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Cytochrome P450 Enzymes in Drug Metabolism: From In Vitro Profiling to Clinical Drug Interaction Risk

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Abstract. Cytochrome P450 (CYP) enzymes plays a key role in most clinical drug metabolism, among which six isoenzymes, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, are involved in about 70% to 80% of drug metabolism reactions. The change of CYP activity will affect the clearance rate, bioavailability and toxicity of drugs, and many serious drug-drug interactions, DDIs) are also caused by the inhibition or induction of CYP. This paper reviews the main in vitro methods of CYP profiling, including metabolic stability test, single substrate inhibition test, cocktail assays and time-dependent inhibition research, and explains how these methods can be combined with LC-MS/MS platform and automation technology for compound screening and early DDI risk assessment. The article also analyzes the distribution of CYP in liver, small intestine and kidney, focusing on the differences in pharmacogenomics and the synergistic effect of CYP3A and P-glycoprotein in intestine. Subsequently, this paper explains how to integrate the relevant data to predict the clinical outcome in the basic pharmacokinetic modeling of physiology, and analyzes the cases of DDI such as azole antifungal agents, statins, tacrolimus and rifampicin. Finally, the article points out the new problems brought by biological agents. Although these drugs are not CYP substrates, they can affect CYP expression through cytokine-mediated mechanism, so the existing mechanism framework needs to be further expanded to explain this kind of interaction.

Keywords: cytochrome P450, drug-drug interactions, in vitro metabolism, pharmacokinetics, physiologically based pharmacokinetic modeling

1. Introduction

Cytochrome P450 (CYP) enzymes plays a key role in drug therapy. This kind of heme-containing monooxygenase is mainly distributed in liver and other tissues, and is responsible for the oxidative metabolism of many small molecular drugs. More than 50 kinds of CYP isoenzymes have been found in human body, among which six isoenzymes, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, are responsible for about 70% to 80% of drug metabolism reactions [1]. Therefore, the change of CYP activity will affect drug clearance, bioavailability and toxicity, and may cause important clinical risks. Many serious drug-drug interactions, DDIs) are related to the inhibition or induction of CYP. For example, the combination of voriconazole and tacrolimus, or the combination of cerivastatin and gemfibrozil may significantly change the plasma drug

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concentration, and then lead to the decline of toxicity or efficacy [1]. Therefore, regulators such as FDA and EMA require that CYP inhibition and induction research be carried out in the early stage of drug development to assess the potential risk of DDI in advance. This article will summarize the main in vitro methods for evaluating CYP activity, explain the distribution of CYP enzyme in important metabolic tissues, and discuss how the mechanism model can integrate relevant data to support clinical DDI risk management. At the same time, this paper will also pay attention to the new challenges brought by biological agents, because these drugs adopt different metabolic pathways and need to further adjust the existing CYP evaluation framework.In vitro methods for CYP profiling

Early systematic evaluation of CYP-related metabolism depends on a set of mature in vitro experimental methods. These methods are fast and reproducible, and can provide enough information to guide compound prioritization and support early decision-making, so as to complete screening before expensive animal or clinical studies begin.

1.1. Metabolic stability and CYP inhibition assays

Human liver microsome (HLM) metabolic stability experiments are usually the first step, determining the metabolic rate of compounds by hepatic CYP enzymes and ranking compounds by intrinsic clearance rate. The data can be used to calculate in vitro half-life and predict hepatic clearance in vivo, enabling research teams to eliminate chemical scaffolds with excessively rapid metabolic turnover before animal pharmacokinetic studies begin [1]. For example, simvastatin and lovastatin are easily influenced by microsomal metabolism mediated by CYP3A4, while pravastatin and rosuvastatin have less CYP-related metabolism. This difference is consistent with the clinical manifestations of DDI [2].

After completing the metabolic stability screening, single-substrate CYP inhibition experiments can be used to evaluate the inhibitory effects of new compounds on six major CYP isoenzymes, and preliminarily judge the risk of DDI by IC50 value [1]. Ketoconazole is a typical case: as a potent CYP3A inhibitor, it can significantly reduce the maintenance dose of tacrolimus required by renal transplant patients, and long-term combined use is related to a higher incidence of acute rejection [3].

