DMD Fast Forward. Published on April 23, 2009 as doi:10.1124/dmd.109.027029

In Vitro and *In Vivo* Induction of Cytochrome P450: A Survey of the Current Practices and Recommendations: A Pharmaceutical Research and Manufacturers of America (PhRMA) Perspective

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Running Title:

Cytochrome P450 Induction – PhRMA Perspective

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Number of text pages: 50

Number of tables: 2

Number of figures: 2

Number of references: 120

Number of words in abstract: 176

Number of words in introduction: 977

Number of words in concluding remarks (Discussion): 503

ABBREVIATIONS: % Act, percent activation. [Ind], inducer concentration. 3-MC, 3-methylcholanthrene. AhR, aryl hydrocarbon receptor. BNF, β-naphthoflavone. CAR, constitute androstane receptor. CITCO, (6-(4-chlorophenyl)imidazo[2,1-beta][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime). CL_{int}, intrinsic clearance. CYP, cytochrome P450. DDI, drug-drug interaction. Da, Dalton. DMEM, Dulbecco's modified Eagle's medium. DMSO, dimethyl sulfoxide. EC_{50}, concentration to reach half the maximal induction effect.

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E_{max}, maximal in vitro induction effect. E_{min}, background level of catalytic activity. F_2, concentration of inducer leading to a 2-fold increase of CYP3A4 mRNA in hepatocytes in vitro. FBS, fetal bovine serum. fu, fraction unbound in blood (plasma). FXR, farnesyl X receptor. GST, glutathione S-transferase. hPXR, human PXR. ITS, insulin, transferrin and selenium as supplements in hepatocyte culture medium. IVIVC, in vitro – in vivo correlation. LBD, ligand binding domain. LXR, liver X receptor. MDR-1, multidrug resistance -1 gene encoding P-glycoprotein expression. MTT, 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. NME, new molecular entity. NOEL, no observed effect level. P-gp, P-glycoprotein. PPAR, peroxisome proliferator-activated receptor. PXR, pregnane X receptor. RIS, relative induction score. RXR, retinoic X receptor. TCDD, 2,3,7,8-tetrachlorobenzo-p-dioxin. UGT, Uridine diphosphate glucuronosyl transferase. VDR, vitamin D receptor. XREM, xenobiotic responsive enhancer module.

Abstract

Cytochrome P450 (CYP) induction is one of the factors that can affect the pharmacokinetics of a drug molecule upon multiple dosing, and can result in pharmacokinetic drug-drug interactions with coadministered drugs causing potential therapeutic failures. In recent years, various *in vitro* assays have been developed and used routinely to assess the potential for drug-drug interactions due to CYP induction. There is a desire from the pharmaceutical industry and regulatory agencies to harmonize assay methodologies, data interpretation, and the design of clinical drug-drug interaction studies. In this paper, a team of ten scientists from nine Pharmaceutical Research and Manufacturers of America (PhRMA) member companies conducted an anonymous survey among PhRMA companies to query current practices with regards to the conduct of *in vitro* induction assays, data interpretation, and clinical induction study practices. The results of the survey are presented in this paper, along with reviews of current methodologies of *in vitro* assays and *in vivo* studies, including modeling efforts in this area. A consensus recommendation regarding common practices for the conduct of CYP induction studies is included.

Introduction

The convergence of recent advances in molecular biology and genomics, higher throughput chemical synthesis and automated high-throughput *in vitro* enzyme and cell-based assays to assess biological activity has led to shorter lead identification and optimization times in drug research. However, these breakthroughs have not yet translated into success in drug development. Surveys suggest that in the last several years, the number of New Drug Applications submitted to regulatory agencies has declined and that the poor success rate during drug development is to some extent due to late stage failures. It is important that novel and innovative approaches are used for assessing the risks and benefits of new molecular entities (NME) early in the Research and Development (R&D) life cycle to minimize late stage attrition.

Based on a survey conducted in 2004, the major factors for compound attrition during clinical development are lack of efficacy, toxicity and safety, and sub-optimal pharmacokinetics and/or bioavailability, with the remaining failures due to financial and/or commercial reasons (Kola & Landis, 2004). In that survey, one notable observation was the reduction of compound attrition due to pharmacokinetic and/or bioavailability issues from pre-1991 to the period between 1991 and 2001. This could be, in part, due to the development and adaptation of *in vitro* drug metabolism assays using human biomaterials in the lead optimization stage, thereby minimizing the potential for unacceptable disposition profiles in humans. However, there is still a need to develop better predictive models for further minimization of compound attrition due to pharmacokinetic issues.

Metabolism by the cytochrome P450 (CYP) monooxygenases is an important determinant of the

pharmacokinetic disposition of a majority of marketed drugs. The expression of this diverse group of enzymes varies widely in the human population. These enzymes are subject to inhibition, and some of them to induction by xenobiotics, leading to pharmacokinetic drug-drug interactions between two or more coadministered drugs (Tucker et al., 2001). The earliest observations that the drug metabolizing enzymes are inducible were made in animals. Polyaromatic hydrocarbons and phenobarbital were found to increase the catalytic activities of liver microsomal enzymes (Conney et al., 1956, Richardson et al., 1952). In humans, many drugs, such as rifampicin, phenobarbital and carbamazepine, are known to cause clinically significant decreases in the systemic exposure of certain coadministered drugs, which has been shown to be due to induction of the CYP isoforms responsible for the metabolism of those drugs. A representative set of examples is summarized in Table 1. In addition to drugs, ethanol, cigarette smoking and herbal products such as St. John's wort are also capable of inducing certain CYP enzymes. Several review articles have been published that detail the biochemical mechanisms involved in CYP induction and the clinical importance of CYP induction (Bresnick et al., 1984, Park et al., 1996; Ma and Lu, 2007).

CYP induction can potentially lead to decreased systemic exposure of the inducing compound, if it is metabolized by the induced enzyme (auto-induction, e.g., carbamazepine) or decreased systemic exposure of a coadministered compound that is subject to metabolism by the induced enzyme. The magnitude of *in vivo* inductive effects varies widely among the different CYP isoform-mediated activities, i.e., minor effects seen with CYP1A2 inducers to major effects seen with CYP3A4 inducers (Table 1). Enzyme induction can potentially lead to sub-therapeutic concentrations of the affected drug. In addition, if the affected drug is metabolized to an active

or toxic metabolite, the pharmacological and toxicological outcomes in the induced state will be different from that in the uninduced state. Hence, it is important to assess the potential for CYP induction early in the R&D cycle and to plan appropriate clinical drug-drug interaction studies.

Recognizing the importance of drug-drug interactions in the overall clinical safety profile of medications, the United States Food and Drug Administration (US FDA) issued a *Guidance to Industry* in 1997 for *in vitro* and in 1999 for *in vivo* studies, and a draft Guidance for comments in 2006. The PhRMA Drug Metabolism and Clinical Pharmacology Technical Groups published a White Paper on the conduct of *in vitro* and *in vivo* drug-drug interaction studies in 2003 (Bjornsson et al., 2003). Also, Hewitt et al. (2007a, 2007b) recently published two articles summarizing an informal survey and recommendations for the conduct of CYP induction assays. Pursuant to the Critical Path Initiative by the US FDA, PhRMA formed a Pharmaceutical Innovation Steering Committee (PISC), which is charged with working on various scientific topics of interest to the pharmaceutical industry in regard to *Predictive Models of Safety, Efficacy, and Compound Properties.* The present topic focuses on *Predictive Models of Compound Properties*, outlining work to assess the predictability of various *in vitro* experimental models currently used across the industry to predict drug-drug interactions due to CYP induction. The key objectives of this effort were to: 1) identify the current practices employed by PhRMA member companies; 2) collate information on methods and models; 3) assess the success/failure of predictability based on current methods and models; 4) identify areas with the greatest need for better predictive methods and models; 5) stimulate interest and promote research for the development of better predictive methods; and 6) foster the development of general methodologies and frameworks which may decrease compound attrition during drug

development.

The PhRMA Drug Metabolism Technical Group has formed an expert team from member companies to steer this effort. An anonymous survey, comprising 61 questions, was conducted to gather information about the current practices for nuclear receptor assays, immortalized hepatocyte assays, human hepatocyte assays, clinical induction studies and new technologies. The responses for the survey were received from fourteen PhRMA member companies, which included ten large companies (> 10000 employees) and four medium-sized companies (1000-10000 employees). Information gathered from this survey is presented in this paper, along with a set of consensus recommendations surrounding the conduct of these assays and data interpretation. Detailed survey data are presented as supplemental information associated with this paper.

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Nuclear Receptor Assays

The primary mechanism by which drugs or xenobiotics can cause enzyme induction is by the activation of gene transcription. Most commonly, a compound is considered an inducer if it activates a nuclear receptor, thereby causing increased expression of the receptor's target genes. The nuclear receptors that have the broadest ligand selectivity and are most commonly involved in the activation of transcription of drug-metabolizing enzymes and transporters are the aryl hydrocarbon receptor (A*h*R), pregnane X receptor (PXR, NR1I2), and constitutive androstane receptor (CAR, NR1I3). Whereas the AhR is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family, CAR and PXR are members of the nuclear hormone receptor superfamily. Target genes for these receptors of interest to the study of drug metabolism include various Phase I (e.g., members of the CYP1A, -2B, -2C and -3A subfamilies) and Phase II enzymes (e.g., uridine diphosphate glucuronosyl transferases (UGTs), glutathione S-transferases (GSTs), and sulfotransferases (SULTs), and drug transporters including P-glycoprotein (Maglich et al., 2002; Kast et al., 2002; Madan et al., 2003; Girault et al., 2005; Donato et al., 2005; Jigorel et al., 2006; Hartley et al., 2006). Activation of other nuclear receptors such as the farnesyl X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs) and the vitamin D receptor (VDR) has been associated with induction of genes relevant to drug metabolism. In addition, induction of Phase II enzymes and NAD(P)H:quinone oxidoreductase (NQO1) in response to antioxidants and chemopreventive chemicals can be mediated by a transcription factor known as nuclear factor erythroid 2-related factor 2 (Nrf2) (see Ripp, 2008, and references therein). However, since FXR, LXR, PPAR, VDR, and Nrf2 have much narrower substrate specificity and activate a more limited set of

genes, they are not typically associated with DDIs and therefore are not covered further in this paper.

