrations, 8% use the dilution method, and 8% employ the Austin liver microsome fraction unbound prediction method (Fig. 3).[10] It is well established that nonspecific protein binding depends on protein concentration in the system. Mathematical equations had been developed and routinely applied to correct dilution of protein concentration to get undiluted $f_{u,inc}$: undiluted $f_u = (1/D)/[(1/\text{diluted } f_u - 1) + 1/D]$; D is dilution factor.[22,23] However, it would be appropriate strategy to use the same protein concentration or hepatocyte density for experimental $f_{u,inc}$ determination for good confidence in estimation of unbound potency and DDI potential for drug candidates. In line with this strategy, most IQ survey respondents use the same protein concentration or hepatocyte density if $f_{u,inc}$ is measured (Fig. 3). Overall, most companies are incorporating $f_{u,inc}$ in DDI assessment and when experimental $f_{u,inc}$ values are determined, most of the companies conduct experiments at the same protein concentration or hepatocyte density used in inhibition studies. This is considered appropriate best practices to better estimate the potential for DDI, especially for very highly bound drug candidates.

### 4.3. In vitro enzyme kinetics

Equations used to calculate the DDI risk by reversible P450 inhibition require the inhibition constant $K_i$ as an input parameter. However, the survey revealed that only a few companies determine this value experimentally (Fig. 4). Instead, most companies measure $IC_{50}$ values and then mathematically convert it to the $K_i$ value. The validity of this approach was demonstrated by Haupt et al[24] using a retrospective analysis of 343 experimentally derived $K_i$ and $IC_{50}$ values obtained within the same laboratory. In this study, 92% of $K_i$ values obtained from dividing $IC_{50}$ values by 2 were found to be within 2-fold of those determined experimentally, with only 1 value outside of 3-fold. Experiments were conducted with the probe substrate concentration equal to the $K_m$ of the reaction and following the Cheng-Prusoff equation (ie, $K_i = IC_{50}/2$ for competitive inhibitors).[5] In cases of noncompetitive inhibition, $K_i$ is equal to $IC_{50}$, and for mixed inhibition, $K_i$ is between $IC_{50}/2$ and $IC_{50}$.[24] Thus, calculating $K_i$ without knowing the inhibition mechanism may lead to underestimation of the $K_i$ value and overestimation of inhibition potency, and therefore represents a more conservative estimate of the DDI risk.

Almost all companies (96%) indicated that once a compound progresses into development, CYP3A inhibition is tested in vitro using 2 structurally different probe substrates, typically midazolam and testosterone. Therefore, when more than one substrate is used for the same enzyme, clinical DDI predictions are generated from the more potent $K_i$ value. However, in cases where testosterone CYP3A metabolism is inhibited more strongly than midazolam, direct translation into clinical DDI assessment is not possible because testosterone cannot be used as a clinical probe substrate. In these cases, a clinical DDI study might use midazolam as probe substrate acknowledging that this may not reflect the worst case or use a different CYP3A probe like triazolam or simvastatin[25] with less well understood relevance to the in vitro testosterone results. The ICH M12 guideline indicates that midazolam or triazolam can be used as appropriate CYP3A index substrates[3]; however, other substrates can be proposed and utilized.

Occasionally, when testing a new drug candidate for P450 inhibition in vitro, enzyme activation, ie, a concentration-dependent increase in probe substrate metabolite, rather than inhibition, is observed. This effect is often attributable to cooperative binding with substrate in the active site or to allosteric interaction at a site distinct from the active site.[13,26,27] Evidence of whether allostery occurs in humans in vivo is limited,[28] and therefore observations of occurrence in vitro are generally considered negligible in the clinic.

More insidious than reversible inhibition is TDI, where an increase in P450 enzyme inhibition potential is observed upon drug preincubation. This can manifest in vivo as enzyme destruction and persistence of DDI even after clearance of the precipitant drug. A comprehensive survey of industry practices to assess TDI was conducted by the Pharmaceutical Research Manufacturer's association and many of the common practices identified in 2009 are relevant and remain in place today and represented in the M12 guidance.[29] Nevertheless, an assessment of the most recent practices may represent an opportunity for a subsequent IQ survey.

