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Common Approaches of Cytochrome P450 (CYP) Induction Assays

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

The induction of enzymes is a defensive mechanism for some xenobiotics, but it may alter the drug's safety and efficacy by altering the activity of metabolic enzymes. One of the major families of enzymes involved in phase I metabolism is Cytochrome P450 (CYP) enzymes which may get induced by certain drugs. Concomitant administration of drugs due to chronic disease or polypharmacy, inducers among them may cause toxicity or reduce the plasma concentration at a sub-therapeutic level. This is one of the dangerous types of drug-drug interactions, but predictable & preventable. The CYPs get induced by three nuclear receptors, including the aryl hydrocarbon receptor (AhR); constitutive androstane receptor (CAR); the pregnane X receptor (PXR). Without identification during drug development, enzyme induction phenomenon of a new drug molecule may get noticed only during pharmacovigilance. Though, this CYP induction may not be a barrier for drug development, it may cause possible DDI and treatment failure. According to FDA guidelines, pharmaceutical industries adopted *In-vitro*, *Ex-vivo* and *In-vivo* techniques based on different

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developmental stages. The results are also interpreted based on regulatory bodies guidelines. For *In-vitro* assay best accepted method is using primary hepatocytes either fresh or cryopreserved, for *Ex-vivo* liver slices of different species and in-vivo, clinical investigations are the extreme option. This paper reviews current industry approaches of CYP induction assays to evaluate potentiality for a new drug molecule as an inducer.

Keywords: Cytochrome P450; CYPs; DDI; induction; FDA; EMA; PK.

1. INTRODUCTION

Drug-drug interaction (DDI) is an undesirable but preventable adverse event of drugs occur due to interaction of two or more drugs with each other. It may increase or decrease the expected known effects of one drug in systemic circulation by the action of another co-administered drug [1-3]. DDI can alter the pharmacokinetic (PK) profile by interfering with either absorption, distribution, metabolism, or elimination [4]. PK-DDI can be one of two possibilities: inhibition or induction of enzyme or transporters causing either elevated blood availability of a drug or diminished blood availability. In contrast, pharmacodynamic (PD) interaction is associated with altered receptor activity by additive, potentiation, synergistic or antagonistic action [5,6]. The ultimate result of DDI is altered therapeutic efficacy which may be characterized by treatment failure or developing toxicity [7]. It is considered the most common cause of adverse drug events (ADR) of a drug and mainly attributed by poly-therapy, which means prescribing at least 5 or more drugs together [5,8]. Multiple factors like age of the patients, co-morbid disease state, impact of disease on drug biotransformation, pharmacological nature of drugs, continuation of multiple prescriptions and poor knowledge on potential DDI can contribute along with polypharmacy in the occurrence of DDI [7,9]. Comparing both PK and PD interventions of DDI, PK found more frequent & predictable whether PD required case by case evaluations [10]. Significant pharmacokinetic DDI can be occurred via altered hepatic metabolism (cytochrome P_{450}/ glucuronidation) or drug transportation (P-glycoprotein) [11].

Cytochrome P_{450} (CYPs) are the major family of enzymes involved in enzymatic hepatic biotransformation of 70-80% clinical drugs in the liver by oxidation [12,13]. Frequently involving and abundant isoforms of this family are 3A4, 2C9, 2C8, 2C19,2D6, 2E1, and 1A2 [10,14]. During phase 1 metabolism, this CYP enzymes initiates the most dangerous type DDI by getting induced or inhibited by concomitant administered drug [15]. Those drugs either referred as inducers or inhibitors based on their action over drug metabolizing CYPs [16]. Thus, CYP induction is the increased gene expression of CYP enzymes through elevated protein synthesis and stabilization on exposure to certain drugs [17].

CYP induction may be resulted in i) reduced therapeutic activity of victim drug when parent compound is the active metabolite or ii) toxicity by increasing the plasma concentration of reactive metabolite/intermediates from inactive parent molecule as a consequence of inducive metabolism [18,19]. CYP enzymes can be induced via receptor mediated mechanisms that involve activation of different ligand activated nuclear receptors hence function as transcription factors followed by increased transcription of targeted genes. These intracellular nuclear receptors are aryl hydrocarbon receptor (AhR); constitutive androstane receptor (CAR); the pregnane X receptor (PXR) and subsequently targeting increased protein synthesis resulted in upregulated CYPs expression. Another way is enzyme stabilization by stabilizing mRNA of targeted gene [20-23]. The general mechanism for receptor-mediated induction is, without the presence of drug (ligand), these receptors are inactivated and associated with co-repressors. Upon binding of inducers on the ligand binding domain, receptors get activated by conformational changes on releasing co-repressors and bind with respective co-factors. Selective dimerization component is available and get attached with respective receptor-cofactor complex. Together the complex locates themselves on specific DNA promoter region through DNA binding domain of receptors hence initiate transcription and translation of required gene for upregulated expression of CYPs [22,24].