Recently, the term "Xenobiotic-Activated Receptor" (XAR) has been proposed to describe the receptors that are involved in the activation of transcription of genes responsible for the metabolism and disposition of drugs and xenobiotics, including CYPs (Ma, 2008).

In silico models of nuclear receptors - Human PXR

PXR consists of an N-terminal DNA-binding domain and a C-terminal ligand binding domain. It forms a heterodimer with the retinoic X receptor (RXR, NR1B2). Several crystal structures of the human PXR (hPXR) with xenobiotic ligands have demonstrated that the hPXR binding site is unusually large and flexible. The structure of the hPXR ligand binding domain consists of a three-layered α-helical sandwich with a unique five-stranded anti-parallel β-sheet and a very large cavity (Poso and Honkakoski, 2006). The large and flexible binding pocket explains why human PXR responds to a wide range of drugs, xenobiotics and endogenous compounds, both varying in structure and molecular weight (e.g., paclitaxel (854 Da) and phenobarbital (232 Da)). It is important to note that the nature of the ligand binding pocket allows compounds to bind in different configurations (three were observed for SR12813; Watkins et al., 2001), although it appears that flexibility was reduced in the presence of the transcriptional co-activator SRC-1 (Watkins et al., 2003).

Detailed knowledge of the structure of PXR and the understanding of how ligands bind to PXR have resulted in attempts to develop a structure-activity relationship (SAR) for PXR binding or activation. Responses to the survey indicated that only two of the participating companies (14%)

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have evaluated Quantitative Structure-Activity Relationship (QSAR) and/or structure based approaches to predict and/or attenuate PXR activity. The challenge thus far has been that it is difficult to use *in silico* models prospectively or to establish SAR, especially in attempts to apply models to new chemical space not included in the training sets. In addition, a lack of consistency has been observed between structure and activity. A number of studies have been published applying modeling approaches to identify PXR agonists. Using literature data for EC_{50} values for twelve PXR ligands, a pharmacophore model for hPXR was developed (Ekins and Erickson, 2002). This model was also used to predict the binding affinity for molecules not in the model but known to be PXR ligands with different potencies. Based on the limited number of compounds assessed, the authors concluded that the model could be used as a filter to identify compounds with high hPXR activation potential. An example where a docking approach was used to study the SAR for PXR to a series of closely related analogs was published by Gao et al. (2007). In another recent paper, Ung et al. (2007) used a combination of machine learning methods and support vector machines as a potential tool to identify novel PXR activators from structurally diverse compounds.

The same companies that evaluated PXR modeling approaches also applied in silico docking and energy minimization techniques for other nuclear receptors such as the A*h*R, estrogen receptor, VDR, PPAR, and glucocorticoid receptor. It was indicated that these approaches were useful and that guidance could be provided to medicinal chemists by identifying novel agonists and antagonists.

In summary, application of modeling approaches to identify agonists/antagonists of nuclear receptors is an emerging field where more research is needed to evaluate the applicability of various strategies.

PXR binding assays

Several *in vitro* PXR binding assays have been described (Jones et al., 2002; Zhu et al., 2004), which are based on the displacement of a radio- or fluorescently labeled high affinity PXR ligand by test compounds. Assays are conducted with the purified PXR ligand binding domain (LBD). Interestingly, it has been reported that stability and solubility of the LBD are enhanced by co-expression with a fragment of the transcriptional co-activator SRC-1 (Jones et al., 2002).

Although *in vitro* binding assays can be employed in high-throughput mode and can be automated relatively easily, the majority of companies (93%) do not routinely employ these assays to assess the potential of compounds to cause enzyme induction. One company indicated that if binding assays are used, it is for mechanistic studies only. Most likely, the lack of routine application of binding assays is due to the lack of a strong correlation between receptor binding and transactivation. An extensive comparison between PXR binding and transactivation was conducted by Zhu et al. (2004). From 616 compounds tested, 8% demonstrated both high binding and trans-activation, 9% high binding but low trans-activation, 82% were low in both assays, and 1% showed significant transactivation but low binding. The lack of correlation between binding and transactivation for some compounds was most likely explained by the fact that there is not always a linear correlation between the binding affinity and the downstream

response generated by the binding as this correlation depends on the conformational change caused by the inducer (Zhu et al., 2004).

Nuclear receptor transactivation assays

Of the survey respondents, 64% routinely employ nuclear receptor transactivation assays to assess the potential of test compounds to cause enzyme induction. In the majority of cases PXR is being evaluated, whereas some companies also determine the activation of the A*h*R. Typically, such determinations are performed in the drug discovery phase. Activation of CAR is not assessed by any company due to the current lack of availability of an *in vitro* assay.

Whereas cell based transactivation assays are available to most companies to identify PXR agonists, the way data are expressed is variable. EC_{50} values are determined by the majority of companies, but in addition, data are expressed as a percent of a positive control, maximum stimulation as percent of a positive control, or fold increase above a positive control. Currently, 57% of the survey participants do not assess or interpret whether compounds are partial agonists in transactivation assays. One company assesses the E_{max} and ignores the EC_{50} value whereas others do not consider partial vs. full agonists.

Currently, the study of antagonism of hPXR by drugs and dietary components is an area of intense academic research. For example, sulforaphane, a biologically active phytochemical found in broccoli represses hPXR activity *in vitro* at concentrations close to those achieved in humans (Zhou et al., 2007). In addition, the phytoestrogen coumestrol (Wang et al., 2008), and members of the azole family (e.g., ketoconazole) have been identified as PXR antagonists (Ekins

et al., 2007). Although nuclear receptor antagonists have been observed by 50% of survey respondents, it is currently not clear how these data should be interpreted from a pharmaceutical industry perspective. A deeper understanding is needed whether antagonism results in downregulation of the basal expression level of PXR target genes and whether this could result in clinically relevant outcomes like toxicity or drug-drug interactions.

It is well established that species specificity exists with respect to gene response(s) to pharmaceutical agents. For example, rifampin is a potent inducer of CYP3A in rabbit and human liver, but is a poor inducer of Cyp3a in rats (Moore et al., 2003). Conversely, the anti-glucocorticoid pregnenolone 16α-carbonitrile and glucocorticoid dexamethasone are potent inducers of Cyp3a in rats and mice but not of CYP3A in human liver. The species-specific induction of CYP3A/Cyp3a has been attributed in part to the differences in amino acid sequence in the ligand binding domain of PXR in different species. In contrast, across various species such as mice, rats, rabbits and humans, the DNA-binding domain of PXR is well conserved with >96% amino acid sequence homology. Due to species specificity in PXR response, it is not possible to use human PXR data to predict the likelihood that autoinduction will be found in safety studies performed in preclinical species. Therefore, some companies (17%) employ, or are in the process of developing, PXR assays for preclinical species. Rat Pxr is of interest to many as rats are widely used in safety assessment studies and repeat dosing of Pxr agonists could result in time-dependent decreases in exposure due to auto-induction.

PXR assay formats and recommended assay conditions

PXR assays are generally conducted with carcinoma cell-lines such as HepG2 or HuH7 transiently or stably transfected with PXR expression and reporter constructs (El Sankary et al., 2001; Yueh et al., 2005; Sinz et al., 2006; Jones et al., 2002; Gao et al., 2007). As response elements in the reporter constructs, the CYP3A4 proximal promoter region (e.g., bp -362 to +64 or bp -568 to +50) linked to the distal xenobiotic responsive enhancer module (XREM; e.g., bp -7839 to -7208 or bp -7836 to 7106) is generally used. These regulatory elements are positioned just upstream of reporter genes such as luciferase, chloramphenicol acetyltransferase, or secreted placental alkaline phosphatase (SEAP).

After challenging the transfected cell lines with test compounds for 24-48 hours, PXR activation is determined by measuring reporter gene activity. The number of replicates and test compound concentrations used vary between the various companies, and depend on the stage of drug development that the assay is conducted, and the variability of the assay. Replicates vary from 2-6 and the number of test compound concentrations tested vary from 5-10. Most companies are using a 96-well based plate format, although 24- or 384-well formats are also being used.

PXR transactivation: data interpretation

PXR assay results have been used to generally rank order compounds (El Sankary et al., 2001), or qualitatively categorize the potency of PXR activators (e.g., low, moderate and high DDI potential) (Persson et al., 2006; Sinz et al., 2006). PXR activation data is often expressed as percent activation (% Act) compared to a positive control, where the "total signal" is the signal from the positive control (e.g., 10 μM rifampicin) and the "blank signal" from the solvent treated

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wells (Sinz et al., 2006). Data are then plotted as percent activation *vs.* log[inducer concentration] as described by Eq. 1.

$$\%Activation = \frac{CompoundSignal - BlankSignal}{TotalSignal - BlankSignal} \times 100\% \qquad \text{Eq. 1}$$

It is recommended to also calculate the activation data as a percent of the positive control response to compare inter-day study results. As cytotoxicity and solubility issues will be encountered with some compounds, EC_{50} curves cannot always be derived. In such cases, the maximum percentage of transactivation, and the compound concentration at which this activation is measured are helpful in interpreting the induction potential of a compound (Sinz et al., 2006). It is recommended that knowledge about limits of cytotoxicity, metabolic stability and solubility are considered when interpreting transactivation assay results.

