Egypt. J. Chem. Vol. 67, SI: M. R. Mahran, pp. **1507 - 1517** (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Biochemical Pathways in Drug-Drug Interactions: A Pharmacological Perspective for Enhanced Drug Safety and Efficacy-Cytochrome Inhibition Mechanisms

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Abstract

Background: Cytochrome P450 (CYP) enzymes play a pivotal role in the metabolism of many drugs, and their inhibition can lead to significant drug-drug interactions (DDIs). Understanding the biochemical pathways involved in CYP inhibition is essential for improving drug safety and efficacy. This review explores the mechanisms of CYP inhibition, reaction phenotyping, and predictive models used to assess drug interactions, with a focus on high-throughput screening, probe assays, and modeling approaches.

Aim: This review aims to provide a comprehensive understanding of CYP inhibition mechanisms, with an emphasis on assessing and predicting drug-drug interactions. It examines in vitro methods, the implications of CYP inhibition in clinical practice, and the use of predictive models to identify potential drug interactions early in the drug development process.

Methods: We conducted a literature review on current methodologies for assessing CYP inhibition, including early high-throughput screening using fluorescent and luminescent assays, probe assays with human liver microsomes (HLM), and model-based approaches. Emphasis was placed on the validation of these methods, limitations associated with each approach, and their predictive capabilities. The use of CYP inhibition assays in combination with predictive modeling techniques, such as ligand- and structure-based models, was also explored.

Results: The review highlighted several methods for assessing CYP inhibition, including the use of cocktail assays, recombinant enzymes, and high-throughput screening. Despite the advantages of these techniques, challenges remain in ensuring substrate selectivity and overcoming limitations such as metabolic pathway complexity and the potential for non-specific inhibition. The application of predictive models, using databases and structural simulations, was found to offer promising tools for early DDI prediction and risk assessment.

Conclusion: Understanding CYP inhibition mechanisms is crucial for optimizing drug safety and efficacy. High-throughput screening, probe assays, and predictive modeling techniques provide valuable insights into potential DDIs. However, challenges remain in refining these methods to enhance their predictive accuracy and applicability in clinical settings. Continued advancements in these areas are essential for improving drug development processes and minimizing adverse drug interactions.

Keywords: Cytochrome P450, drug-drug interactions, CYP inhibition, high-throughput screening, probe assays, predictive modeling, drug safety, pharmacokinetics.

1. Introduction

Drug interactions are a significant problem, and the increasing use of polypharmacy in clinical settings poses a continuing difficulty [1]. When a perpetrator drug changes the plasma concentrations of a victim drug by either blocking or promoting its metabolism or transport, these interactions—known as drug-drug interactions, or DDIs—usually occur [1,2]. DDIs may thereby alter the pharmacokinetic (PK) profile, which could result in unexpected toxicities

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Receive Date: 14 November 2024, Revise Date: 06 December 2024, Accept Date: 10 December 2024
DOI: 10.21608/ejchem.2024.336321.10804

and/or decreased efficacy. Elevated values of the area under the curve (AUC), maximum plasma concentration (Cmax), and half-life (t1/2) can occur when enzymes or transporters inhibit the removal of drugs [3]. Conversely, induction tends to lower t1/2, Cmax, and AUC. The need to assess DDI potential during drug development and post-marketing surveillance is highlighted by the numerous medications that have been taken off the market as a result of side effects associated with DDIs, such as terfenadine, mibefradil, cisapride, and nefazodone [1,4]. There are three phases involved in determining a drug's DDI potential [5]. Finding the main metabolic routes by which the medication is eliminated from the body is the first stage. The second is assessing how transporters and enzymes that break down drugs affect how the medication is disposed of. Understanding how the medication may affect the way these enzymes and transporters function is the final step in the process. When evaluating DDIs, pharmacokinetic interaction studies concentrate on the drug's connection with metabolizing enzymes because many medicinal medicines undergo hepatic metabolism [2,6]. The oxidative biotransformation of 70–80% of commercially available medications is catalyzed by cytochrome P450 (CYP) enzymes, a superfamily of enzymes that are mostly found in the liver but are also found in the intestines, kidneys, lungs, and brain [7]. The most common types of the 57 functioning human CYPs in the liver are CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5, which are commonly implicated in drug metabolism and DDIs [2,7]. Because of safety concerns about these interactions, regulatory bodies frequently update DDI study criteria, especially with relation to CYP enzymes. Recommendations on test systems, probe substrates, and positive controls are included in these guidelines [2]. The Food and Drug Administration (FDA) of the United States released an updated guideline in 2020 called "In vitro Drug Interaction Studies-Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions." Several modeling strategies can be used to extrapolate in vitro results to in vivo predictions or clinical research designs [2,5].

Reaction Phenotyping

Overview of Reaction Phenotyping:

It is crucial to establish whether a drug candidate is a substrate for a Cytochrome P450 (CYP) enzyme before administering it to patients, in order to avoid potential drug-drug interactions (DDIs) that could have clinical implications. Additionally, understanding the primary enzymes involved in metabolism can provide valuable insights into pharmacogenetic factors, disease states, or environmental influences that might affect drug metabolism [68]. Reaction phenotyping is a widely utilized in vitro method designed to identify the specific enzymes and pathways responsible for the metabolism of a drug [69,70,71,72]. This approach is essential for evaluating the potential for DDIs, as certain metabolic pathways may compete for the same enzymes. The U.S. Food and Drug Administration (FDA) recommends this process for industry sponsors in its guidelines [5]. One of the initial steps in determining the necessity of clinical DDI studies is to understand the contributions of CYP enzymes to the overall metabolism of a drug. The primary objectives of reaction phenotyping are to: (1) quantify the proportion of the drug metabolized by each CYP enzyme involved in metabolic clearance, (2) characterize the enzyme kinetics and other relevant in vitro parameters, and (3) conduct early screenings for potential DDIs [68,69,73,74,75].

Fraction Metabolized (fm):

The fraction metabolized (fm) represents the extent to which a drug is metabolized by a specific enzyme in the liver. This value is unique to each enzyme and substrate. A high fm value (greater than 0.9) indicates that a single enzyme is predominantly responsible for the metabolism of the drug, which can raise concerns regarding DDIs, especially if the primary route of elimination for the compound involves metabolism. To mitigate this risk, the pharmaceutical industry generally aims to reduce the fm value of a compound by introducing structural modifications. Reaction phenotyping techniques, explained further in subsequent sections, are used to determine the fm value, which is a critical aspect of evaluating a drug's susceptibility to DDIs [74].

In Vitro Pharmacokinetic Parameters:

Evaluating in vitro pharmacokinetic (PK) parameters is essential for understanding the in vivo effects of a drug [2,76]. At steady state, the concentration of the enzyme-substrate complex remains constant, and any variations in substrate concentration are considered negligible [76]. This assumption forms the basis for maintaining minimal substrate turnover during experiments aimed at determining enzyme kinetic parameters. In these experiments, it is assumed that the substrate concentration greatly exceeds the enzyme concentration, making any fluctuations in substrate levels due to complex formation insignificant. Therefore, it is important to keep enzyme concentrations as low as possible to meet this requirement [76]. Key in vitro PK parameters include the maximum velocity (Vmax), the substrate concentration at half-maximal enzyme velocity (Km), intrinsic clearance (CLint), the half-life (t1/2), and the fraction of metabolic clearance (fCL) attributed to specific pathways [11]. It is vital to measure the unbound drug concentration since only the unbound form is pharmacologically active and capable of crossing cell membranes. Although total drug concentration is typically measured, determining the unbound drug concentration is often more challenging. For drugs with high protein binding, FDA guidance suggests using a default unbound fraction in plasma of

1% (fu,p = 0.01), unless experimental data indicates a lower value. Additionally, intrinsic clearance can also be evaluated through substrate depletion experiments using microsomes or hepatocytes [78].