AhR is an intracellular polycyclic receptor, and the dimerization partner is Ah receptor nuclear translocator (Arnt). Therefore, the expression of CYPs mostly CYP1A1 and CYP1A2 elevated on complex binding as mentioned above in the enhancer regions [20,22,25]. CAR, another nuclear receptor, found to induce the metabolism

by CYP2B family on being mediated by phenobarbital. Specifically, two promoter components, phenobarbital-responsive enhancer module (PBREM) and the xenobiotic-responsive enhancer module (XREM), get engaged with CAR binding site and cause upregulation of mostly CYP2B6 along with CYP3A4, CYP2Cs and CYP2A6. These upregulated enzymes are responsible for biotransformation of 70% drugs. This CARs have unique DNA binding and ligand binding domain, which is recognizable by specific modulator of targeted gene as PBREM specific for upregulation of CYP2B6 and CYP2B1 [26-28].

PXR, a ligand activated orphan receptor of nuclear receptor superfamily were first activated by the pregnanes 21-carbon steroids and now by many marketed clinical drugs (rifampicin) [29]. After activation by PXR agonists, like the mechanism of other nuclear receptors, PXR response element (PXRE) gets attached with promoter region of CYP3A4 gene, namely distal xenobiotic responsive element modulator and 9-*cis* retinoic acid receptor α (RXRα) in the proximal modulator. Resulted in overexpression of CYP3A4, the enzyme responsible for 50% drugs [30,31]. Other than CYP3A4, PXR can target the CYP2A, CYP2B, CYP2C genes [23]. PXR can be repressed through phosphorylation by kinases and CYPs are downregulated [32].

Evaluation for potentiality of a new molecule or clinical drug (substrate for above mentioned receptors) for CYP induction is very important to predict possible drug-drug interactions.

However, without prior detection, DDI can only be identified after marketization and during pharmacovigilance, which can be risky sometimes [33,34]. Therefore, during pre-clinical development many pharmaceutical companies employed different approaches to assess CYP induction potential for predicting, decreasing, or possibly eradicating dangerous DDI with new drug candidate. In this study, different FDA-approved CYP induction assay methods will be reviewed along with their possible advantageous or disadvantageous outcomes.

2. DIFFERENT APPROACHES OF CYP INDUCTION ASSAY

Probability of CYPs induction in the liver (as CYPs are abundant in hepatocytes), can be studied by in-vivo, in-vitro and ex-vivo techniques [21]. Among these, different pharmaceutical company, or different sites of same one, may have their own choice of method for screening based on the phase of development [35]. During evaluation, a new molecular entity can be identified either as the substrate of CYP or can itself be an inducer and may potentiate DDI if it contributes to >25% in metabolism/clearance [1].

2.1 *In vitro* Cytochrome P450 Induction Assays

During in-vitro methods, gene expression of CYP enzymes are assessed on exposure to drugs by using hepatocytes with an expectation of in-vivo scenario reflection but in lesser complexity than direct animal study system. Hepatocytes preferably collected from human but other species as rats, dogs, monkeys, and pigs are also choice by some scientists for in vitro assays [35,36]. Various models are prepared according to USFDA guidance, using primary hepatocytes (including fresh and cryopreserved); stable transfected & transiently transfected immortalized hepatocytes; stem-cell derived hepatocytes; hepatic cell line, and reporter gene contrasts [37-40]. Pharmaceutical industries interpreted their results based on USFDA guidelines. Accordingly, for considering a new molecule positive in in-vitro induction assay, it needs to exploit the CYP enzymes >40% compared to positive control and clinical DDI & in-vivo studies are suggested afterwards. In some cases, development process can be discontinued due to large induction potential of that drug candidate [19].