1.2. Cocktail assays and time-dependent inhibition

In cocktail experiments, multiple probe substrates can be incubated together, so as to detect the CYP activity or inhibition of various isoenzymes at the same time, and effectively improve the experimental efficiency. Abnormal or inconsistent results are confirmed by single-substrate experiments [1]. A phenotypic identification cocktail experiment based on dextromethorphan applied to dogs revealed large individual and breed-related differences in CYP2D family activity, indicating that multiple probe designs can capture not only inhibitory signals but also population variation [4].

Time-dependent inhibition (TDI) is evaluated by IC50 shift experiments with and without NADPH preincubation. Compounds showing enhanced inhibition after pre-incubation are flagged as potential mechanism-based inhibitors with high clinical DDI risk [1]. Erythromycin and clarithromycin clearly exhibit this pattern: without pre-incubation they only slightly inhibit midazolam metabolism, but after NADPH pre-incubation they cause obvious, concentration-dependent loss of midazolam hydroxylation activity. Inactivation parameters derived from these

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experiments can accurately predict a 2- to 3-fold increase in oral midazolam AUC in clinical studies, while azithromycin shows little effect under the same conditions [5].

1.3. Analytical platforms and automation

Because of its high specificity and sensitivity, LC-MS/MS has become a commonly used standard analytical method for CYP experiments. Midazolam and its main metabolite, 1'-hydroxymidazolam, can be rapidly detected in human plasma at a level lower than ng/mL, and the single running time is less than six minutes, so it can support the phenotypic judgment of CYP3A in clinical and preclinical studies [6]. At the same time, sample pretreatment and automation of orifice plate operation also improved the efficiency, repeatability and process consistency of metabolic stability and CYP inhibition experiments [7]. Together, these technologies form a layered experimental system with both detection speed and mechanism analysis, which can be used for CYP risk assessment.

2. Tissue distribution of CYP enzymes

CYP exists in many organs, among which the distribution of liver, small intestine and kidney will affect drug absorption, metabolism and excretion. Therefore, it is very important to know the expression level of different parts for predicting oral bioavailability and setting the parameters of mechanism model.

2.1. Hepatic CYP expression

Liver is the core part of CYP-mediated first-pass metabolism and systemic drug metabolism. Individual differences in liver CYP expression will directly affect the first-pass extraction score and oral bioavailability. The unlabeled global protein omics study of human liver microsomes has been able to quantitatively analyze 17 kinds of CYP isoenzymes, drug transporters and binding enzymes, and provide a basis for predicting the first-pass extraction scores of CYP-sensitive drugs by in vitro-in vivo extrapolation (IVIVIVE) and physiological pharmacokinetics (PBPK) models [8].

Pharmacogenomics is an important reason for this difference. The expression level of CYP3A5 in people with active CYP3A5*1 allele is about 16 times that of people with *3/*3 nearly inactivated genotype, so the metabolic capacity of CYP3A5 in liver of different populations will be different, which will further affect the oral bioavailability of CYP3A5 substrate [8]. The distribution of CYP3A5*1 allele is also closely related to the ancestral background, and it is more common in African-American population than in European or Asian population, so race can be used as an important variable for dose prediction of CYP3A5 sensitive drugs. Tacrolimus is a typical case: in different ethnic groups, the expression of CYP3A5 is usually associated with higher maintenance dose, faster clearance rate and higher risk of early acute rejection [9]. Warfarin also shows a similar problem: functional variation of CYP2C9 will reduce warfarin clearance rate and maintenance dose requirements, so CPIC guidelines suggest that the initial dose should be determined according to genotype [10].

Liver CYP will be affected by induction, inhibition, inflammation, diseases and pharmacogenomics, which will change the first-pass metabolism and clinical drug exposure. It is helpful to predict the bioavailability and population differences of CYP-sensitive oral drugs more accurately by analyzing the measured liver CYP abundance and its variation, layering according to CYP3A5 expression status, and testing the induction or inhibition situation [8, 11].