When evaluating pharmacokinetic (PK) drug-drug interactions (DDIs), a common method involves calculating a ratio that accounts for the fraction of metabolism (fm), the inhibitor concentration ([I]), and the inhibition constant (KI) to predict the potential for DDIs. As fm increases, the likelihood of a DDI also rises, underlining the importance of determining the fm value in assessing DDI risks. The FDA recommends conducting in vivo clinical studies when a single enzyme is responsible for 25% or more of a drug's clearance. However, the primary pathways of drug clearance are best determined through in vivo radiolabeled studies [74]. There are three main strategies for reaction phenotyping: (1) selective inhibition using chemical inhibitors or antibodies, (2) recombinant CYP (rCYP) enzyme panels, and (3) correlation analyses with CYP activities in human liver microsomes (HLM) from different donors [69]. These methods can be used independently or in combination, with results often compared to improve accuracy and agreement [80,81]. The chemical inhibition approach involves using specific inhibitors in human-derived in vitro hepatic systems, with the inhibitor's selectivity for a target enzyme being a crucial factor. Chemical inhibitors recommended by the FDA are selected for their potency and specificity [82]. Additionally, inhibitory antibodies can be employed, although they may not always achieve maximal inhibition [84]. The recombinant CYP panel method involves incubating a set of rCYP enzymes with the drug of interest, applying a scaling factor to account for total liver metabolism rather than just the individual enzymes. The scaling factor adjusts for differences between the recombinant enzymes and human liver microsomes, using either the intersystem extrapolation factor (ISEF) or relative activity factor (RAF) [73,85]. These scaling factors, however, can vary depending on the probe substrate used. Lastly, the correlation analysis approach assesses the relationship between the rate of metabolite formation and the marker activity of specific CYP enzymes in a panel of HLMs from various donors [2]. While this method is useful for identifying significant enzyme contributions, it does not provide fm values and is typically employed when a single enzyme's contribution is substantial [80].

Qualitative-then-Quantitative Approach:

Recently, a novel approach was presented that combines the utilization of a recombinant cytochrome P450 (rCYP) panel with specific chemical inhibitors at different concentrations in pooled human liver microsomes (HLM) in a sequential manner [75]. To qualitatively determine which CYP enzymes are able to convert the parent drug into metabolites of interest, this qualitative-then-quantitative method starts with a rCYP screening panel. Following this first qualitative stage, the medication is incubated with progressively higher quantities of selective inhibitors that target the identified CYP enzymes, enabling the measurement of the degree of inhibition. This methodology is different from earlier approaches, which usually used one or two methodologies and compared the results to see if they agreed. To get more accurate estimates of the percentage metabolized (fm), it uses a pre-selected group of CYPs that are known to metabolize the drug, followed by the use of selective inhibitors to measure the fraction of metabolic clearance (fCL) through particular pathways. By avoiding overestimating the contribution of specific CYP enzymes to total metabolism, the qualitative-then-quantitative technique provides more accurate estimates of fm. It also discusses the inconsistencies that may occur when fm values derived from various response phenotyping techniques do not match. Studies looking at the metabolism of the antibiotic linezolid have used this method, identifying more intricate metabolic processes and involving more CYP enzymes than were previously known [75,87]. typically implicated CYPs (CYP1A2, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) as well as less typically found enzymes (CYP1A1, 2A6, 2B6, 2E1, and 4A11) were included in Wyndalda et al.'s enlarged rCYP screening [88]. A finding not previously reported in the literature, this wider rCYP screening revealed that CYP2J2, 4F2, and 1B1 also metabolize linezolid [87]. The precise contributions of each CYP were ascertained by employing selective inhibitors for each of the discovered enzymes, proving the usefulness of the qualitative-then-quantitative method in revealing hitherto unnoticed enzymatic contributions to drug metabolism.

A number of additional strategies can be applied in addition to the ones previously mentioned. Because they provide more exact data than non-labeled drugs, radioactive ([14C]-labeled) compounds are frequently utilized in vitro systems and provide a more accurate assessment of fm and scaling factors (RAF/ISEF) during pre-clinical research [81]. Bioanalytical investigations are frequently needed to validate results when non-radiolabeled substances are utilized [80]. Moreover, in vitro-to-in vivo extrapolation (IVIVE), a difficult component of forecasting in vivo clearance and fm, is being improved by machine learning techniques. The enzyme contribution ratios involved in metabolism can be predicted from a compound's structure, as recent research has shown, and these predictions closely match in vitro results [89]. Other machine learning models have been created to forecast in vivo clearance from in vitro data. For example, one model used chemical structures, ionization, logP, and other in vitro parameters to successfully predict intravenous clearance values for 16 Pfizer compounds based on data from over 600 molecules [90]. In order to forecast drug-drug interaction (DDI) potential, such with ketoconazole, before clinical trials, SIMCYP®

software has also been used to estimate the fraction and rate of metabolism of compounds using in vitro data [91]. These modeling frameworks, which are based on in vitro data, can be used to anticipate the disposition of drugs prior to first-in-human trials and provide useful insights for directing the planning and scheduling of clinical DDI investigations.

It is important to consider the limitations of reaction phenotyping approaches notwithstanding their advantages. The lack of specificity of certain inhibitors is a major issue with the selective inhibitor method [58,92,93]. By taking into consideration overlapping inhibition profiles, a six-parameter inhibition curve fitting approach has been suggested as a way to address this problem and produce more precise estimates of enzyme contributions [73]. The use of hepatocytes in suspension, which have a short incubation time of 4–6 hours [95,96], and HLM systems, which normally retain activity for only 1–2 hours after thawing [94], present another drawback. Studying low-turnover compounds with prolonged in vitro half-lives is made more difficult by this. With better turnover rates for slower metabolized substances like tolbutamide and alprazolam when compared to suspended hepatocytes, new models like the HepatoPac® co-culture system have demonstrated promise in getting around this restriction [97]. When assessed in this model, the fm values for 10 of the 13 CYP3A4 substrates were found to be within two times their in vivo values in a different investigation [98]. As a result, alternative validated systems can effectively address the shortcomings of HLM systems. Furthermore, the microsomal system itself is restricted since it does not contain cytosolic enzymes like aldehyde oxidase (AO), monoamine oxidase (MAO), xanthine oxidase (XO), and alcohol dehydrogenase (ADH) and only contains enzymes that are found in the endoplasmic reticulum membrane, such as CYPs, flavin-containing monooxygenase (FMO), and UDP-glucuronyltransferases (UGTs) [5]. Relying exclusively on a microsomal system may ignore the roles that cytosolic enzymes play in metabolism, even if they are rarely the main enzymes responsible for drug metabolism [101].