Values of E_{max} and EC_{50} from *in vitro* data are typically calculated by non-linear regression analysis of the fold-activation response above the vehicle control vs. inducer concentration. A common equation used for transactivation assays is described by the sigmoidal E_{max} model (Eq. 2; Ripp et al., 2006; El Sankary et al., 2001; Hariparsad et al., 2008).

$$Effect = \frac{E_{max} \times [\text{Ind}]^{\gamma}}{EC_{50}{}^{\gamma} + [\text{Ind}]^{\gamma}} \qquad \text{Eq. 2}$$

where E_{max} is the maximal *in vitro* induction effect, EC_{50} is the concentration to reach half the maximal *in vitro* induction effect, also referred to as the *in vitro* induction potency, [Ind] is the concentration of inducer in the *in vitro* assay, and γ is the Hill or sigmoidicity coefficient that accommodates the shape of the curve. Alternately, the sigmoidal dose-response Hill model has

been used to describe induction data (Eq. 3; Persson et al., 2006; Kanebratt and Andersson, 2008).

$$Effect = E_{\min} + \frac{E_{\max} - E_{\min}}{1 + \left(\frac{EC_{50}}{[Ind]}\right)^{k}} \qquad \text{Eq. 3}$$

where E_{max}, EC_{50}, and [Ind] are as described above and E_{min} is considered to describe the background level of the effect, and k is the slope of the curve. This model takes into account the background signal in the assay, which becomes more important if the dynamic range is low. The models described by equations 1 and 2 will result in similar values if there is a large magnitude in difference between E_{min} and E_{max} (~≥ 10-fold). The γ or k values have potential significance in predictions of clinical DDI, however its use in current DDI prediction models has not been reported.

In comparing PXR transactivation data with actual clinical DDI effects, Persson et al. (2006) used several approaches to rank order clinical DDI potential with respect to three categories: inducers, weak inducers, or non-inducers *in vivo* (or lack of evidence for induction in the literature). Some of the models used were based upon EC_{50} only, E_{max}/EC_{50} or C_{max}/EC_{50} values. Although the data were not presented in this publication, it was discussed that these three models did not successfully group the 25 compounds examined according to known CYP3A induction properties *in vivo*. When compounds were ranked based upon AUC_{tot}/EC_{50}, the CYP3A *in vivo* inducers were grouped together with the exception of primidone. Ranking the compounds by $AUC_{unbound}/EC_{50}$ clustered all *in vivo* inducers, except primidone and troglitazone. Taking into consideration the extent of the induction response (Eq. 3), did not further improve the classification of compounds.

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Sinz et al. (2006) categorized PXR transactivation data from 170 drugs and natural products with high, moderate, or low clinical induction potential based upon the percentage of transactivation response of the compound (relative to the percentage of maximal RIF response) at the efficacious $C_{max,total}$. For compounds where the percent transactivation relative to rifampicin reached a plateau, an EC_{50} was calculated from the curves. For all other compounds, the following parameters were reported: maximum percentage of transactivation, and the concentration at which maximum trans-activation was measured. The criteria for having a high potential for clinical DDI was defined as a value of >40%, between 15-40% for moderate potential, and <15% as having a low DDI potential. It should be noted that applying these rules may be confounded by cell toxicity or limited solubility at compound concentrations used in hPXR assays. Overall, the approach was found to be useful as it identified all of the potent known inducers and several moderate inducers with no false positive compounds in the high risk category.

The incorporation of clinical drug concentrations of the inducer (e.g., C_{max} or AUC) in addition to the transactivation response parameters is essential to avoid the prediction of false positives. For example, based on transactivation data, simvastatin was ranked as having a high induction propensity based on the E_{max}/EC_{50} ratio (El Senkary et al., 2001), yet simvastatin does not induce CYP3A4 at clinically relevant exposures (Prueksaritanont et al., 2000). The magnitude of hPXR activation therefore needs to be viewed in light of the drug concentrations that will be needed in the clinic for target engagement. Overall PXR data is useful for categorizing DDI potential of compounds with respect to CYP3A induction. It is amenable to screening in early drug

discovery to identify compounds causing induction via activation of PXR, as it is used by the majority of PhRMA companies surveyed.

A*h*R Assays

In the unliganded, inactive state, A*h*R resides in the cytoplasm, in complex with two molecules of the chaperone protein heat shock protein 90, the immunophilin-like X-associated protein-2, and a 23 kDa co-chaperone protein referred to as p23 (for reviews see Kawajiri and Fujii-Kuriyama, 2007; Beischlag et al., 2008). Upon ligand binding, the A*h*R is believed to undergo a conformational change that exposes a nuclear localization sequence resulting in translocation of the complex into the nucleus. Release of the ligand-AhR from this complex and its subsequent dimerization with an A*h* nuclear translocator (Arnt) protein converts the A*h*R into its high affinity DNA binding form. Binding of the complex to its specific DNA recognition sites, the xenobiotic response elements, upstream of CYP1A2 and other target genes, stimulates transcription of these genes. Known strong A*h*R ligands are 2,3,7,8-tetrachlorobenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC), and β-naphthoflavone (BNF). Interestingly, some compounds have been shown to induce A*h*R target genes but do not appear to competitively bind to the A*h*R. The reason for this is not completely clear, but includes well known A*h*R activators such as omeprazole (Daujat et al., 1992).

Most companies (89%) consider activation of the A*h*R as somewhat important but only 23% assess the induction potential of compounds in a reporter gene assay. Since HepG2 cells contain relatively high endogenous levels of the A*h*R, overexpression of the receptor by transfection is not required in these cells. Activation can be measured in a format similar to the PXR

transactivation assay by transfecting cells with a reporter construct containing the xenobiotic response element linked to luciferase or another suitable reporter gene. Positive controls typically used in such experiments are TCDD, 3-MC, BNF, and the proton pump inhibitors omepazole and lanzoprazole.

Although activation of the A*h*R is viewed as an undesirable characteristic of drugs, a current challenge is to predict what EC_{50} or E_{max} value measured *in vitro* in relation to clinical concentrations of inducer will result in a clinically significant induction response as illustrated by the example of omeprazole. In cultured hepatocytes, omeprazole induces CYP1A2 mRNA, protein and catalytic activity (Diaz et al., 1990), and the EC_{50} for activation of the A*h*R was 100 μM in a reporter assay (Quattrochi and Tukey, 1993). In a study conducted with cancer patients, omeprazole induced both CYP1A2 protein and catalytic activity significantly after a 4 day treatment period (Diaz et al., 1990). However, studies conducted in healthy Caucasian subjects showed a weak or no reduction in the exposure of CYP1A2 substrates after administration of omeprazole at therapeutic dose (Xiaodong et al., 1994; Andersson et al., 1998; Ko et al., 1999). In studies done with patients who demonstrate a poor metabolizer phenotype for CYP2C19 substrates, omeprazole decreased the exposure of the CYP1A2 substrate caffeine. Since omeprazole clearance is largely determined by CYP2C19, omeprazole plasma concentrations are markedly higher in poor than in extensive metabolizers (C_{max} 554 ng/mL and 2782 ng/mL in extensive and poor metabolizers, respectively; Uno et al., 2007). Although the plasma level of omeprazole in poor metabolizers is still markedly lower than the EC_{50} measured in an A*h*R transactivation assay, the example of omeprazole reinforces the importance of interpreting *in vitro* derived induction data in the context of systemic exposure of a drug.

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CAR assays

The relevance of activation of the constitutive androstane receptors (CAR) was valued from "don't know" to "very important" by survey respondents. This is likely a reflection of the fact that at present no company has an assay available to monitor activation of CAR *in vitro* and therefore the significance of CAR activation is difficult to assess on a routine basis. Whereas in unchallenged hepatocytes or *in vivo* CAR is sequestered in the cytoplasm, in continuously cultured cell lines it is localized in the nucleus where it is constitutively active, even in the absence of a ligand. In hepatocytes treated with CAR activators, CAR translocates to the nucleus by a complex and not completely understood mechanism (Kawamoto et al., 1999; for review see Kodama and Negishi, 2006), where it activates transcription of certain cytochrome P450, UDP-glucuronosyl transfererase, glutathione transferases and other genes (Maglich et al., 2002; Ueda et al., 2002). The activation mechanism of CAR is different from PXR, as the latter has a low basal activity and is highly activated upon ligand binding. CAR response elements have been mapped in a number of human genes, including CYP2B6, CYP3A4, members of the CYP2C family, and UGT1A1 (Swales and Negishi, 2004). Known activators of CAR are CITCO, artemisin, and phenobarbital. Interestingly phenobarbital does not bind to CAR directly, but stimulates its translocation to the nucleus by dephosphorylation of a serine residue (Kawamoto et al., 1999; Hosseinpour et al., 2006). Analysis of CAR is further complicated by the identification of a number of alternatively spliced transcripts, encoding variants with insertions or deletions in the ligand-binding domain (Auerbach et al., 2003). One of the variants, CAR3, demonstrated low basal activity and could be activated by a number of both direct and indirect CAR activators in a transactivation assay (Faucette et al., 2007). Although less

promiscuous than PXR, based on the information available, activation of CAR can cause induction of a wide range of genes but currently measuring activation of this receptor is difficult due to the lack of the availability of a *bona fide* transactivation assay. Faucette et al. (2007) have reported that CAR agonists produce stronger CYP2B6 induction compared to CYP3A4 induction.

Summary – Nuclear receptor assays

The relevance of the various nuclear receptors during drug discovery and development was rated differently by the various companies. Use of PXR activation assays to predict enzyme induction potential was considered by a majority of companies to be very important (83%), whereas CAR (54% somewhat important, 31% very important) and AhR (89% somewhat important, 11% very important) assays were generally considered somewhat important.