### 4.4. Translation DDI risk prediction

As demonstrated in Fig. 8 and further supported by the comments, availability of data often dictates the timing of when prediction models will be utilized. For example, early in discovery, static models, while conservative in nature (as demonstrated by ~70% of cases overpredicted), are employed to provide initial guidance before more data are available to implement PBPK models. In this survey, 100% of the PBPK cases with mispredictions were due to underprediction. Unfortunately, with only 4 of 25 responders providing examples of PBPK DDI predictions and responses being blinded, the survey could not assess root causes. Some explanations proposed by responders included potential inhibitory contributions from metabolites or that substrate and inhibitor models were not properly verified with clinical data.

The high number of clinical DDI studies may be due to the size of the company's portfolio or historical ways of working where the tendency was to run DDI studies to refine the drug label. However, as demonstrated in the literature, the latter is now often replaced by modeling.[30] This is also in agreement with the responses noting that since 2010, there has been a shift from utilizing basic static models to predict clinical DDIs by focusing on PBPK models.[30]

Overpredictions by the static model are likely the result of the conservative nature of the equation (ie, utilization of a 50-fold multiple). The static model is based on $C_{max}$ and dose while the dynamic approach incorporates concentration changes over time as well as the incorporation of active metabolites and multiple inhibitors, resulting in a more informed DDI evaluation.

When predicting intestinal DDI potential, 92% of companies utilize the dose/250 mL as the input as per regulatory guidance. Companies are now expanding their targets to include classes of proteins once considered undruggable due to their large and complex functions[31] by pursuing research into new chemical

modalities. These new modalities are often larger and more lipophilic in nature and test the dynamic range of traditional absorption, distribution, metabolism, excretion screening approaches which might result in higher numbers of solubility limited evaluation of DDIs. It is possible that respondents predicting DDIs based on solubility limitations (6%) may be linked to the modalities they are working with. Possible DDI due to intestinal P450 inhibition rather than hepatic has been observed by 32% of respondents; however, 40% were unable to confirm the differentiation. In several examples, systemic exposures at tested doses appeared too low to fully account for the interaction. Often teams relied on mechanistic modeling and inspection of PK profiles—looking for a greater effect on the $C_{max}$ than on the area under the concentration-time curve (AUC) or half-life, as indicators that the interaction was predominantly intestinal.

### 4.5. Best practices recommendations

Accurate predictions of DDI can be challenging. Although significant progress has been made through increasingly complex, data-rich PBPK models, respondents still reported several cases where predictions were contrary to or did not match the outcomes in the follow-on clinical studies (Fig. 8). Best practices recommendations are provided below to help improve the continuous efforts in DDI translation and predictions based on the evolving regulatory guidance, emerging literature, and survey responses. To offer context, we have also identified case studies from the literature that highlight some of these best practices and show how predictions from the in vitro data compare with the results observed in clinical DDI studies.

#### 4.5.1. Ensure DDI assessment aligns with the most recent regulatory guidelines

It is indubitable that industry best practices will continue to evolve, even more so considering the impact artificial intelligence and machine learning models have started to have in the last few years since this survey was circulated. Industry guidelines (as the ICH M12) and other regulatory guidance documents have also evolved over the years, and while proposing to be "non-prescriptive," they have been universally adopted across the industry as the gold standard to follow in study design and data analysis. This has been widely confirmed by the results of this survey, and the authors of this manuscript strongly propose adherence to the most recent guidelines. The survey was conducted prior to the finalization of the ICH M12 guidelines, and we anticipate companies will modify their practices to align with these new guidelines if needed.