CYP Inhibition:

Mechanisms of CYP Inhibition:

The assessment of a drug's potential to inhibit cytochrome P450 (CYP) enzymes is an intricate process that begins early in the preclinical stages of drug development. CYP enzymes feature both active and allosteric sites capable of binding multiple ligands, which may serve as substrates, inhibitors, or activators. CYP inhibition can be broadly categorized into reversible, quasi-irreversible, and irreversible inhibition.

Reversible Inhibition:

Competitive, non-competitive, uncompetitive, and mixed competitive/non-competitive inhibition are the four main categories of reversible inhibition [102]. After the inhibitor separates from the active or allosteric site, enzyme function is restored in each instance. Therefore, the inhibitor's elimination of half-life determines how long reversible inhibition lasts in vivo. The equilibrium dissociation constant (Ki) controls how a reversible inhibitor separates from an enzyme. Competitive inhibition, in which two compounds "compete" for binding to the same active site on a CYP enzyme, is the most often observed and well-understood type of reversible inhibition. When a competitive inhibitor is present, this competition decreases the enzyme's availability to metabolize a medication [102,103]. While the reaction's maximum velocity (Vmax) is unaltered, the competition for the active site raises the Michaelis constant (Km) [104]. If an inhibitor attaches to an allosteric site without a substrate occupying the active site, this is known as non-competitive inhibition [102]. After non-competitive binding, substrates can still attach to the active site, but the resulting enzyme-substrate-inhibitor complex is no longer active [103]. Km stays the same while Vmax decreases as a result of inhibition because non-competitive inhibitors do not impact substrate binding [102,104]. Only when the enzyme is already attached to a substrate, creating an enzyme-substrate complex, can an uncompetitive inhibitor bind to an allosteric site; it has no affinity for the free enzyme [102,103]. The enzyme-substrate-inhibitor combination is rendered inactive, similar to non-competitive inhibition. As the reaction gets closer to equilibrium, uncompetitive inhibitors lower Vmax by reducing the amount of functional enzyme-substrate complexes, which lowers Km [102].

A specific kind of non-competitive inhibition known as mixed competitive/non-competitive inhibition occurs when the inhibitor's affinity for the allosteric site varies based on whether a substrate is bound in the active site, which is indicated by a factor α [102]. The inhibitor dissociation kinetics must be described using two terms: αKi for dissociation from the enzyme-substrate-inhibitor complex and Ki for dissociation from the enzyme-inhibitor complex [102]. As a result, mixed inhibitors lower Vmax and, depending on the value of α, may raise or lower Km [102]. Classic Michaelis-Menten enzyme kinetic tests, which measure Vmax and Km both with and without the inhibitor, can be used to experimentally identify the type of reversible inhibition imposed by an inhibitor [102,104]. By plotting the inverse of the metabolite synthesis rate against the inverse of the substrate concentration, the outcomes of these studies can be displayed on a Lineweaver-Burk plot. As seen in **Figure 1**, each type of reversible inhibition causes a distinctive change in the Michaelis-Menten and Lineweaver-Burk plots. In the past, changes in slope (equivalent to Km/Vmax), x-intercept (equivalent to −1/Km), and y-intercept (corresponding to 1/Vmax) on the Lineweaver-Burk plot were used to visually

estimate enzyme kinetic constants and the mechanism of inhibition [104]. Nonlinear regression, which fits the Michaelis-Menten model directly to non-transformed data, is now the most popular and accurate technique for calculating kinetic constants [102]. The model that best fits the experimental data (shown by the equations in **Figure 1**) can then be used to identify the type of inhibition. For these investigations, statistical software like GraphPad Prism is frequently utilized.

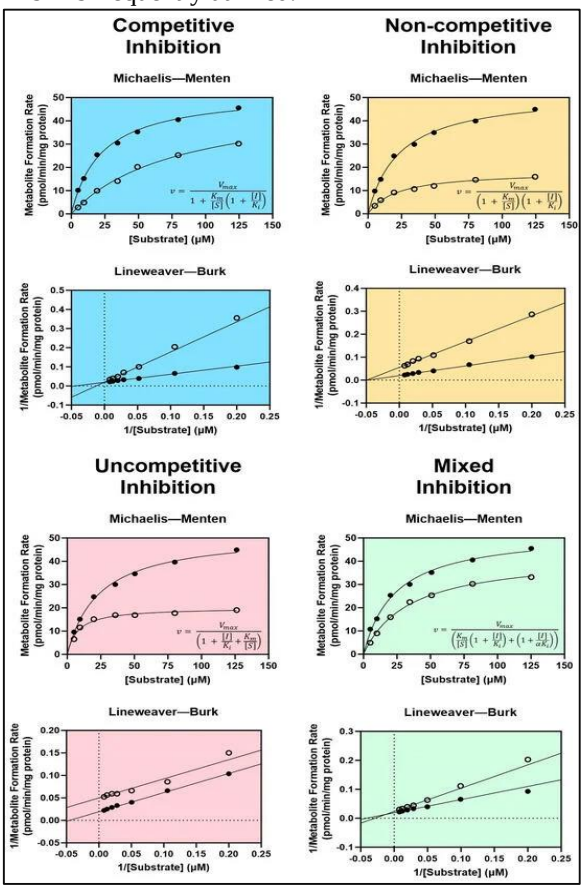

Figure 1: Enzyme kinetics of reversible inhibition.

Before performing enzyme kinetic experiments for inhibition, experimental conditions must be optimized. The linearity of metabolite synthesis with respect to both incubation time and protein concentration must be established beforehand in order to guarantee precise measurement of kinetic parameters. The substrate concentration used in these studies should be close to the Km. To reduce substrate depletion, the shortest incubation period and the lowest protein concentration within the linear range have to be chosen [102]. Moreover, the inhibitor's concentration need to correspond with the CYP active site's in vivo concentration [102].

Differentiating Forms of Inhibition

The degree of inhibition, whether reversible, quasi-irreversible, or irreversible, can be measured and differentiated experimentally based on specific enzyme kinetic tests and the nature of the interaction between the inhibitor and the CYP enzyme. Here are some key methods:

- **Reversible Inhibition**: The R1 value, calculated as R1=1+Imax,/Ku is used to assess the potential for drug interactions via reversible inhibition. If R1≥1.02R_1 \geq 1.02R1≥1.02, further studies, such as predictive modeling or clinical DDI studies, are recommended. The R1 value is calculated using the maximal unbound plasma concentration of the interacting drug (Imax,u) and the unbound dissociation constant (Ki,u).
- **Time-Dependent Inhibition**: Time-dependent and mechanism-based inhibitors are distinguished by preincubating the inhibitor with the CYP enzyme before adding the substrate. This preincubation step reveals whether the inhibition is time-dependent, with time-dependent inhibitors showing increasing inhibition with longer preincubation times. The "dilution method" is typically employed, where enzyme-inhibitor complexes are diluted into a second incubation containing the substrate. The kinact and Ki values are calculated, allowing for the assessment of time-dependent inhibition.
- **Quasi-Irreversible Inhibition**: Some inhibitors form metabolite intermediate complexes that remain bound to the enzyme's heme group under physiologic conditions, a process known as quasi-irreversible inhibition. Recovery of enzyme activity can sometimes be achieved using specific reagents like potassium ferricyanide, which helps displace the metabolic intermediate and restore enzyme activity.
- **Irreversible Inhibition**: Irreversible inhibition, often referred to as mechanism-based or suicide inhibition, occurs when an inhibitor permanently inactivates the CYP enzyme, typically through covalent binding to the heme group or the apoprotein. In some cases, this inhibition can be reversible, and enzyme activity can be restored over time as the affected tissue synthesizes new CYP enzymes. The recovery half-life for CYP enzymes is typically 20-50 hours, depending on the enzyme and the inhibitor.