There are currently no regulatory requirements to measure activation of nuclear receptors in reporter assays and pharmaceutical companies mostly use these assays to guide their discovery programs. Since reporter assays are relatively high throughput and cost effective, they can be a valuable tool in drug discovery. In addition, an understanding of the molecular mechanism by which a drug causes a clinically significant positive induction response is advantageous in that it can be used as guidance to predict which other enzymes or transporters could be induced other than for instance CYP3A4. This is especially the case for PXR and A*h*R for which reliable transactivation assays are available. Due to the complexity of CAR-mediated gene regulation, *in vitro* data for this receptor are difficult to interpret at present. The use of receptor activation assays is not recommended as the sole guidance to assess the induction potential of NMEs, as it

cannot be excluded that receptors other than the ones routinely assessed could cause induction or that metabolites not formed in immortalized cell lines could be inducers. In addition, it should be realized that crosstalk between the receptors causing enzyme induction with many signal transduction pathways has been reported, suggesting that xenobiotics may affect a broad range of biological processes (for review see Pascussi et al., 2008).

DMD Fast Forward. Published on April 23, 2009 as DOI: 10.1124/dmd.109.027029
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Hepatocyte Assays

Cultured primary hepatocytes and immortalized hepatocytes are the two models used to evaluate *in vitro* induction of drug metabolizing enzymes. The experimental conditions, advantages and disadvantages of these systems are discussed below.

Primary hepatocytes assays

With appropriate culture conditions, human hepatocytes remain differentiated and retain the ability to respond to inducers for a period of time. Cultured primary human hepatocytes are the most accepted (industry, academia, regulatory) *in vitro* system for assessing the potential for drug candidates to induce human CYP expression (Hewitt et al., 2007c, LeCluyse et al., 2005). Human hepatocytes are a cellular system comprised of human receptors, co-activators and repressors, target genes and promoters, as well as human drug metabolizing enzymes capable of biotransforming drugs. These properties are analogous to the liver and are essential to effectively model the inducibility of drug candidates and their metabolites. Recently our knowledge and understanding of species-specificity of the mammalian CYP isoforms and their interactions with other drugs have increased significantly. The failure in using animal data to routinely predict enzyme induction in humans is well documented (Kocarek et al., 1995); hence the need for a human–relevant model system becomes more apparent. Primary human hepatocyte culture systems have been shown to effectively model human *in vivo* induction responses and are recognized as an effective tool for assessing induction potential (Hewitt et al., 2007c). The enzyme induction data from *in vitro* methods are known to correlate well with clinical observations, provided the *in vitro* experiments are performed at pharmacologically relevant concentrations of drug (LeCluyse et al., 2000). When hepatocytes are cultured using

appropriate conditions which facilitate liver-like cell morphology and expression of liver-specific proteins, CYP enzymes are effectively induced *in vitro* analogous to the *in vivo* situation in terms of the magnitude and specificity of induction (LeCluyse et al., 2000, Runge et al., 2000). In addition to CYP enzymes, numerous studies have been reported using primary hepatocyte culture systems to assess induction of a variety of gene targets, such as, Phase II enzymes and transporters (Kodama et al., 2004, Raucy et al., 2002, Raucy, 2003, Raucy et al., 2004). While fresh human hepatocytes are the standard for evaluating *in vitro* induction of CYP enzymes, attachable cryopreserved hepatocytes have also been used. The drug metabolizing enzymes remain inducible after cryopreservation, and due to the significant variation in activities of drug metabolizing enzymes between individual human livers, certain lots of cryopreserved cells can generate results essentially indistinguishable from freshly isolated cells (Hewitt et al., 2007c, Schehrer et al., 2000). Catalytic activities, mRNA and protein expression of CYP1A2, 2B6, 2C9, 2E1 and 3A4 in cryopreserved hepatocytes have been shown to be inducible by standard CYP inducers (Roymans et al., 2005). The advantage cryopreserved cells offer over fresh isolates is that experiments can be planned in advance and are not dependent on the sporadic availability of fresh primary hepatocytes.

In general, a test compound is evaluated in hepatocyte cultures from several different donors, in addition to a vehicle control and a positive control for 2-3 days, with medium changed every 24 hours during incubation. Test compounds are evaluated at or around anticipated therapeutic concentrations and the positive control incubation is used to assess the suitability of each donor preparation and often as a comparator to induction observed by the test compound. Enzyme induction potential can be evaluated by measuring increases in enzyme activities, mRNA levels

or protein expression (Western immunoblotting). The data from cultured hepatocyte experiments can take several forms, such as, fold or percent increase compared to the vehicle control, percent increase compared to the positive control, or EC_{50} determination based on data generated from enzyme activity or mRNA expression.

The survey indicates that nearly all companies use primary human hepatocytes to characterize the enzyme induction potential of compounds in drug development and the majority of companies also use them to evaluate enzyme induction in drug discovery. As noted in the past, the major limitation to the use of primary human hepatocytes continues to be cost and availability. Many companies conduct hepatocyte experiments in-house with cells purchased from commercial vendors, moreover most companies indicated that outsourcing to contract research organizations was commonly used in combination with in-house experiments. A few companies indicated that they isolated human hepatocytes in-house or obtained cells from hospitals or universities.

Researchers indicated that they are comfortable using both freshly isolated hepatocytes and cryopreserved-attachable hepatocytes because it has been shown that induction responses are similar between fresh hepatocytes and attachable lots of cryopreserved hepatocytes (Hewitt et al., 2007c, Roymans et al., 2005). Respondents did indicate that in drug discovery cryopreserved cells were more often used due to increased availability while combinations of fresh and cryopreserved were used in drug development. Further, three donors were routinely evaluated when assessing enzyme induction of compounds in development while only one donor was typically used in drug discovery. Donor characteristics that should be avoided because they were

considered undesirable and could affect the outcome of the experiment or viability of the cells included: a high BMI or fatty livers; liver disease, such as viral infections; and very young or elderly donors (< 6 mo or >60 yr of age). The most common culture format was equally distributed between collagen monolayer and collagen-matrigel sandwich culture. Depending on the endpoint (RNA expression or enzyme activity) the plate formats varied slightly, although the most common format for both applications was the 24-well plate. When only RNA expression was considered, 24- and 96-well plates were the most common, whereas when only enzyme activity was measured, 24-, 48-, and 96-well formats were most commonly employed. However, when enzyme activity was to be measured from microsomes isolated from cultured hepatocytes, Petri dishes or 6-well plates were often employed. Once plated, the cells were generally allowed 1-2 d to recover or adapt to the cell culture conditions.

A variety of cell culture media were described in the survey responses (William's E, InVitroGRO, DMEM, hepatocyte maintenance, Chee's, HepatoSTIM) yet the most common were William's E and InVitroGRO HI. Regarding media supplements, common additions included ITS (insulin, transferrin, selenium), dexamethasone, and antibiotics (penicillin-streptomycin), similar to what is commonly reported in the literature (LeCluyse et al., 2005). Other less commonly described media additions included FBS, linoleic acid, and anti-fungals. In some instances there was mention of different media/supplements for plating and culturing cells. Most common was the addition of serum to the media after plating which was then removed and the experiment continued with serum-free media. Test compounds or positive controls were typically dissolved in DMSO and incubated at a final concentration of 0.1% DMSO. Other solvents, such as acetonitrile, methanol, ethanol, and higher concentrations of DMSO were noted

as alternative dissolution methods; however they were typically employed only when 0.1% DMSO was not applicable. The minimum number of testing concentrations was three but ranged up to ten, and duplicate or triplicate replicates were generally employed.

The enzyme most routinely evaluated was CYP3A4 while enzymes such as CYP1A2, CYP2B6, and CYP2C9 were also often evaluated. Other enzymes and transporters such as CYP2C8, CYP2C19, UGT1A1 and MDR1 were occasionally evaluated. The most frequently used positive controls were as follows: CYP1A2 (omeprazole, 20-50 μM); CYP2B6 (phenobarbital, 750-1000 μM); CYP2C9 and CYP3A4 (rifampicin, 10-25 μM). Other less common positive controls were β-naphthoflavone (10-30 μM) for CYP1A2 and CITCO (2 μM) or rifampicin (10 μM) for CYP2B6. The length of cell treatment with positive controls and test compounds overlapped between RNA expression and enzyme activity measures, but were generally 2 and 3 day treatments, respectively. In order to have confidence in the data generated from test compounds, researchers have adopted various levels of acceptance criteria for donor preparations based on the RNA or activity response of positive controls. In general, 2-10 fold increases in RNA expression or 2-4 fold increases in enzyme activity were noted as acceptable donor responses to positive controls; however the minimum of 2 fold was the most common response by far for enzyme activity.

The most common endpoint for assessing enzyme induction was measuring enzyme activity (100% of respondents) with the majority of respondents also measuring RNA expression (77%). When measuring enzyme activity, the single probe substrate incubated *in situ* was the most common method with a much smaller percentage of researchers using cassette probes *in situ*.

The second most common method of measuring enzyme activity was by means of microsomal incubations (i.e., microsomes isolated from plated hepatocytes). As to measuring RNA expression, the most common method was RT-PCR followed by several other methods, such as branched DNA, nuclease protection, Invader®, and Affymetrix®. Both RNA expression and enzyme activity data require normalization to an endogenous component or cell number. In the case of mRNA expression, housekeeping genes, such as GAPDH, 18S, or actin are recommended. For normalization of the catalytic activity, cell number, total protein, or DNA content can be used.

Immortalized hepatocytes assays

Primary human hepatocytes are commonly used in enzyme induction studies; however, their limited supply and significant donor-to-donor variation complicate their application in early drug discovery. Consequently, there is a need to generate human hepatocyte-like cells that provide a continuous supply while maintaining stable expression of necessary enzymes, transporters, and nuclear hormone receptors for routine screening and characterization of enzyme induction. Immortalized hepatocytes are one example of cells that can grow and divide indefinitely under optimal culture conditions and can occur naturally (e.g., tumorigenic cells isolated from hepatocarcinomas) or by converting primary hepatocytes into non-tumorigenic immortalized cells (Sinz and Kim, 2006).