#### 4.5.2. Utilize a tiered approach to evaluate DDI risk

Basic and mechanistic static models are still widely used to assess potential risks at the earlier discovery and pre-new molecular entity stages. The basic model is conservative, as evidenced by ~70% of cases where utilization overpredicts DDI risk. If these early evaluations trigger a potential red flag, then more advanced, data-rich models clearly need to be implemented to further evaluate the risks and potential mitigation pathways. PBPK modeling can be used to further inform on the risk for clinically relevant inhibition.[32]

Example: Capivasertib, a kinase inhibitor, inhibited CYP2D6 in vitro with an $IC_{50}$ value of 5.3 $\mu$M. The basic static model suggested an in vivo DDI potential ($C_{max,u}/K_{i,u} = 0.304$, above the 0.02 cut-off value). However, PBPK modeling using desipramine, a sensitive CYP2D6 substrate, predicted that coadministration of capivasertib would increase desipramine AUC no more than 1.5-fold.[33] This classified capivasertib as a weak CYP2D6 inhibitor, and FDA waived the requirement for a clinical DDI study.

#### 4.5.3. Correct for incubational binding ($f_{u,inc}$) and plasma protein binding ($f_{u,p}$)

Survey results indicated that correcting drug binding in the in vitro assay is now standard practice, as required by the guideline to accurately assess DDI risk. However, the survey results did not show clear, conclusive consensus as to whether the free fraction should be measured experimentally or determined using predictive tools. There is no conclusive evidence that indicates one methodology is more reliable or accurate than the other, but further evaluation would be useful. Although the authors do not always advocate for $f_{u,inc}$ to be measured experimentally, determination of $f_{u,inc}$ at the same protein concentration as in the in vitro inhibition assay would be optimal. The measured $f_{u,p}$ should also be applied to calculate $C_{max,u}$. Historically, if drugs were 99% or more bound, agencies recommended $f_{u,p}$ be set at a fixed value of 0.01. In a departure from this precedent, the ICH M12 now allows utilization of $f_{u,p}$ values below 0.01 (assuming the method is sufficiently validated).[3] This is notable since this may reduce the number of required clinical DDI studies of highly bound drugs.

Example: Regorafenib, approved for the treatment of metastatic colorectal cancer, was found to be an in vitro reversible inhibitor of several P450 isoforms, but primarily CYP2C8 and CYP2C9, with calculated $K_i$ values of 0.6 and 4.7 $\mu$M, respectively, not corrected for binding. An N-oxide metabolite was also a potent CYP2C9 inhibitor with $K_i$ values of 0.8 $\mu$M.[34] When regorafenib was approved, regulatory guidance did not include correction for unbound fraction in plasma when using the basic static model, and in its older version, the risk for DDI was flagged. However, in subsequent clinical trials, daily dosing (160 mg) of regorafenib for 21 days showed no significant impact on the AUC of the CYP2C8 substrate, and a weak 1.25-fold increase in warfarin AUC (the sensitive CYP2C9 substrate). Therefore, regorafenib can increase the risk of bleeding when taken with warfarin due to a drug interaction that elevates the anticoagulant effects of warfarin.[35] More recent analyses suggest a low risk of DDI with warfarin via P450 inhibition with the parent drug regorafenib (AUC $\leq$1.2-fold), but potential contributions to DDI from the metabolite were not considered.[36] The potential contribution of the metabolite to the DDI with warfarin is unclear. Since subsequent plasma protein binding studies indicated very high binding for regorafenib (ie, 99.5%),[37] using the basic static model risk assessment under the current guidelines, which incorporate unbound plasma $C_{max,u}$ and incubational unbound $K_i$, the DDI risk would not have been flagged. This illustrates how incorporation of the protein binding values in the risk assessment could affect the decision whether to conduct a clinical DDI trial.