3.1.5. Predictive Models and Clinical Implications

To determine the clinical relevance of CYP inhibition, further investigation is necessary when the R1 or R2 values exceed certain thresholds. The FDA recommends predictive modeling or clinical DDI studies to assess the impact of potential drug-drug interactions (DDIs) in vivo.

- **R2 Value**: For time-dependent inhibition, the R2 value is calculated as R2=(kobs+kdeg)/ kdeg where kobs represents the observed

inactivation rate, and kdeg is the constant enzyme degradation rate. If R2≥1.25R_2 \geq 1.25R2≥1.25, it indicates a significant risk for drug interactions, necessitating further investigation using clinical DDI studies or model-based predictions. These kinetic models and experimental approaches are critical in drug development, particularly when assessing the potential for DDIs and ensuring the safe and effective use of medications that may alter CYP enzyme activity.

Methods for Assessing CYP Inhibition

Early High-Throughput Screening:

High-throughput screening (HTS) techniques, including fluorescent and luminescent assays, are used to assess the inhibitory potential of drugs in the early stages of development. These assays typically use recombinant cytochrome P450 enzymes (rCYPs) in a 96-well plate format, where a pro-fluorescent or pro-luminescent substrate is metabolized to generate detectable signals. The half-maximal inhibitory concentration (IC50) is calculated from the inhibitor concentration that reduces signal by 50% compared to control reactions. These assays offer high throughput and sensitivity, though they are generally limited by the non-selectivity of the substrates for individual CYP enzymes. Moreover, substrates must generate specific fluorescent signals to avoid nonspecific interference. To improve throughput and broaden the scope of inhibition studies, cocktail assays with multiple selective CYP substrates are used. These are often combined with LC-MS for metabolite profiling, such as the Basel cocktail which includes substrates like caffeine (CYP1A2), efavirenz (CYP2B6), and midazolam (CYP3A). Additionally, radiolabeled substrates can also be used to detect metabolites through solid phase extraction or scintillation proximity assays.

Probe Assays for CYP Inhibition:

After initial screening, more detailed probe assays using human liver microsomes (HLM) are conducted. These assays typically involve the use of validated CYP substrates that serve as markers for each CYP enzyme. The U.S. FDA has published a list of index substrates, inhibitors, and inducers for various CYP enzymes to aid in DDI screening. However, probe assays are not without limitations. Many substrates are not perfectly selective for a single CYP enzyme, and the presence of multiple metabolites can complicate interpretation, especially if the inhibitor is also metabolized by the same enzymes. To minimize potential confounding effects, alternative substrates may be used, or recombinant enzymes can be employed. While recombinant enzymes provide high specificity, they may not fully represent the complexity of the microsomal system, which contains additional drug-metabolizing enzymes that may affect results. Therefore, after screening with recombinant enzymes, further validation with microsomal assays is typically recommended. For CYP3A, which has a flexible active site, inhibition should ideally be tested using multiple marker reactions, such as midazolam 1′-hydroxylation and testosterone 6β-hydroxylation. In clinical DDI studies, an ideal index substrate should exhibit a measurable increase in exposure when co-administered with an inhibitor. A strong inhibitor should increase the AUC (area under the curve) of a sensitive substrate by at least five-fold, while moderately sensitive substrates should show a two- to five-fold increase in AUC.

Model-Based Approaches for Predicting CYP Inhibition:

The advancement of high-throughput screening has also led to the use of predictive modeling to assess CYP-mediated drug-drug interactions (DDIs). These models can be used early in the drug development process and offer a low-cost approach to assess many compounds, even before they are synthesized. Predictive models can be developed using ligand-based or structure-based methods and validated using external datasets of known CYP substrates and inhibitors. In ligand-based models, large chemical databases are screened for compounds that may bind to and inhibit CYP enzymes based on quantitative structure-activity relationships (QSAR). Structure-based models, on the other hand, use 3D protein structures of CYP enzymes obtained through techniques like X-ray crystallography or NMR and predict binding interactions via docking simulations. Both approaches utilize statistical and machine learning techniques, such as multiple linear regression, to predict the inhibitory potential of compounds. These model-based methods allow for rapid and cost-effective DDI assessments, which are particularly useful for compounds that have not yet been synthesized or tested in vitro. By using computational predictions, researchers can identify potential CYP interactions early in the drug development pipeline and prioritize candidates for further testing.

Conclusion:

Cytochrome P450 (CYP) enzymes are integral to drug metabolism and are involved in many drug-drug interactions (DDIs) that can alter drug efficacy and safety. Inhibition of these enzymes, whether through reversible or irreversible mechanisms, can significantly impact pharmacokinetics and lead to adverse outcomes. The understanding of CYP inhibition mechanisms and their assessment is crucial for minimizing these risks and optimizing therapeutic strategies. This review highlights the different methods used to assess CYP inhibition, such as early high-throughput screening, probe assays, and model-based approaches. High-throughput screening offers a rapid means of testing drug interactions and can assess multiple CYP enzymes in parallel. However, challenges such as substrate selectivity and the complexity of metabolic

pathways remain. Probe assays with human liver microsomes (HLM) are considered the industry standard for measuring CYP inhibition but still face limitations, particularly with substrates that are not fully selective for specific CYP enzymes. These assays also require careful consideration of the metabolic pathways involved, as inhibitors may also affect the metabolism of the substrate itself. Predictive models have become an invaluable tool for DDI prediction, allowing for early assessment of potential interactions before clinical trials. These models, which include ligand-based and structure-based approaches, use large databases and advanced computational techniques to predict the inhibitory effects of compounds on CYP enzymes. While promising, the use of predictive models must be refined to improve their accuracy and applicability across different drug classes and therapeutic contexts. In conclusion, the development of more accurate and reliable methods for assessing CYP inhibition is essential for the future of drug development and personalized medicine. Combining high-throughput assays, probe tests, and predictive models will enable more effective risk management of DDIs, enhancing drug safety and efficacy. Continued research and innovation in these areas will drive better clinical outcomes and inform regulatory guidelines for drug development.