Several lines of hepatocarcinoma cells (e.g., HepG2, BC2, HepaRG) have been evaluated for their ability to mimic the enzyme induction response of primary human hepatocytes. Among them, the HepG2 cell line has been well characterized and is widely used throughout drug

metabolism and toxicology testing. HepG2 cells have demonstrated robust induction response to CYP1A inducers; however, due to the low basal expression of enzymes and little to no induction response to known CYP3A4 inducers, HepG2 cells are not considered an appropriate model to study drug-drug interactions (Harmsen et al., 2008, Westerink et al., 2007). Gomez-Lechon et al. (2001) performed an extensive analysis of the biotransformation properties of BC2 cells by measuring basal and inducible expression of Phase I and II enzymes. Their results showed an increase in CYP1A1/2 enzyme activity (8-fold) by methylcholanthrene, CYP2B6 activity (1.7-fold) by phenobarbital, and CYP3A4 activity (5-fold) by dexamethasone. HepaRG cells, when cultured to confluency for several weeks under specific culture conditions, develop into a highly differentiated hepatocyte-like cell line (Gripon et al., 2002). A comprehensive expression analysis performed by Aninat et al. (2006) showed that RNA expression of major transcription factors (PXR, AhR) were similar between primary human hepatocytes and differentiated HepaRG cells and the expression of CAR was 20-30% of that found in human hepatocytes. Kanebratt and Andersson (2008) have demonstrated increases in mRNA and activity using the HepaRG cell line and prototypical inducers. However, the expression of enzymes and nuclear receptors, as well as the response to enzyme inducers has been shown to vary depending on the media composition and culture conditions (Aninat et al., 2006).

The Fa2N-4 cell line is derived through immortalization of primary human hepatocytes using the SV-40 large T antigen. Using known inducers of CYP enzymes, Mills et al. (2004) reported concentration-dependent increases in both transcript levels and enzyme activities of CYP3A4, CYP2C9 and CYP1A2. These changes were comparable to induction responses observed in primary human hepatocytes. In addition, UGT1A and MDR-1 were also induced by rifampicin

treatment in this cell line. Unfortunately, Fa2N-4 cells have very low expression of CAR and several drug transporters, therefore CAR-mediated induction of CYP2B6 cannot be evaluated, the induction of CYP3A4 may be attenuated, and cellular uptake of drugs may be reduced or altered (Hariparsad et al., 2008).

LS180 is a human colon carcinoma cell line routinely used and well-characterized in studying intestinal absorption of drugs and regulation of CYP3A4 and MDR-1. In LS180 cells, CYP3A4 and MDR-1 have been shown to be responsive to induction stimuli by reserpine, rifampicin, phenobarbital and verapamil, as well as induction of CYP1A2 when exposed to the prototypical CYP1A inducer, omeprazole (Brandin et al., 2007, Schuetz et al., 1996). Overall, LS180 cells appear to be a suitable model to study the regulation of CYP3A4 and MDR-1 in the intestine, although there is evidence to suggest that this cell line may not recapitulate the induction response in liver tissue or hepatocytes due to altered levels of the nuclear receptor corepressor (NCoR) (Zhou et al., 2004).

The survey results indicate that a majority of companies do not use immortalized hepatocytes for routine screening, however ~30% of companies use the Fa2N-4 cell line in drug discovery to assess CYP1A and CYP3A4 enzyme induction. As an example, Ripp et al. (2006) developed a higher-throughput induction assay using Fa2N-4 cells in 96-well plates and produced detailed concentration-response curves for known inducers of CYP3A4. Although the Fa2N-4 cell line has the ability to evaluate induction of other enzymes, as well as transporters, none of the companies indicated evaluation of induction beyond CYP1A and CYP3A4. The most common experimental conditions were 3 day treatment of cells with about 5 concentrations of test

compound (in triplicate) in a 24-well plate format. The common positive controls used were rifampicin (10 μM) and omeprazole (10 μM). Enzyme activity using probe substrates and RNA expression by RT-PCR were the common endpoints measured in each assay. Interpretation of results was generally characterized by percent activity or RNA expression compared to the positive control.

In summary, immortalized hepatocytes appear to be useful in evaluating well characterized induction mechanisms (e.g., PXR- or AhR-mediated). However, no one cell line affords an exact reproduction of a hepatocyte and most companies felt that these cell lines are not fully understood or characterized. For example, unknown expression of nuclear receptors or transcription factors, as well as drug transporters and cytotoxic effects were noted as variables that are not fully understood across cell lines. Therefore, due to these limitations, we do not consider immortalized hepatocyte cell lines are an adequate replacement for primary human hepatocytes; however, they may be used to complement the use of primary human hepatocytes, especially for higher-throughput applications in drug discovery and early stages of drug development.

Hepatocyte assays - Interpretation of data

Interpretation of hepatocyte induction data is a highly debated topic with respect to predicting clinical outcome even though several methods exist for assessing the drug-drug interaction potential of enzyme inducers. All of the respondents indicated they used the 'percent of positive control' in the interpretation of enzyme induction data and nearly half of the respondents also used 'fold induction above vehicle control' as well.

The "40% of positive control" criteria, originating initially from the PhRMA position paper (Bjornsson et al., 2003), was set in order to establish a true positive induction signal in *in vitro* assays. The value was set particularly for activity data as, typically, the dynamic range of mRNA induction is greater. In the case where the maximal induction levels of the positive control are only 4- to 5-fold (in the range of acceptability), anything less than 40% of this would be in the background of the assay. In some cases where positive control inducers (e.g. for CYP1A2) elicit a very high induction response, discretion must be taken as to whether the 40% cut-off criteria is appropriate for induction of CYP activity *in vitro*. From the current survey, it is clear that better predictive models are needed to aid in understanding the correlations between *in vitro* data and *in vivo* effect with respect to induction. In particular, those models that relate the *in vitro* induction response to clinically relevant exposures, either as a correlation (described later) or more physiologically-based models, which take into account the pharmacokinetics of the inducer and affected substrate, transporters, and mixed mechanisms (CYP reversible and time-dependent inhibition and induction) would be of most value. In addition to the 'percent of positive control', 15% of the respondents also determined EC_{50} values in this assay (be it mRNA, activity or protein level; the endpoint assay was not discerned). From the comments made, most companies are, however, relating the concentration-dependent induction response to known or expected clinical exposures.

When assessing 'percent of positive control' or other ratio methods that incorporate therapeutic drug concentrations, most often the C_{max} total drug concentration was used (71%) although some researchers indicated the use of unbound C_{max} drug concentration (36%). Even though the

unbound C_{max} drug concentration conforms to the "free drug hypothesis" and theoretically may be more accurate, the more conservative approach of using total C_{max} drug concentration appears to be predominant when assessing enzyme induction potential. The section on *in vitro* to *in vivo* correlations (IVIVC) contains further discussions on data analysis and interpretation. Finally, the actual therapeutic drug concentration may not be known with a great deal of confidence during preclinical testing or early human clinical trials, therefore it is common to evaluate enzyme induction over a range of drug concentrations. The survey indicates that most researchers would consider enzyme induction as being significant when increases in enzyme activity are observed within 2- to 5-fold of the 'anticipated' C_{max} at therapeutic dose.

Several common situations were noted in the survey responses when interpreting enzyme induction data. They include: 1) increases in RNA or enzyme activity at low drug concentrations with decreases in RNA or activity at higher concentrations (inverted U-shaped response curve); 2) increases in RNA expression with little or no increase in enzyme activity, and 3) an enzyme induction response is observed in only one of several donor preparations. The first situation (inverted U-shaped response) is typical of compounds that exhibit poor solubility in culture media or cytotoxicity. Although drug dissolution in the original neat DMSO solution is readily discernable, the survey indicates that only 46% of researchers confirmed the solubility of test compounds in culture media. However, most survey respondent's indicated that cytotoxicity was commonly assessed during the course of an experiment. The most common methods for assessing cytotoxicity were cell morphology, enzyme leakage, ATP content, or mitochondrial function (MTT). Even though solubility and/or cytotoxicity are often used to explain inverted U-shaped dose response curves, there have been several cases reported for which solubility or

toxicity were not the apparent cause (Raucy, 2003, Ripp et al., 2006). Therefore, the inverted U-shaped dose response curve may in some cases represent a true underlying pharmacology that is incompletely understood at this time. In the second case of increased RNA expression with little to no increase in enzyme activity, this example is common when the test compound is both an inducer and inhibitor of the same enzyme. The RNA expression is unaffected by the enzyme inhibition properties, however the enzyme activity measurements can be confounded by concomitant inhibition and induction. This situation is commonly encountered when enzyme activity is measured *in situ* and the test compound is not adequately removed from the hepatocyte culture, however this situation can often be overcome by measuring enzyme activity with isolated microsomes. Also, removing the drug from the culture system will eliminate the inhibition properties of reversible inhibitors, but often the test compounds are both reversible and irreversible inhibitors (mechanism-based or time-dependent inhibitors). In this case the enzyme becomes inactivated or non-functional due to the affects of the irreversible inhibitors. The interpretation of this phenomenon is more difficult and complex; the drug is clearly an inducer (based on RNA expression information) however the prediction of anticipated drug-drug interactions will be affected by the simultaneous induction-inhibition properties of the test compound. The most well known drug that exhibits these properties is ritonavir which predominantly presents as an inhibitor of CYP3A4 yet in some patients evidence for drug interactions due to enzyme induction have been reported (Hsu et al., 1998). Due to this interplay between inhibition and induction of CYP enzymes, we recommend using both enzyme activity and RNA expression endpoints to fully understand and interpret enzyme induction in cultures of primary human hepatocytes. Ultimately, the drug-drug interaction (pharmacokinetic changes), if needed, should be evaluated in well designed clinical drug interaction studies, as the *in vitro* data

will be difficult to extrapolate to *in vivo* effects. Finally, when only one of several donor hepatocyte preparations indicates a positive induction response, the result cannot be ignored or discounted as an outlier. However, this type of result will typically elicit additional mechanistic studies or repeat hepatocyte studies to better understand the spurious nature of the positive result.