#### 4.5.4. Conduct in vitro inhibition assays with probe substrate concentrations at $K_m$ and utilize resulting $IC_{50}$ values to calculate $K_i$

Most early-stage models, whether static models or more advanced mechanistic models, are predicated on kinetic data generated in vitro. As the ICH guideline indicates, predictive equations compare the $K_i$ value corrected for incubational binding with the maximum unbound systemic concentration to determine whether a potential DDI risk is flagged. However, accurate, experimental $K_i$ determinations are both labor and time intensive, and therefore it has been widely accepted that the $K_i$ value can be estimated from the more readily determined $IC_{50}$ values, as discussed previously in the *Enzyme Kinetics* section. This also leads to a derived $K_i$ value that is the most conservative (ie, lowest), allowing for more stringent predictions and should be the approach to follow even as compounds transition into development.

*4.5.5. Understand your model assumptions and clinical population*

PBPK modeling of DDI risks is improving with more sophisticated approaches and can be implemented as a compound progresses into development and the clinic. PBPK modeling is not without its challenges though, and verification of the model is critical.[38] Newly built models are likely improve over time as additional in vitro and in vivo data are incorporated.

Example: Dacomitinib, a tyrosine kinase inhibitor, was characterized in vitro as a potent CYP2D6 inhibitor, with an unbound $K_i$ value of 16 nM. Given the 45 mg daily steady-state $C_{max,u}$ value of 4.4 nM (108 ng/mL; $f_{u,p}$ 0.0192), the basic static model predicted the possibility of DDI risk when dacomitinib is coadministered with a sensitive CYP2D6 substrate ($1+C_{max,u}/K_{i,u} = 1.28$). Initial simulations in Simcyp (V17) using dextromethorphan as the substrate predicted an AUC increase of 1.2-fold, but in a subsequent clinical trial the observed AUC increase was actually 9.6-fold.[39,40] This unexpected difference was later attributed in part to the high variability in baseline dextromethorphan AUC, stemming from CYP2D6 polymorphism, possible intestinal interactions, and the high clearance of dextromethorphan.[40] These findings contributed to subsequent Simcyp (V18/V19) updates of population CYP2D6 enzyme abundance values from meta-analyses, eventually resulting in the Simcyp (V24) baseline dextromethorphan model that more accurately reflect CYP2D6 phenotypes (I. Gardner, personal communication, December 10, 2025). Additionally, this study highlights how crucial it is to consider each patient's phenotype separately from their genotype when modeling DDI interactions and in determining if dose adjustments are needed for CYP2D6 substrates with a narrow therapeutic index during coadministration in clinical settings.

## 5. Conclusion

Since the late 1990s, health agencies have issued guidance documents to guide the pharmaceutical industry regarding the development of safer medicines with fewer potential DDIs. To this end, we sought to review and understand the methods currently in use to identify and advance such drugs through the early phases of evaluation and clinical development. In vitro inhibition assays that consider protein binding, consistent with the free drug hypothesis, are prevalent during early discovery although inhibition kinetics endpoints dominate in the development phase. We also attempted to understand the prevalence of the various methods used to predict clinical DDI from the aforementioned in vitro data and present case studies where the in vitro to in vivo translation is lacking. It is our hope that this report serves to inform broadly on current industry practices for conducting reversible inhibition studies while also providing consensus commentary from this IQ working group on optimum experimental and data analyses approaches.

## Abbreviations

AUC, area under the concentration-time curve; BSA, bovine serum albumin; DDI, drug-drug interaction; EIWG, Enzyme Inhibition Working Group; EMA, European Medicines Agency; FDA, Food and Drug Administration; $f_{u,inc}$, fraction unbound in incubation; ICH, International Council for Harmonization; HLM, human liver microsome; IQ, International Consortium for Innovation and Quality in Pharmaceutical Development; PBPK, physiologically based pharmacokinetic; TDI, time-dependent inhibition; UGT, uridine 5′-diphosphoglucuronosyltransferase.

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## Conflict of interest

The authors declare no conflicts of interest.

## Data availability

The authors declare that all the data supporting the findings of this study are available within the paper and Supplemental Material.

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## Supplemental material

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