References:

1. Tornio, A.; Filppula, A.M.; Niemi, M.; Backman, J.T. Clinical Studies on Drug-Drug Interactions Involving Metabolism and Transport: Methodology, Pitfalls, and Interpretation. *Clin. Pharmacol. Ther.* **2019**, *105*, 1345–1361.
2. Lu, C.; Di, L. In vitro and in vivo methods to assess pharmacokinetic drug- drug interactions in drug discovery and development. *Biopharm. Drug Dispos.* **2020**, *41*, 3–31.
3. Sinz, M.; Wallace, G.; Sahi, J. Current industrial practices in assessing CYP450 enzyme induction: Preclinical and clinical. *AAPS J.* **2008**, *10*, 391–400.
4. Wienkers, L.C.; Heath, T.G. Predicting in vivo drug interactions from in vitro drug discovery data. *Nat. Rev. Drug Discov.* **2005**, *4*, 825–833.
5. *In Vitro Drug Interaction Studies—Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry*; U.S. Food and Drug Administration: Silver Spring, MD, USA, 2020.
6. Sun, L.; Mi, K.; Hou, Y.; Hui, T.; Zhang, L.; Tao, Y.; Liu, Z.; Huang, L. Pharmacokinetic and Pharmacodynamic Drug-Drug Interactions: Research Methods and Applications. *Metabolites* **2023**, *13*, 897.
7. Zanger, U.M.; Schwab, M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol. Ther.* **2013**, *138*, 103–141.
8. Yoshida, K.; Maeda, K.; Sugiyama, Y. Hepatic and intestinal drug transporters: Prediction of pharmacokinetic effects caused by drug-drug interactions and genetic polymorphisms. *Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 581–612.
9. Kot, M.; Daniel, W.A. The relative contribution of human cytochrome P450 isoforms to the four caffeine oxidation pathways: An in vitro comparative study with cDNA-expressed P450s including CYP2C isoforms. *Biochem. Pharmacol.* **2008**, *76*, 543–551.
10. Wojcikowski, J.; Daniel, W.A. Perazine at therapeutic drug concentrations inhibits human cytochrome P450 isoenzyme 1A2 (CYP1A2) and caffeine metabolism—An in vitro study. *Pharmacol. Rep.* **2009**, *61*, 851–858.
11. Wojcikowski, J.; Danek, P.J.; Basinska-Ziobron, A.; Puklo, R.; Daniel, W.A. In vitro inhibition of human cytochrome P450 enzymes by the novel atypical antipsychotic drug asenapine: A prediction of possible drug-drug interactions. *Pharmacol. Rep.* **2020**, *72*, 612–621.
12. Takahashi, E.; Fujita, K.; Kamataki, T.; Arimoto-Kobayashi, S.; Okamoto, K.; Negishi, T. Inhibition of human cytochrome P450 1B1, 1A1 and 1A2 by antigenotoxic compounds, purpurin and alizarin. *Mutat. Res.* **2002**, *508*, 147–156.
13. Niwa, T.; Inoue-Yamamoto, S.; Shiraga, T.; Takagi, A. Effect of antifungal drugs on cytochrome P450 (CYP) 1A2, CYP2D6, and CYP2E1 activities in human liver microsomes. *Biol. Pharm. Bull.* **2005**, *28*, 1813–1816.
14. Uehara, S.; Murayama, N.; Higuchi, Y.; Yoneda, N.; Yamazaki, H.; Suemizu, H. Comparison of mouse and human cytochrome P450 mediated-drug metabolising activities in hepatic and extrahepatic microsomes. *Xenobiotica* **2022**, *52*, 229–239.
15. Burnham, E.A.; Abouda, A.A.; Bissada, J.E.; Nardone-White, D.T.; Beers, J.L.; Lee, J.; Vergne, M.J.; Jackson, K.D. Interindividual Variability in Cytochrome P450 3A and 1A Activity Influences Sunitinib Metabolism and Bioactivation. *Chem. Res. Toxicol.* **2022**, *35*, 792–806.
16. Guo, J.; Zhu, X.; Badawy, S.; Ihsan, A.; Liu, Z.; Xie, C.; Wang, X. Metabolism and Mechanism of Human Cytochrome P450 Enzyme 1A2. *Curr. Drug Metab.* **2021**, *22*, 40–49.
17. Lee, K.S.; Kim, S.K. Direct and metabolism-dependent cytochrome P450 inhibition assays for evaluating drug-drug interactions. *J. Appl. Toxicol.* **2013**, *33*, 100–108
18. Walsky, R.L.; Obach, R.S. Validated assays for human cytochrome P450 activities. *Drug Metab. Dispos.* **2004**, *32*, 647–660.
19. McDonald, M.G.; Au, N.T.; Rettie, A.E. P450-Based Drug-Drug Interactions of Amiodarone and its Metabolites: Diversity of Inhibitory