Hepatocyte Assays- Recommendations

The most commonly used and recommended experimental protocol for assessing enzyme induction in primary human hepatocytes for regulatory submissions is as follows.

- Fresh or platable cryopreserved hepatocytes, as either monolayer or sandwich culture, with a 1-2 day recovery period after plating.
- Treatment with NME and positive controls for 2-3 days (changing medium with test compounds every 24 hours) in media containing ITS, dexamethasone, and penicillin-streptomycin as media supplements.
- Test compounds dissolved preferably in DMSO (v/v 0.1%) whenever possible and incubated at 3 or more different concentrations (in triplicate), spanning anticipated or known therapeutic concentration range including a concentration at least an order of magnitude higher. Alternate solvents and NME concentration ranges may be employed, as appropriate.
- The recommended positive controls include omeprazole (25-50 μM), phenobarbital (1000 μM) and rifampicin (10 μM) for CYP1A2, 2B6, and 3A4, respectively, at concentrations known to elicit maximal induction response.
- Assessment of cytotoxicity of the NME under the experimental conditions.

- Knowledge of aqueous solubility characteristics and visual assessment of solubility in the culture medium at the concentrations tested.
- Catalytic activity and mRNA assessments for CYP1A2, CYP2B6 and CYP3A4.
- For a hepatocyte induction experiment to be acceptable, CYP1A2, CYP2B6 and CYP3A4 positive controls should exhibit ≥2-fold vehicle control catalytic activity and ≥6-fold vehicle control mRNA level.
- At least three donor hepatocytes, with experiment meeting the acceptable criteria for each donor.
- A positive result in at least one of the three donor hepatocytes is considered an indication of induction.
- Interpretation of results are conducted by an empirical approach such as 'percent change compared to the positive control' or a mathematical or correlation based approach, using the therapeutic C_{max} drug concentration at steady-state as benchmark. The use of free (unbound) or total (bound and unbound) drug concentrations for data interpretation may be based on the available historical data and/or models employed at each laboratory.

In Vitro/ In Vivo Correlations (IVIVC)

Some common approaches to data interpretation and IVIVC have been briefly discussed in the previous sections on transactivation and hepatocyte assays. Since IVIVC is a topic of critical importance, a more in depth analysis is presented below. This discussion is based on the review of the current literature, implicit information from the survey, and analyses by the authors, and is not based on specific survey results.

DMD Fast Forward. Published on April 23, 2009 as DOI: 10.1124/dmd.109.027029
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It can be appreciated that whatever *in vitro* end-point used to assess induction potential (e.g., nuclear receptor transactivation, mRNA or enzyme activity from human hepatocytes), the magnitude of the maximal *in vitro* induction effect (E_{max}), *in vitro* induction potency (EC_{50}), character of the interaction (e.g. full or partial agonists, etc.), and an idea about actual clinical exposure are all needed to determine whether or not a compound will likely be an inducer of CYP enzymes in humans *in vivo* (Smith et al., 2007). The number of different published models to predict the clinical outcome of CYP induction are limited; however the numbers of reports, particularly of modeling CYP3A induction *in vivo*, have increased very recently. Those models that have been reported are based upon the same concept, that being the law of mass action for receptor binding (Ripp et al., 2006; Kato et al., 2005; Kanebratt and Andersson, 2008; Shou et al., 2008; Fahmi et al., 2008a; Fahmi et al., 2008b). The E_{max} model (Eq. 4) is generally used to describe an induction effect based upon EC_{50} and E_{max} values obtained in *in vitro* assays in relation to an inducer concentration ([Ind]), i.e. a therapeutic effective *in vivo* concentration.

$$Effect = \frac{E_{max} \times [Ind]}{EC_{50} + [Ind]} \qquad \text{Eq. 4}$$

The endpoints measured in primary (or immortalized) human hepatocytes are typically CYP3A mRNA and/or enzymatic activity. As to what *in vitro* end-point is more amendable to predictions of clinical outcomes, mRNA data is most often used and is considered appropriate based upon several factors: 1) the induction process involves receptor binding and transactivation of the gene transcription, and hence mRNA production is a more direct measure of this event than enzyme activity, 2) measurement of mRNA provides a facile measure with better dynamic range than enzyme activity measurements, 3) measurement of CYP activity *in situ*, which is the most common means for assessing enzyme activity, can potentially result in false negatives in the case of time-dependent or mechanism-based inhibitors, or potent reversible

inhibitors with high metabolic stability in hepatocyte cultures. To this point, a good correlation between CYP3A4 mRNA and activity increases with inducers has been found in human hepatocytes when time-dependent inhibitors (mechanism-based inhibitors or formation of metabolites that are potent reversible inhibitors) are excluded (Fahmi et al., 2008a).

PXR transactivation data

As described in a previous section, PXR transactivation assays have been used to generally rank order (El Sankary et al., 2001) or qualitatively categorize the *in vivo* potency of CYP3A inducers (e.g. high, moderate, low DDI potential) with respect to known clinical DDI (Persson et al., 2006; Sinz et al., 2006). Direct extrapolation of PXR activation data for quantitative prediction of DDI magnitude *in vivo* has not been reported at this time. It remains to be determined if it has quantitative DDI prediction potential with respect to CYP3A induction, for instance, as it may have its limitations due to the induction of CYP3A by other mechanisms, e.g., CAR.

Human hepatocyte induction data

Modeling approaches to predict clinical DDI magnitude of CYP inducers using *in vitro* data, have only recently been reported. The models have been almost exclusively for predictions of CYP3A induction, but may be amendable to the induction of other CYPs. The relatively limited reports of clinical induction with respect to other CYP enzymes is likely the reason that most models have been validated against CYP3A. There have been, in general, two types of approaches taken: Mathematical Prediction Approaches and Correlation (calibration curve) Approaches, both of which are described below.

DMD Fast Forward. Published on April 23, 2009 as DOI: 10.1124/dmd.109.027029
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Mathematical Prediction Approaches

Prediction models have been reported that incorporate *in vitro* induction data expressed in the sigmoidal E_{max} model (Eq. 1) or E_{max} model (Eq. 4) into mathematical equations to predict changes in AUC of CYP3A substrates based upon the assumptions of the "well-stirred liver" model for hepatic blood clearance. The first report to quantitatively predict clinical DDI magnitude with respect to CYP induction using *in vitro* induction data in the E_{max} model was by Kato et al. (2005). More recent reports have incorporated the fraction of the substrate metabolized by the affected CYP enzyme (f_{mCYP}) into the prediction model, as the magnitude of the DDI is dependent upon this value in addition to the properties of the inducer (Shou et al., 2008; Fahmi et al., 2008b). The more recent equations used to predict change in AUC of an orally administered affected CYP3A substrate by a CYP3A inducer are shown below in Eq. 5 (Shou et al., 2008) and in Eq. 6 (from Fahmi et al., 2008b, showing only the induction model). Parameters incorporated into the DDI predictions from these reports include: EC_{50} and E_{max} of CYP3A4 induction in hepatocytes (mRNA and/or activity), relevant clinical *in vivo* concentrations of inducer either as unbound or total ([Ind]), fractions of the victim drugs cleared by CYP3A (f_{mCYP}) and, in Shou et al. (2008), the fraction unbound of the inducer in hepatocytes (fu_{hept}). Fahmi et al. (2008b) also incorporated a model to account for intestinal availability of CYP3A substrates in the presence and absence of a CYP3A inducer, as well as models to incorporate concurrent CYP inhibition or inactivation (not shown). The model in Eq. 5 was used to predict the actual clinical DDI of 6 inducers in 103 clinical DDI trials from *in vitro* induction data (enzyme activity, 2 hepatocyte donors) and it was found that the best correlations (r^2 = 0.578-0.624) of the modeled outcome to actual clinical DDI were found when using unbound [Ind] and fu_{hept}. The predictability of Eq. 6 was dependent upon the value "d", a parameter

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needed for *in vitro*-to-*in vivo* scaling of the induction data (mRNA), which was optimized by linear least-squares regression that maximized the accuracy of the global data set. It was noted in Shou et al. (2008), that using mRNA data, for instance, over-predicted the DDI with RIF, and it was deemed due to the much greater E_{max} values observed for mRNA induction compared to enzyme activity. The need for a scalar or normalization to a positive control maximal E_{max} value is apparent, and mRNA data may be more affected as the dynamic range is greater than that of enzyme activity. Normalizations of E_{max} should be performed to predict clinical DDI, due to the large inter-individual variability in maximal CYP induction *in vitro* and to scale the *in vitro* effect to the *in vivo* clinical effect.

$$\frac{AUC_{po,i}}{AUC_{po}} = \frac{CL_{int}}{CL_{int,i}} = \frac{1}{f_{m,CYP} \times \left(1 + \frac{E_{max} \times [Ind]^n}{(EC_{50} \times fu_{hept})^n + [Ind]^n}\right) + (1 - f_{m,CYP})} \quad \text{Eq. 5}$$

$$\frac{AUC_{po,i}}{AUC_{po}} = \frac{CL_{int}}{CL_{int,i}} = \frac{1}{f_{m,CYP} \times \left(1 + \frac{d \times E_{max} \times [Ind]}{(EC_{50}) + [Ind]}\right) + (1 - f_{m,CYP})} \quad \text{Eq. 6}$$

In addition to the mathematical models, described above, the physiologically-based drug interaction model, Simcyp® Population-Based ADME Simulator (Simcyp Ltd, Sheffield, UK) has recently incorporated a model to predict clinical induction from *in vitro* induction parameters (Rostami-Hodjegan, 2009). This model accounts for normalization of the E_{max} value by the maximal positive control response, as well as, concurrent CYP inhibition and inactivation.