A preliminary, high throughput screening model can be used to assess increased nuclear receptor activation, which is subsequently responsible for increasing CYP enzyme synthesis. This evaluation can also be performed by integrating hepatoma cells (cell-based reporter assay) with CYP3A4 enhancer region & a luciferase reporter gene or CYP3A4-luciferase structure with human PXR. Current regulatory guidelines suggested that the industry perform in-vitro induction assay for CYP3A4 preclinically as it is the most responsive PXR mediate enzyme. If the result is negative, induction potential for CYP2C can be excluded as both are mediated by PXR [23,41,42]. Another sophisticated technique used in pharmaceutical industries is utilizing hepatic cell lines or immortalized cell lines which preserved hepatocyte morphology with consistent results in reproducible manner. The most approved alternate of primary hepatocytes is HepaRG cell

lines. Immortalized cell lines can be prepared by transforming hepatocytes with plasmid encoding immortalizing genes and currently Fa2N-4 (non-tumerogenic) is most employed by pharmaceutical industries [24,43-45]. While using immortalized cell line, acceptable endpoint is measuring the mRNA as marker for induction [46]. Hepatoma cell lines including HepG2, HepaRG, and BC2 are broadly utilized by pharmaceutical industries [47].

The use of primary hepatocytes is most accepted by industry & regulatory bodies accordingly considered as the high standard as the hepatocytes retain their in-vivo CYP metabolism qualities in a certain level even after isolation [48,49]. A useful approach for retaining this phenotype behavior for long time is preparing 3D structures preferably over 2D monolayers [49]. CYP mRNA, protein and microsomal activity can be measured using models prepared from primary cultures of human hepatocytes. Sandwiched human primary hepatocytes (at least from three individual donor) are also usable due to inter-individual differences of metabolic systems [1,50].

Some difficulties of using primary or cryopreserved human hepatocytes, including less availability, loss of enzyme activity after cryopreservation, and the ability to target only single receptor-mediated process at a point and batch to batch variations, leads to the use of immortalized cells and hepatic cell line. Advantages of immortalized & hepatic cell lines over primary and transfected cell systems are (a) capable to interpret multiple nuclear hormone receptor-mediated pathways; (b) easy access to unlimited sources;(c) continuous supply by propagation and (4) stable response to inducers [17,24,39,49]. In addition, according to current guideline, the endpoint evaluation of in vitro assay is changing mRNA levels instead of previous enzyme activity measurement [51].

2.2 *Ex vivo* Cytochrome P450 Induction Assays

Ex vivo assays are investigations or measurements performed in or on tissue from a species in an environment different from its natural condition with minimal changes to the environmental factors [52]. In vitro studies use primary cultures of hepatocytes from human and/or other species, however, the species with the most similar induction profiles to humans can be found using ex vivo cytochrome P450 (CYP) enzyme induction assays in monkey, dog, rat, and mouse liver microsomes [53]. These assays can also explain unexpected outcomes in animal pharmacokinetic/pharmacodynamic (PK/PD), absorption, metabolism, distribution, excretion, and toxicology studies [53].

D. D. Surry and his colleagues developed a method called Rat Hepatocyte Induction Potential (RHIP) test for CYP3A, 1A1, 1A2, and 4A1 which are sensitive and selective, and show a strong qualitative correlation with ex-vivo CYP induction data [54]. It assesses the CYP-inductive capacity of xenobiotics in rat hepatocytes cultured on Cytostar-T™ scintillating 96-well plates, in which hepatocytes are fixed and then hybridized in situ with a set of four specific [^{35}S]-dATP-labelled oligonucleotide probes antisense to discrete regions of CYP mRNA [54].

Drug-induced changes in the expression of the cytochrome P450 genes are a significant problem in the preclinical development of pharmaceuticals because they impact safety studies by reducing the systemic exposure of a compound undergoing toxicological evaluation. For this purpose, the induction potential of candidate drugs will be studied using catalytic end points as part of the drug development process [55-57]. The chance to investigate this problem is provided by TaqMan technology, which has the advantages of a better dynamic range and precise enzyme identification where relative differences in mRNA expression are measured based on PCR cycling threshold values [55,58,59]. Because of the assay reliability, cheap cost, and availability of commercially accessible assays, TaqMan® technology is frequently employed in clinical and research settings for genotype analysis [60]. Though it was developed to test a drug's potential to alter the expression of the P450 gene in rat ex vivo livers [55,60,61], it can be modified to detect mRNA changes in various tissues [62], species [63], in vitro systems, and gene targets [60].

nCounter platform is another new technology for quantifying CYP induction ex-vivo developed by NanoString Technologies, provide a cost-effective multiplex analysis of more than eight hundred targets [64]. The NanoString technology offers an alternative method for measuring CYP induction and cross-validating the Taqman findings and provides directly assesses gene expression from liver slice homogenates without

the need for prior RNA extraction and c-DNA synthesis, or amplification [65]. To evaluate the NanoString approach, seventeen commercial medicines were incubated overnight with rat liver slices, and the induction of CYP1A1 and 2B1/2 was measured [65].