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2.2. Intestinal CYP expression

The small intestine is an important extrahepatic site for CYP expression and a critical checkpoint for oral drug absorption. CYP3A is the most important isoenzyme in intestinal epithelial cells and, together with P-glycoprotein (P-gp), forms a metabolic-transport barrier that can significantly reduce the fraction of drug absorbed through the intestinal wall [11]. In a crossover study of midazolam, oral bioavailability was approximately 50 percent lower than that predicted by hepatic extraction alone, and the intestinal extraction fraction was of a similar magnitude to hepatic extraction, confirming that the intestinal wall is a true first-pass metabolic organ for CYP3A substrates [12]. Immunoquantitative studies of human small intestine P450 showed that CYP3A is the predominant isoenzyme, with CYP2C9, CYP2C19, CYP2J2, and CYP2D6 also expressed to varying degrees, and that expression levels of each isoenzyme vary greatly among individuals [13].

Intestinal CYP does not operate independently. The apical efflux transporter P-gp and CYP3A work synergistically in intestinal epithelial cells: apical efflux reduces net absorption but increases the number of drug molecules cycling across the enterocyte membrane, subjecting them to repeated oxidation cycles and amplifying first-pass loss and variability [14]. This shows that when the two systems are inhibited or induced at the same time, synergistic DDI effect may occur. Clinical evidence of Simvastatin shows that its oral exposure will be limited by the metabolism of CYP3A4 in the first pass of intestine, and inhibition of CYP3A4 will obviously increase its systemic exposure level [2].

In addition to CYP-related barriers, early calculation methods can also help identify compounds with poor absorption capacity. QSAR model based on molecular weight, logP and other physical and chemical characteristics can find low absorption chemical skeleton in the early stage of research and development, and can be used to guide structural optimization, thus reducing the risk of clinical failure in the later stage [15].

2.3. Renal CYP expression

Renal clearance drugs mainly rely on glomerular filtration, renal tubular secretion and reabsorption, but the kidney also expresses extrahepatic CYP enzyme, so it may also affect local physiological function and systemic pharmacokinetics [11]. Among them, CYP2C and CYP2J families are more important in the kidney, which can convert arachidonic acid into epoxyeicosatrienoic acids, EETs). EETs is involved in the regulation of vascular tension, inflammation and angiogenesis, and further affects renal hemodynamics and renal tubular transport function [16].

CYP activity in kidney is also affected by redox state. Protein omics and functional studies of human liver and kidney microsomes show that oxidative stress can lead to cysteine sulfinylation of drug metabolism CYP enzyme and change its catalytic activity [17]. In chronic nephropathy, uremia can down-regulate CYP enzymes and drug transporters in liver and intestine, thus increasing the in vivo exposure of some drugs that are not mainly excreted through kidney. This effect is more obvious for drugs with narrow treatment window and complicated clearance route, so dose adjustment and closer monitoring may be needed [18].

3. Mechanistic modeling: PBPK and IVIVE

Oral bioavailability can be expressed as $F = Fa \times Fg \times Fh$, where Fa reflects dissolution and epithelial permeation, Fg represents intestinal wall extraction, and Fh represents hepatic first-pass

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metabolism. PBPK models use this framework to integrate organ-level CYP data and predict systemic exposure and DDI risk under varied conditions [11].

These models parameterize CYP abundance in intestinal epithelial cells and hepatocytes using proteomics data, incorporate intrinsic clearance values from microsomal and recombinant CYP experiments, and include reversible inhibition, TDI, and induction modules. They can simulate the contribution of each organ compartment to overall first-pass loss and the separate effects of a given inhibitor or inducer on Fg or Fh. Reference values of Fg and Fh for probe drugs such as midazolam are well documented [12, 13]. Scenarios involving potent inducers such as rifampicin or potent inhibitors such as azole antifungals are used to test whether models can reproduce observed clinical exposure changes.

These model frameworks also apply to clinical therapeutic drugs. Tacrolimus exposure and dose requirements depend on CYP3A5 phenotype and the combined regulation of CYP3A and P-gp [9, 3]. Statin pharmacokinetics are governed by multiple factors: the interplay between CYP3A metabolism and hepatic uptake and efflux transporters determines exposure and DDI sensitivity within the same drug class [2]. Integrating quantitative tissue expression data, in vitro kinetic data, and clinical probe results into a PBPK framework helps predict population variability more reliably, guides formulation design, and supports evidence-based DDI risk labeling [12, 13, 14, 8, 11].