Correlation (calibration curve) Prediction Approaches

DMD Fast Forward. Published on April 23, 2009 as DOI: 10.1124/dmd.109.027029
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There has been success with more empirical approaches to predict clinical DDI with respect to CYP3A induction from *in vitro* human hepatocyte data. Induction studies using immortalized cells (Fa2N-4 and HepaRG cells) and cryopreserved human hepatocytes have been used to correlate *in vitro* CYP3A4 mRNA induction results (as a "Relative Induction Score" (RIS) or AUC/F_2 value, described in more detail below) with the actual magnitude of *in vivo* CYP3A induction (Ripp et al., 2006; Kanebratt and Andersson, 2008; Fahmi et al., 2008a). These more empirical approaches require calibration curves with positive and negative controls. This calibration curve becomes very important when using different hepatocyte donors or to account for inter-experimental variability when using immortalized cells or primary hepatocytes. It may be established that the EC_{50}s do not vary greatly and it is probable that running positive and negative controls at the concentration at E_{max} would suffice in populating a curve. In the immortalized cell line (Fa2N-4 cells) the EC_{50} and E_{max} values varied with a coefficient of variation of 28 and 37%, respectively (Ripp et al., 2006). These values are, however, likely to vary more with different primary hepatocyte donors. It is well recognized that the E_{max} value can vary considerably with donor. It is also likely that differences in cell membrane or protein binding, expression levels of nuclear receptors (e.g. PXR and CAR), co-activators, repressor proteins, and active drug transporters of different hepatocyte donors may also influence EC_{50} values. It is recommended to run full calibration curves to obtain EC_{50} and E_{max} until it is established that EC_{50}s do not vary considerably in the particular cell-line or a particular cryopreserved hepatocyte donor.

RIS Approach

The use of the RIS approach is described in Ripp et al. (2006). In this study, the researchers used Fa2N-4 cells to assess induction of CYP3A4 mRNA with various inducers and used the E_{max} model (Eq. 4) as the RIS. The RIS was plotted versus the actual clinical mean percent decrease in AUC of the CYP3A4 probe substrate, midazolam, or ethinylestradiol from the particular study referenced in Ripp et al. (2006). Compounds that were both inducers and time-dependent inhibitors of CYP3A4 within a therapeutically relevant concentration range were deliberately excluded from the Ripp et al. dataset. The IVIVC correlation was excellent using $C_{max,u}$ in the model ($r^2 \geq 0.92$) for both midazolam and ethinylestradiol. If total C_{max} was used instead of free C_{max}, the correlation dropped ($r^2 < 0.55$). Recently, the RIS approach was also evaluated using human cryopreserved hepatocytes (Fahmi et al., 2008a). The correlation between RIS calculated using E_{max} and EC_{50} values from CYP3A4 mRNA induction data and percent decrease in midazolam or ethinylestradiol exposure was also excellent ($r^2 = 0.96$ and 0.82, respectively). A benefit of the cryopreserved hepatocytes in this evaluation was a greater signal-to-noise ratio for induction, compared to the Fa2N-4 cells. In addition, Fa2N-4 cells do not express CAR and have altered expression of some hepatic transporters, which may effect intracellular inducer concentrations.

AUC/F_2 Approach

The AUC/F_2 approach, as described by Kanebratt and Anderson (2008), was also successful in correlating an *in vitro* induction response to the actual clinical DDI magnitude of CYP3A substrates. In this method, F_2 was defined as the concentration of inducer leading to a 2-fold increase of the base-line levels of CYP3A4 mRNA in the hepatocytes (in this study, HepaRG cells were used). CYP3A4 mRNA induction data were fit to a Hill equation for one site dose-

response (Eq. 2) to obtain the F_2 value. The F_2 value may also be obtained by visual inspection of the data of where a 2-fold increase is observed. This approach may be of benefit when an E_{max} value cannot be reached, such as in the case of compounds with low solubility and/or cell toxicity.

In relation to exposure (AUC), the AUC/F_2 value was used to rank highest to lowest DDI potential. The correlation between *in vitro* and *in vivo* induction in this study was assessed by plotting AUC/F_2 vs. the clinical % decrease in AUC of a CYP3A probe substrate. The data was fit to an equation, analogous to the E_{max} model (Eq. 4):

$$Effect = \frac{\%\ \text{decrease of}\ \textit{in vivo}\ \text{AUC}_{max} \times (AUC / F_2)}{\%\ \text{decrease of}\ \textit{in vivo}\ \text{AUC}_{50} + (AUC / F_2)} \qquad \text{Eq. 7}$$

A good correlation was reached when using AUC_{total} ($r^2 = 0.863$). Using unbound AUC values gave only a slightly lower correlation ($r^2 = 0.859$). When the maximum plasma concentration *in vivo* ($C_{max,\ total}$ and $C_{max,u}$) was used instead of AUC and AUC_u, the r^2 values were slightly lower (0.710 and 0.812, respectively). This approach seems to be promising, but will need further assessment with data from primary human hepatocytes.

Comparisons of Correlation (calibration curve) Approaches to Predict Clinical CYP3A induction

In an effort to compare different correlation approaches, it was desirable to evaluate these different models using a single dataset. Therefore, we have used the data from Ripp et al. (2006) to compare different correlation methods to correlate *in vitro* induction response data of CYP3A4 mRNA to actual *in vivo* clinical DDI with the CYP3A probe substrate, midazolam. The calibration approaches that were compared were the E_{max} model (Eq. 4) (or RIS) as described in Ripp et al. (2006), C_{max}/EC_{50} (used previously for rank-ordering PXR

transactivation data (Persson et al., 2006), AUC/F_2 model as described in (Kanebratt and Andersoon, 2008) and the E_{max} model multiplied by the ratio of NOEL (no observed effect level) to C_{max}. Recently, an addition to the E_{max}-based IVIVC model has been suggested (Hewitt et al., 2007a; Hewitt et al., 2007b). Hewitt et al. proposed that the highest concentration at which no induction response is observed (NOEL) *in vitro* should be incorporated in predictions of induction risk; however no IVIVC using this model has yet been published (Hewitt et al., 2007b). The E_{max}, EC_{50}, and free and total C_{max} values of the compounds evaluated were as described by Ripp et al. (2006). The AUC values used in the AUC/F_2 model were as described by Kanebratt and Andersson (2008) and for rosiglitazone (8 mg) and pioglitazone (45 mg) the AUC values used were 8.3 and 27 μM·h, respectively (Malinowski et al., 2000; Christensen et al., 2005). The AUC values of Compound A (40 and 120 mg) were 0.21 and 0.88 μM·h, respectively. The F_2 values were calculated for this data set as described by Kanebratt and Anderson (2008) or by visual inspection of the concentration vs. induction effect data.

As reported previously (Ripp et al., 2006), there was an excellent correlation with predictions of clinical DDI using the E_{max} model (RIS) and $C_{max,u}$ ($r^2 = 0.927$, Figure 1A), however using $C_{max, total}$ there was a poor correlation ($r^2 = 0.503$, data not shown). Using the same data set, the AUC/F_2 and C_{max}/EC_{50} correlation models using unbound $C_{max,u}$ also found excellent correlations with *in vivo* induction results ($r^2 = 0.932$ and 0.927, Figure 1B and 1C). Poor correlations were found using $C_{max, total}$ in these models ($r^2 = 0.663$, AUC_{total}/F_2; $r^2 = 0.537$, $C_{max,total}/EC_{50}$, data not shown). Poor correlations were also found with incorporation of the $NOEL/C_{max}$ in the E_{max} equation using total or unbound C_{max} concentrations ($r^2 = 0.644$ and 0.571, respectively, data not shown).

Overall Recommendations for IVIVC Approaches and Future Directions

As discussed above, there are numerous mathematical and correlation approaches for establishing IVIVC for induction. Many of these approaches have demonstrated utility in IVIVC, but also have their disadvantages. Therefore, the authors are not recommending any single approach for use in all situations, but rather recommend understanding the pros and cons of each approach and leave it to the investigator to decide on the best tool for a given situation. The Correlation Approaches are quite straightforward and amenable to predictions of clinical DDI with respect to CYP3A induction; however they require an extensive amount of data to establish calibration curves. They may also not be appropriate in cases where compounds are both inhibitors and inducers of CYP3A. Mathematical Model Approaches, that have the capability to capture concurrent CYP inhibition, are likely more amenable for more comprehensive DDI predictions. The donor-to-donor or cell-line variability in the maximal inducibility of the *in vitro* test systems (e.g. immortalized cell-lines, primary hepatocytes) lead to the requirement for a calibrator (e.g. positive controls) in the Mathematical Model Approaches to relate the *in vitro* response to the actual clinical response. Physiologically-based models, although relatively new for the induction field, are promising tools for the future of in vitro to in vivo extrapolation of induction. Additionally, most current IVIVC models were tested specifically for CYP3A; development of IVIVC models for induction of enzymes other than CYP3A is an area that requires more investigation.

Clinical Induction Studies

An NME may be a precipitant (inducer) and/or an object (substrate) of a pharmacokinetic drug-drug interaction due to enzyme induction. This paper has focused on the assessment of NME as an inducer of CYP enzymes. *In vitro* induction studies with cultured human hepatocytes will provide guidance for the clinical induction studies to assess if coadministration of the NME will result in decreased exposure of CYP substrates due to enzyme induction. The survey responses indicated that the company clinicians are very interested in knowing the *in vitro* data (72% very interested, 28% somewhat interested). The *in vitro* induction data is generally presented to the clinicians in the context of clinical concentrations, comparison to other therapeutics, clinical relevance, implications for labeling, and safety and efficacy in the context of reduced NME exposure. The *in vitro* information appears to be widely used for prioritizing clinical DDI studies in the development programs, providing guidance for clinical study designs, and for recommending the inclusion/exclusion criteria and contraindicated medications.