Mechanisms. *Drug Metab. Dispos.* **2015**, *43*, 1661–1669.
20. Dinger, J.; Woods, C.; Brandt, S.D.; Meyer, M.R.; Maurer, H.H. Cytochrome P450 inhibition potential of new psychoactive substances of the tryptamine class. *Toxicol. Lett.* **2016**, *241*, 82–94.
21. Shih, H.; Pickwell, G.V.; Guenette, D.K.; Bilir, B.; Quattrochi, L.C. Species differences in hepatocyte induction of CYP1A1 and CYP1A2 by omeprazole. *Hum. Exp. Toxicol.* **1999**, *18*, 95–105.
22. Martinez, C.; Albet, C.; Agundez, J.A.; Herrero, E.; Carrillo, J.A.; Marquez, M.; Benitez, J.; Ortiz, J.A. Comparative in vitro and in vivo inhibition of cytochrome P450 CYP1A2, CYP2D6, and CYP3A by H2-receptor antagonists. *Clin. Pharmacol. Ther.* **1999**, *65*, 369–376.
23. Sanderink, G.J.; Bournique, B.; Stevens, J.; Petry, M.; Martinet, M. Involvement of human CYP1A isoenzymes in the metabolism and drug interactions of riluzole in vitro. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 1465–1472.
24. Weiss, J.; Sawa, E.; Riedel, K.D.; Haefeli, W.E.; Mikus, G. In vitro metabolism of the opioid tilidine and interaction of tilidine and nortilidine with CYP3A4, CYP2C19, and CYP2D6. *Naunyn Schmiedebergs Arch. Pharmacol.* **2008**, *378*, 275–282.
25. Gallagher, E.P.; Wienkers, L.C.; Stapleton, P.L.; Kunze, K.L.; Eaton, D.L. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. *Cancer Res.* **1994**, *54*, 101–108
26. Granfors, M.T.; Backman, J.T.; Laitila, J.; Neuvonen, P.J. Tizanidine is mainly metabolized by cytochrome p450 1A2 in vitro. *Br. J. Clin. Pharmacol.* **2004**, *57*, 349–353.
27. Juvonen, R.O.; Jokinen, E.M.; Javaid, A.; Lehtonen, M.; Raunio, H.; Pentikainen, O.T. Inhibition of human CYP1 enzymes by a classical inhibitor alpha-naphthoflavone and a novel inhibitor N-(3,5-dichlorophenyl)cyclopropanecarboxamide: An in vitro and in silico study. *Chem. Biol. Drug Des.* **2020**, *95*, 520–533.
28. Reid, J.M.; Kuffel, M.J.; Miller, J.K.; Rios, R.; Ames, M.M. Metabolic activation of dacarbazine by human cytochromes P450: The role of CYP1A1, CYP1A2, and CYP2E1. *Clin. Cancer Res.* **1999**, *5*, 2192–2197.
29. Faucette, S.R.; Hawke, R.L.; Lecluyse, E.L.; Shord, S.S.; Yan, B.; Laethem, R.M.; Lindley, C.M. Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab. Dispos.* **2000**, *28*, 1222–1230.
30. Desta, Z.; Saussele, T.; Ward, B.; Blievernicht, J.; Li, L.; Klein, K.; Flockhart, D.A.; Zanger, U.M. Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro. *Pharmacogenomics* **2007**, *8*, 547–558.
31. Nishiya, Y.; Hagihara, K.; Ito, T.; Tajima, M.; Miura, S.; Kurihara, A.; Farid, N.A.; Ikeda, T. Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab. Dispos.* **2009**, *37*, 589–593.
32. Walsky, R.L.; Astuccio, A.V.; Obach, R.S. Evaluation of 227 drugs for in vitro inhibition of cytochrome P450 2B6. *J. Clin. Pharmacol.* **2006**, *46*, 1426–1438.
33. Faucette, S.R.; Wang, H.; Hamilton, G.A.; Jolley, S.L.; Gilbert, D.; Lindley, C.; Yan, B.; Negishi, M.; LeCluyse, E.L. Regulation of CYP2B6 in primary human hepatocytes by prototypical inducers. *Drug Metab. Dispos.* **2004**, *32*, 348–358.
34. Gerbal-Chaloin, S.; Daujat, M.; Pascussi, J.M.; Pichard-Garcia, L.; Vilarem, M.J.; Maurel, P. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J. Biol. Chem.* **2002**, *277*, 209–217.
35. Li, L.; Welch, M.A.; Li, Z.; Mackowiak, B.; Heyward, S.; Swaan, P.W.; Wang, H. Mechanistic Insights of Phenobarbital-Mediated Activation of Human but Not Mouse Pregnane X Receptor. *Mol. Pharmacol.* **2019**, *96*, 345–354.
36. Madan, A.; Graham, R.A.; Carroll, K.M.; Mudra, D.R.; Burton, L.A.; Krueger, L.A.; Downey, A.D.; Czerwinski, M.; Forster, J.; Ribadeneira, M.D.; et al. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab. Dispos.* **2003**, *31*, 421–431.
37. Hesse, L.M.; Venkatakrishnan, K.; Court, M.H.; von Moltke, L.L.; Duan, S.X.; Shader, R.I.; Greenblatt, D.J. CYP2B6 mediates the in vitro hydroxylation of bupropion: Potential drug interactions with other antidepressants. *Drug Metab. Dispos.* **2000**, *28*, 1176–1183.
38. Turpeinen, M.; Nieminen, R.; Juntunen, T.; Taavitsainen, P.; Raunio, H.; Pelkonen, O. Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes in vitro. *Drug Metab. Dispos.* **2004**, *32*, 626–631.
39. Walsky, R.L.; Obach, R.S. A comparison of 2-phenyl-2-(1-piperidinyl)propane (ppp), 1,1′,1″-phosphinothioylidynetrisaziridine (thioTEPA), clopidogrel, and ticlopidine as selective inactivators of human cytochrome P450 2B6. *Drug Metab. Dispos.* **2007**, *35*, 2053–2059.
40. Lai, X.S.; Yang, L.P.; Li, X.T.; Liu, J.P.; Zhou, Z.W.; Zhou, S.F. Human CYP2C8: Structure, substrate specificity, inhibitor selectivity,

inducers and polymorphisms. *Curr. Drug Metab.* **2009**, *10*, 1009–1047.
41. Li, X.Q.; Bjorkman, A.; Andersson, T.B.; Ridderstrom, M.; Masimirembwa, C.M. Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: A new high affinity and turnover enzyme-specific probe substrate. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 399–407.
42. Kim, M.J.; Lee, J.W.; Oh, K.S.; Choi, C.S.; Kim, K.H.; Han, W.S.; Yoon, C.N.; Chung, E.S.; Kim, D.H.; Shin, J.G. The tyrosine kinase inhibitor nilotinib selectively inhibits CYP2C8 activities in human liver microsomes. *Drug Metab. Pharmacokinet.* **2013**, *28*, 462–467.
43. Wang, J.S.; Neuvonen, M.; Wen, X.; Backman, J.T.; Neuvonen, P.J. Gemfibrozil inhibits CYP2C8-mediated cerivastatin metabolism in human liver microsomes. *Drug Metab. Dispos.* **2002**, *30*, 1352–1356.
44. Raucy, J.L.; Mueller, L.; Duan, K.; Allen, S.W.; Strom, S.; Lasker, J.M. Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 475–482.
45. VandenBrink, B.M.; Foti, R.S.; Rock, D.A.; Wienkers, L.C.; Wahlstrom, J.L. Evaluation of CYP2C8 inhibition in vitro: Utility of montelukast as a selective CYP2C8 probe substrate. *Drug Metab. Dispos.* **2011**, *39*, 1546–1554.
46. Polasek, T.M.; Elliot, D.J.; Lewis, B.C.; Miners, J.O. Mechanism-based inactivation of human cytochrome P4502C8 by drugs in vitro. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 996–1007.
47. Leemann, T.; Transon, C.; Dayer, P. Cytochrome P450TB (CYP2C): A major monooxygenase catalyzing diclofenac 4′-hydroxylation in human liver. *Life Sci.* **1993**, *52*, 29–34. [
48. Murayama, N.; Yajima, K.; Hikawa, M.; Shimura, K.; Ishii, Y.; Takada, M.; Uno, Y.; Utoh, M.; Iwasaki, K.; Yamazaki, H. Assessment of multiple cytochrome P450 activities in metabolically inactivated human liver microsomes and roles of P450 2C isoforms in reaction phenotyping studies. *Biopharm. Drug Dispos.* **2018**, *39*, 116–121.
49. Mao, J.; Mohutsky, M.A.; Harrelson, J.P.; Wrighton, S.A.; Hall, S.D. Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: Comparison of plasma and protein-free media incubation conditions. *Drug Metab. Dispos.* **2012**, *40*, 706–716.
50. Tang, C.; Shou, M.; Mei, Q.; Rushmore, T.H.; Rodrigues, A.D. Major role of human liver microsomal cytochrome P450 2C9 (CYP2C9) in the oxidative metabolism of celecoxib, a novel cyclooxygenase-II inhibitor. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 453–459
51. Giancarlo, G.M.; Venkatakrishnan, K.; Granda, B.W.; von Moltke, L.L.; Greenblatt, D.J. Relative contributions of CYP2C9 and 2C19 to phenytoin 4-hydroxylation in vitro: Inhibition by sulfaphenazole, omeprazole, and ticlopidine. *Eur. J. Clin. Pharmacol.* **2001**, *57*, 31–36.
52. Chen, Y.; Ferguson, S.S.; Negishi, M.; Goldstein, J.A. Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 495–501.
53. Mori, K.; Hashimoto, H.; Takatsu, H.; Tsuda-Tsukimoto, M.; Kume, T. Cocktail-substrate assay system for mechanism-based inhibition of CYP2C9, CYP2D6, and CYP3A using human liver microsomes at an early stage of drug development. *Xenobiotica* **2009**, *39*, 415–422. [
54. Wrighton, S.A.; Stevens, J.C.; Becker, G.W.; VandenBranden, M. Isolation and characterization of human liver cytochrome P450 2C19: Correlation between 2C19 and S-mephenytoin 4′-hydroxylation. *Arch. Biochem. Biophys.* **1993**, *306*, 240–245.
55. Suzuki, H.; Kneller, M.B.; Haining, R.L.; Trager, W.F.; Rettie, A.E. (+)-N-3-Benzyl-nirvanol and (−)-N-3-benzyl-phenobarbital: New potent and selective in vitro inhibitors of CYP2C19. *Drug Metab. Dispos.* **2002**, *30*, 235–239.
56. Ko, J.W.; Desta, Z.; Soukhova, N.V.; Tracy, T.; Flockhart, D.A. In vitro inhibition of the cytochrome P450 (CYP450) system by the antiplatelet drug ticlopidine: Potent effect on CYP2C19 and CYP2D6. *Br. J. Clin. Pharmacol.* **2000**, *49*, 343–351.
57. Barecki, M.E.; Casciano, C.N.; Johnson, W.W.; Clement, R.P. In vitro characterization of the inhibition profile of loratadine, desloratadine, and 3-OH-desloratadine for five human cytochrome P-450 enzymes. *Drug Metab. Dispos.* **2001**, *29*, 1173–1175.
58. Khojasteh, S.C.; Prabhu, S.; Kenny, J.R.; Halladay, J.S.; Lu, A.Y. Chemical inhibitors of cytochrome P450 isoforms in human liver microsomes: A re-evaluation of P450 isoform selectivity. *Eur. J. Drug Metab. Pharmacokinet.* **2011**, *36*, 1–16.
59. Reese, M.J.; Wurm, R.M.; Muir, K.T.; Generaux, G.T.; St John-Williams, L.; McConn, D.J. An in vitro mechanistic study to elucidate the desipramine/bupropion clinical drug-drug interaction. *Drug Metab. Dispos.* **2008**, *36*, 1198–1201.
60. VandenBrink, B.M.; Foti, R.S.; Rock, D.A.; Wienkers, L.C.; Wahlstrom, J.L. Prediction of CYP2D6 drug interactions from in vitro data: Evidence for substrate-dependent inhibition. *Drug Metab. Dispos.* **2012**, *40*, 47–53.
61. Bertelsen, K.M.; Venkatakrishnan, K.; Von Moltke, L.L.; Obach, R.S.; Greenblatt, D.J. Apparent mechanism-based inhibition of human