4. Clinical DDI evidence

The results of in vitro mechanistic studies are highly consistent with clinical observations. Azole antifungals provide a clear example: a single dose of itraconazole or fluconazole can increase midazolam AUC and Cmax approximately 3.5-fold and 2-fold, respectively, and after multiple doses of itraconazole the AUC can increase approximately 7-fold, with correspondingly enhanced psychomotor effects [19]. These findings are consistent with TDI in vitro data: erythromycin and clarithromycin cause significant NADPH-dependent loss of midazolam hydroxylation activity, and predicted clinical AUC increases align closely with actual observations [5]. This correspondence strongly supports the value of the hierarchical experimental process described in Section 1.

Statin's pharmacokinetics shows that similar drugs have different dependence on CYP pathway. The potent CYP3A4 inhibitors can significantly increase the exposure of simvastatin, lovastatin and atorvastatin, and increase the risk of myopathy. Fluvastatin is mainly metabolized by CYP2C9, so it is less affected by CYP3A regulation. Pravastatin and rosuvastatin rely more on transporter-mediated clearance, so they are more susceptible to OATP and BCRP status [2]. Rifampicin, as a typical potent CYP3A and P-gp inducer, can reduce the systemic exposure of CYP3A substrates such as midazolam and statins, so it may be necessary to increase the dosage or change the dressing in clinic to maintain the curative effect [11]. Tacrolimus also reflects the relationship between CYP3A and P-gp: CYP3A5 phenotype and the joint regulation of the two systems will jointly affect the dosage and clinical results of transplant patients [9, 3].

5. Discussion

The evidence in this review shows that CYP data in vitro can be clearly related to clinical results through tissue-specific expression and mechanism model. However, there are still obvious gaps in the existing process in the evaluation of biological agents.

At present, CYP analysis methods are mainly oriented to small molecule drugs. Biological agents, such as monoclonal antibodies, fusion proteins and peptide therapeutics, are not eliminated by oxidative metabolism of CYP, but rely on proteolysis, intracellular decomposition, receptor-

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mediated elimination and FcRn-mediated circulation. With the increasing proportion of biological agents in approved drugs, their interaction with CYP-related pathways becomes more important.

At present, a relatively clear mechanism is cytochrome-mediated cyp suppression. During the treatment of biological agent, inflammatory cytokines such as IL-6 and tumor necrosis factor may reduce the expression of CYP in liver, thus affecting the clearance rate of small molecule drugs, and even need to adjust the dose if necessary. For example, in patients with rheumatoid arthritis, inhibition of IL-6 by tocilizumab can restore CYP3A4 activity, thus increasing the exposure level of CYP3A substrates. Although the cytokine-CYP interaction has been recorded, it has not been systematically included in the standard DDI evaluation process of biological agent combined with small molecule drug.

An important direction in the future is to extend PBPK frameworks to the modeling of cytokine-driven CYP inhibition. At the same time, there is also a lack of a standardized and lightweight process in practice, which can combine the early Fa absorption screening based on QSAR or machine learning models with the fast Fg and Fh sensitive modules to identify the first-pass risks in the early development of new chemical skeletons. These improvements are helpful to make the existing framework better adapt to the complexity of modern drug development process.

6. Conclusion

CYP enzyme in liver and intestine is the core factor affecting the first-pass metabolic loss and the difference of oral bioavailability. Early in vitro experiments such as metabolic stability, inhibition, TDI and cocktail can provide basic data for compound screening and DDI risk judgment. The information of tissue expression in protein omics and pharmacogenomics is helpful to explain the differences in clearance rate and drug exposure of different individuals. By incorporating these data into the PBPK model, clinical outcomes can be predicted more accurately, and dosage setting, preparation optimization and DDI labeling can be supported. Although kidney plays a small role in CYP-mediated metabolism, redox regulation and changes in disease-related enzymes will still increase the complexity of analysis. With the increasing application of biological agents, cytokine-mediated CYP regulation needs to be incorporated into the existing framework in the future, so as to evaluate the interaction risk in complex treatment schemes more comprehensively.

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