2.3 *In vivo* Cytochrome P450 Induction Assays

After quite success in in-vitro and ex-vivo approaches, there is always a gap and demand of direct evaluation of CYP induction in clinical settings for an absolute view [23]. Thus, in-vivo assays are considered the most accurate and largely applicable method with better approximation of CYP induction. In this method, always it is not feasible to measure the increased amount of enzyme and activity directly (mostly in human), so an indirect approach is considering the AUC (area under plasma concentration-time curve) of a drug, before and after administration of the new drug molecule or the drug that shows potentiality as an inducer. Mice, rats, monkeys, and dogs are commonly usable animals before human trials for in-vivo study, though the enzymatic systems and receptor specifies vary substantially from humans [66]. The conservation of different CYPs varies from animal to animal, even to strains for example, CYP1A2 is induced by omeprazole only in humans, not mice or rats. For this non-predictive phenomenon, animals can be modified in different ways including genetically modified to create humanized mice and transplantation of immunodeficient mice in human hepatocytes. This will eradicate the unwanted effect of mice hepatic protein on metabolism [23,67-69]. Due to numerous evidence of CYPs induction variations among animal species, it is suggested that animal models can be used to retrieve preliminary pharmacokinetic data, but human models are final of choice. For study in human, volunteers or patients can be chosen and enzyme induction can be characterized by using specific CYP probe standards. Standard probes should maintain some criteria as it should be selective for specific enzymes, must not be inhibitory for some other enzymes and pharmacokinetic profile should be in favor including avoid rapid metabolism and shorter half-life. Another suggestion for endpoint measurement for in-vivo is to measure the actual pharmacological (pharmacokinetic) values as EC_{50} (effective concentration) and Emax [19,24,70]. Even in humans, interindividual variations due to polymorphism of CYPs and the effect of suggested inducer probes may vary [71].

According to FDA guidelines, a) before human in-vivo/clinical investigations, results of in-vitro and early clinical investigations data should be considered. In addition, as already stated, for considering a drug for in-vivo studies it need to induce certain enzyme in >40% of the positive control on primary hepatocytes; b) certain enzymes can be excluded from the in-vivo study based on FDA guidelines on negative in-vitro findings [1].

3. CONCLUSIONS

Choice of method for CYP induction assay depends on some integrated factors. In the preliminary phase of development, to identify a new drug molecule as an inducer of certain drugs which may initiate DDI, the industrial approach is to start from in-vitro techniques. This in vitro can be high throughput screening by using different animal cell cultures, but the FDA suggests using fresh, cryopreserved, or immunized human cells. The endpoint measurement is here to quantify increased enzyme activity by elevated mRNA expression. Dose-dependent changes on mRNA expressions are observed (one out of three cell batches need to induce at least one isoform) and a 2-fold to 4-fold increase is considered positive potential for DDI. Another reliable in-vitro measurement is reporter gene assay which measure nuclear receptor activation. Primarily, CYP3A4, CYP2B6, CYP1A2 are assessed for induction probability and selection of positive control are also significant. CYP2C should not be tested during preliminary in vitro, after getting positive data investigations for other enzyme isoforms can be done. Another technique is ex-vivo analysis where it is possible to get similar induction profile as human with less pharmacokinetic complexity. The endpoint measurement measures the level of mRNA using real-time reverse transcriptase-polymerase chain reaction, emphasizing more accuracy. Finally, in-vivo methods are available to get the actual scenario with animal study. Nevertheless, animals' enzyme systems mostly the ligand binding domain for nuclear receptor cannot mimic completely the human neither in vitro nor in vivo, so clinical investigations are the ultimate option to avoid misleading comparisons. In clinical settings, plasma AUC levels are compared before and after co-administered drug with inducer.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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