CYP2D6 in vitro by paroxetine: Comparison with fluoxetine and quinidine. *Drug Metab. Dispos.* **2003**, *31*, 289–293.
62. Madeira, M.; Levine, M.; Chang, T.K.; Mirfazaelian, A.; Bellward, G.D. The effect of cimetidine on dextromethorphan O-demethylase activity of human liver microsomes and recombinant CYP2D6. *Drug Metab. Dispos.* **2004**, *32*, 460–467.
63. Otton, S.V.; Ball, S.E.; Cheung, S.W.; Inaba, T.; Rudolph, R.L.; Sellers, E.M. Venlafaxine oxidation in vitro is catalysed by CYP2D6. *Br. J. Clin. Pharmacol.* **1996**, *41*, 149–156.
64. Lee, J.; Fallon, J.K.; Smith, P.C.; Jackson, K.D. Formation of CYP3A-specific metabolites of ibrutinib in vitro is correlated with hepatic CYP3A activity and 4beta-hydroxycholesterol/cholesterol ratio. *Clin. Transl. Sci.* **2023**, *16*, 279–291.
65. Walsky, R.L.; Obach, R.S.; Hyland, R.; Kang, P.; Zhou, S.; West, M.; Geoghegan, K.F.; Helal, C.J.; Walker, G.S.; Goosen, T.C.; et al. Selective mechanism-based inactivation of CYP3A4 by CYP3cide (PF-04981517) and its utility as an in vitro tool for delineating the relative roles of CYP3A4 versus CYP3A5 in the metabolism of drugs. *Drug Metab. Dispos.* **2012**, *40*, 1686–1697.
66. Bissada, J.E.; Truong, V.; Abouda, A.A.; Wines, K.J.; Crouch, R.D.; Jackson, K.D. Interindividual Variation in CYP3A Activity Influences Lapatinib Bioactivation. *Drug Metab. Dispos.* **2019**, *47*, 1257–1269.
67. Hellum, B.H.; Hu, Z.; Nilsen, O.G. The induction of CYP1A2, CYP2D6 and CYP3A4 by six trade herbal products in cultured primary human hepatocytes. *Basic Clin. Pharmacol. Toxicol.* **2007**, *100*, 23–30.
68. Doran, A.C.; Burchett, W.; Landers, C.; Gualtieri, G.M.; Balesano, A.; Eng, H.; Dantonio, A.L.; Goosen, T.C.; Obach, R.S. Defining the Selectivity of Chemical Inhibitors Used for Cytochrome P450 Reaction Phenotyping: Overcoming Selectivity Limitations with a Six-Parameter Inhibition Curve-Fitting Approach. *Drug Metab. Dispos.* **2022**, *50*, 1259–1271.
69. Zientek, M.A.; Youdim, K. Reaction phenotyping: Advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab. Dispos.* **2015**, *43*, 163–181.
70. Lu, A.Y.; Wang, R.W.; Lin, J.H. Cytochrome P450 in vitro reaction phenotyping: A re-evaluation of approaches used for P450 isoform identification. *Drug Metab. Dispos.* **2003**, *31*, 345–350.
71. Rodrigues, A.D. Integrated cytochrome P450 reaction phenotyping: Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* **1999**, *57*, 465–480.
72. Zhang, H.; Davis, C.D.; Sinz, M.W.; Rodrigues, A.D. Cytochrome P450 reaction-phenotyping: An industrial perspective. *Expert. Opin. Drug Metab. Toxicol.* **2007**, *3*, 667–687.
73. Dantonio, A.L.; Doran, A.C.; Obach, R.S. Intersystem Extrapolation Factors Are Substrate-Dependent for CYP3A4: Impact on Cytochrome P450 Reaction Phenotyping. *Drug Metab. Dispos.* **2022**, *50*, 249–257.
74. Di, L. Reaction phenotyping to assess victim drug-drug interaction risks. *Expert. Opin. Drug Discov.* **2017**, *12*, 1105–1115.
75. Doran, A.C.; Dantonio, A.L.; Gualtieri, G.M.; Balesano, A.; Landers, C.; Burchett, W.; Goosen, T.C.; Obach, R.S. An improved method for cytochrome p450 reaction phenotyping using a sequential qualitative-then-quantitative approach. *Drug Metab. Dispos.* **2022**, *50*, 1272–1286.
76. Nagar, S.; Argikar, U.A.; Tweedie, D.J. Enzyme kinetics in drug metabolism: Fundamentals and applications. *Methods Mol. Biol.* **2014**, *1113*, 1–6.
77. Zientek, M.A.; Goosen, T.C.; Tseng, E.; Lin, J.; Bauman, J.N.; Walker, G.S.; Kang, P.; Jiang, Y.; Freiwald, S.; Neul, D.; et al. In Vitro Kinetic Characterization of Axitinib Metabolism. *Drug Metab. Dispos.* **2016**, *44*, 102–114. [
78. Jones, H.M.; Houston, J.B. Substrate depletion approach for determining in vitro metabolic clearance: Time dependencies in hepatocyte and microsomal incubations. *Drug Metab. Dispos.* **2004**, *32*, 973–982
79. Rowland, M.; Matin, S.B. Kinetics of drug-drug interactions. *J. Pharmacokinet. Biopharm.* **1973**, *1*, 553–567.
80. Bjornsson, T.D.; Callaghan, J.T.; Einolf, H.J.; Fischer, V.; Gan, L.; Grimm, S.; Kao, J.; King, S.P.; Miwa, G.; Ni, L.; et al. The conduct of in vitro and in vivo drug-drug interaction studies: A Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab. Dispos.* **2003**, *31*, 815–832.
81. Bohnert, T.; Patel, A.; Templeton, I.; Chen, Y.; Lu, C.; Lai, G.; Leung, L.; Tse, S.; Einolf, H.J.; Wang, Y.H.; et al. Evaluation of a New Molecular Entity as a Victim of Metabolic Drug-Drug Interactions—An Industry Perspective. *Drug Metab. Dispos.* **2016**, *44*, 1399–1423.
82. Drug Development and Drug Interactions|Table of Substrates, Inhibitors and Inducers. Available online: **https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers** (accessed on 2023).
83. Gelboin, H.V.; Krausz, K. Monoclonal antibodies and multifunctional cytochrome P450: Drug

metabolism as paradigm. *J. Clin. Pharmacol.* **2006**, *46*, 353–372.
84. Polsky-Fisher, S.L.; Cao, H.; Lu, P.; Gibson, C.R. Effect of cytochromes P450 chemical inhibitors and monoclonal antibodies on human liver microsomal esterase activity. *Drug Metab. Dispos.* **2006**, *34*, 1361–1366. [
85. Chen, Y.; Liu, L.; Nguyen, K.; Fretland, A.J. Utility of intersystem extrapolation factors in early reaction phenotyping and the quantitative extrapolation of human liver microsomal intrinsic clearance using recombinant cytochromes P450. *Drug Metab. Dispos.* **2011**, *39*, 373–382.
86. Siu, Y.A.; Lai, W.G. Impact of Probe Substrate Selection on Cytochrome P450 Reaction Phenotyping Using the Relative Activity Factor. *Drug Metab. Dispos.* **2017**, *45*, 183–189.
87. Obach, R.S. Linezolid Metabolism Is Catalyzed by Cytochrome P450 2J2, 4F2, and 1B1. *Drug Metab. Dispos.* **2022**, *50*, 413–421.
88. Wynalda, M.A.; Hauer, M.J.; Wienkers, L.C. Oxidation of the novel oxazolidinone antibiotic linezolid in human liver microsomes. *Drug Metab. Dispos.* **2000**, *28*, 1014–1017.
89. Watanabe, R.; Kawata, T.; Ueda, S.; Shinbo, T.; Higashimori, M.; Natsume-Kitatani, Y.; Mizuguchi, K. Prediction of the Contribution Ratio of a Target Metabolic Enzyme to Clearance from Chemical Structure Information. *Mol. Pharm.* **2023**, *20*, 419–426.
90. Keefer, C.E.; Chang, G.; Di, L.; Woody, N.A.; Tess, D.A.; Osgood, S.M.; Kapinos, B.; Racich, J.; Carlo, A.A.; Balesano, A.; et al. The Comparison of Machine Learning and Mechanistic In Vitro-In Vivo Extrapolation Models for the Prediction of Human Intrinsic Clearance. *Mol. Pharm.* **2023**, *20*, 5616–5630.
91. Youdim, K.A.; Zayed, A.; Dickins, M.; Phipps, A.; Griffiths, M.; Darekar, A.; Hyland, R.; Fahmi, O.; Hurst, S.; Plowchalk, D.R.; et al. Application of CYP3A4 in vitro data to predict clinical drug-drug interactions; predictions of compounds as objects of interaction. *Br. J. Clin. Pharmacol.* **2008**, *65*, 680–692.
92. Eagling, V.A.; Tjia, J.F.; Back, D.J. Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. *Br. J. Clin. Pharmacol.* **1998**, *45*, 107–114.
93. Nirogi, R.; Palacharla, R.C.; Uthukam, V.; Manoharan, A.; Srikakolapu, S.R.; Kalaikadhiban, I.; Boggavarapu, R.K.; Ponnamaneni, R.K.; Ajjala, D.R.; Bhyrapuneni, G. Chemical inhibitors of CYP450 enzymes in liver microsomes: Combining selectivity and unbound fractions to guide selection of appropriate concentration in phenotyping assays. *Xenobiotica* **2015**, *45*, 95–106.
94. Pearce, R.E.; McIntyre, C.J.; Madan, A.; Sanzgiri, U.; Draper, A.J.; Bullock, P.L.; Cook, D.C.; Burton, L.A.; Latham, J.; Nevins, C.; et al. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch. Biochem. Biophys.* **1996**, *331*, 145–169.
95. Elaut, G.; Papeleu, P.; Vinken, M.; Henkens, T.; Snykers, S.; Vanhaecke, T.; Rogiers, V. Hepatocytes in suspension. *Methods Mol. Biol.* **2006**, *320*, 255–263.
96. Stringer, R.; Nicklin, P.L.; Houston, J.B. Reliability of human cryopreserved hepatocytes and liver microsomes as in vitro systems to predict metabolic clearance. *Xenobiotica* **2008**, *38*, 1313–1329.
97. Chan, T.S.; Yu, H.; Moore, A.; Khetani, S.R.; Tweedie, D. Meeting the Challenge of Predicting Hepatic Clearance of Compounds Slowly Metabolized by Cytochrome P450 Using a Novel Hepatocyte Model, HepatoPac. *Drug Metab. Dispos.* **2019**, *47*, 58–66
98. Klammers, F.; Goetschi, A.; Ekiciler, A.; Walter, I.; Parrott, N.; Fowler, S.; Umehara, K. Estimation of Fraction Metabolized by Cytochrome P450 Enzymes Using Long-Term Cocultured Human Hepatocytes. *Drug Metab. Dispos.* **2022**, *50*, 566–575.
99. Lu, A.Y. Liver microsomal drug-metabolizing enzyme system: Functional components and their properties. *Fed. Proc.* **1976**, *35*, 2460–2463.
100. Asha, S.; Vidyavathi, M. Role of human liver microsomes in in vitro metabolism of drugs—A review. *Appl. Biochem. Biotechnol.* **2010**, *160*, 1699–1722.
101. Cerny, M.A. Prevalence of Non-Cytochrome P450-Mediated Metabolism in Food and Drug Administration-Approved Oral and Intravenous Drugs: 2006–2015. *Drug Metab. Dispos.* **2016**, *44*, 1246–1252.
102. Ring, B.; Wrighton, S.A.; Mohutsky, M. Reversible mechanisms of enzyme inhibition and resulting clinical significance. *Methods Mol. Biol.* **2014**, *1113*, 37–56