A decision tree approach based on the available *in vitro* induction data and the therapeutic concentrations of NME is recommended for deciding whether a clinical DDI study is needed (Figure 2). In many cases, negative findings from early *in vitro* studies can eliminate the need for clinical DDI investigations. As discussed elsewhere in this paper, this decision is not straight forward for isoforms other than CYP3A4. It is recommended that a case-by-case analysis be made for other isoforms keeping in view of the magnitude of predicted interaction, the therapeutic indices of potentially coadministered drugs that are metabolized by the CYP isoform of interest and other factors that may impact safety and tolerability during coadministration.

In studying an investigational drug as an inducer, the choice of substrates for initial *in vivo* studies depends on the P450 enzymes affecting the interacting drug (Table 2). If the initial study shows that the NME induces metabolism of a sensitive substrate, further studies using less sensitive substrates, based on the likelihood of coadministration, may be useful. If the initial study is negative with the most sensitive substrate, then interaction with less sensitive substrates can be assumed to be negative. Further, if the NME is not found to be an inducer of CYP3A4 *in vitro* or in a clinical DDI study, it can be assumed that it will not affect other enzymes which are inducible via the PXR-mediated pathway (e.g., CYP2C8, CYP2C9, CYP2C19).

Clinical Study Design Considerations

The HIV protease inhibitor ritonavir offers a good example of the need for a well thought out clinical study design to evaluate the inductive responses of an NME. Ritonavir is not only a potent inhibitor but also an inducer of CYP3A (Hsu et al., 1998). The dual inhibition and induction of CYP3A may produce complex and time-dependent pharmacokinetic interactions with other drugs. The net effect of ritonavir on CYP3A-mediated metabolism *in vivo* represents a balance of inhibition and induction, which can not be easily predicted (Greenblatt et al., 1999). For such compounds, inhibition will likely dominate initially, while on more extended exposure, induction may partially or completely offset inhibition.

The clinical DDI study should be designed with the aim of providing specific recommendations regarding the clinical significance of the interaction based on what is known about the dose–response and/or the pharmacokinetic/pharmacodynamic (PK/PD) relationship for either the NME or approved drugs used in the study. The selection of a particular study design depends on a

number of factors for both the substrate and interacting drug, such as: (1) use of the substrate and/or NME in an acute or chronic condition; (2) therapeutic window of substrate and NME; and (3) PK-PD characteristics of the substrate and NME.

Study Population: The choice of the population in the clinical DDI study will largely be driven by the indication and nature of the likely induction. The ideal population to conduct such studies is the healthy volunteers, as they offer the greatest flexibility for assessments and monitoring. However, in certain indications such as oncology and/or when an NME can not be administered to healthy volunteers, a typical patient population may be needed. The survey responses indicate this as the general scenario (77% using healthy volunteers), with the exception of oncologic and cytotoxic NMEs. Sometimes it is possible to assess the clinical consequences of induction in the context of a clinical study (Fine et al., 2004).

Dose selection: For both the substrate and NME, testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and shortest dosing interval of the NME should be used, and the duration of the treatment should be sufficient to reach the steady-state or a clinically relevant regimen. This survey indicates that almost all the responding companies (93%) dose NME for at least five days or to steady-state when conducting a clinical DDI study. Additional factors that may be taken into consideration include the existence of major and/or pharmacologically active metabolites of the NME, dose- and time-dependency of NME pharmacokinetics, and any safety issues that may ensue when NME is dosed in combination with a probe substrate.

Assessments: Changes in pharmacokinetic parameters together with a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects in the general population or in specific populations can be used to assess the clinical relevance of the drug–drug interaction. Pharmacodynamic endpoints, if available, may be useful complements to pharmacokinetic data in understanding the clinical significance of interactions (e.g., biomarkers, prothrombin time for warfarin, blood glucose assessments with anti-diabetic drugs).

Data Analysis and Reporting: Results of drug–drug interaction studies should be reported as 90% confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with and without the interacting drug. The number of subjects required for a given drug–drug interaction study will depend on how small an effect is clinically important to detect or rule out, the inter- and intra-subject variability in pharmacokinetic measurements, and possibly other factors or sources of variability not well recognized.

Recommendations for Clinical Drug-Drug Interaction Studies

NME as an Inducer

- Based on *in vitro* induction study data and the likely therapeutic concentration, follow the decision tree approach to assess if a clinical DDI is needed or not.
- Choose the most sensitive probe substrate for clinical DDI study (Table 2).

- NME dosed to steady state or a clinically relevant regiment at the highest dose and shortest dosing interval intended for the marketing approval.
- Assess PK of probe before and after NME treatment, and document NME exposure.
- Data analysis and interpretation should include clinical consequences of induction relative to the therapeutic index of the substrate.

Alternate assessments: The measurement of urinary 6β-hydroxycortisol/cortisol urinary ratio provides a simple non-invasive method to monitor CYP3A4 induction, as demonstrated with many drugs including carbamazepine, a known inducer of CYP3A4 (El Desoky et al., 2005). However, 6β-hydroxycortisol/cortisol urinary ratio and midazolam clearance were reported to be poorly correlated, likely due to high intra-individual variability of the 6β-hydroxycortisol/cortisol urinary ratio compared to midazolam clearance (Chen et al., 2007). Based on the observation that CYP3A4 inducer carbamazepine increases plasma 4β-hydroxycholesterol levels, it has been suggested that this could be a biomarker for CYP3A4 induction in humans (Diczfalusy et al., 2008, Wide et al., 2008). Survey responses indicate limited use of these techniques.

The Erythromycin Breath Test (Watkins, 1996) measures liver CYP3A4 catalytic activity and since CYP3A4 represents the major metabolic pathway for many therapeutics, this test provides a phenotypic measurement of enzymatic activity and the effect of putative inducers. A recent clinical study suggests that the role of uptake and efflux transporters in the disposition of erythromycin could complicate the interpretation of the results (Frassetto et al., 2007). A similar

methodology has been tested successfully for CYP1A2-mediated caffeine N-demethylation (Rost et al., 1992). Survey responses indicate occasional use of these techniques.

A novel approach to assess *in vivo* measurement of CYP mRNA expression in peripheral blood lymphocytes as a predictor of enzyme induction has not been successful (Haas et al., 2005). If successfully developed in future, an approach similar to this effort, would be of enormous value in identifying the potential for induction-based DDI, and dose adjustments based on simple diagnostic methods.

Some groups have recommended a cocktail of several isoform-specific CYP substrates as an alternate method of conducting clinical DDI studies (Yeh et al., 2006, Tomalik-Scharte et al., 2005). Though this approach may be a good investigational tool, it may not be an appropriate approach for labeling purposes when an interaction does occur.

Alternate Models and New Technologies

Though cultured human hepatocytes have been accepted as the "gold standard" for non-clinical drug interactions studies, various attempts are being made with alternate methods for assessing CYP induction. The present survey included questions about the appropriateness and adaptation of several new technologies.

Human Liver Slices: Precision-cut liver slices have been proposed as an alternate model to hepatocytes for assessing metabolism of NMEs *in vitro*. Since cellular architecture is intact in liver slices, it is thought that this may be a better representation of the *in vivo* situation. This model has not been widely used for enzyme induction studies due to various shortcomings related to fresh liver availability and difficulties in maintaining enzymatic activities for prolonged periods, although progress has been reported (Lupp et al., 2002, Persson et al., 2006). In a study conducted by Olinga et al. (2008), it was demonstrated that AhR, CAR and PXR-mediated induction of drug metabolizing enzymes (both Phase I and II) as well as drug transporters were inducible by prototypical inducers of these pathways in human liver slices.

Animal Models: The interspecies differences in homology, expression, regulation and substrate specificity of CYPs is well-known (Martignoni et al., 2006). The time-dependent exposure decreases of an NME in nonclinical multiple dose toxicology studies generally are due to auto-induction of NME metabolism. However, due to interspecies differences, this observation may not translate to humans. Based on enzyme homology, overlapping substrate specificities and *in vivo* observations, rhesus monkey has been proposed as an acceptable model for CYP3A-mediated drug interactions (Prueksaritanont et al., 2006). However, the survey responses indicate only one company uses monkey as a model to predict human CYP induction.

Chimeric Animal Models: Transplantation of human hepatocytes into mouse liver to generate chimeric mice with humanized liver has been successful (Tateno et al., 2004). In such chimeric mice, the expression of the major CYP isoforms and their catalytic activities have been found to be similar to those of the donor hepatocytes. In studies conducted by Katoh et al., (2005, 2008), rifampicin increased CYP3A4 mRNA, protein and catalytic activity (dexamethasone 6-hydroxylation) by 8- to 22-, 3- to 10- and 5- to 12-fold, respectively. In the same study, 3-methylcholanthrene increased CYP1A2 mRNA and protein expression by 2- to 9-fold, and 5-fold, respectively. The authors concluded that the chimeric mouse may be an useful model to estimate and predict the *in vivo* induction of P450s in humans. The authors also point out that chimeric mice could be used to proliferate human hepatocytes at a low cost, which would be an advantage in countries where the availability of human hepatocytes is scarce.

Humanized Animal Models: In the last few years, humanized mouse models expressing human genes of CYP isoforms and nuclear receptors, or a combination of both, have been developed and evaluated (Xie et al., 2000, Xie and Evans, 2002, Gonzalez and Yu, 2006; Felmlee et al., 2008). Though promising, the general feeling of the survey respondents is that these models "need more work" for routine use, although they may be helpful for mechanistic studies *in vivo*.

Concluding Remarks

This survey of PhRMA member companies clearly demonstrated that CYP induction has gained wide acceptance as an important factor for consideration during drug discovery and development. Many companies have implemented assays for nuclear receptors, most notably PXR, for early detection of the potential for induction. The greater availability of fresh and cryopreserved hepatocytes in the United States has facilitated the emergence of this *in vitro* model as the "gold standard" for evaluating the potential for CYP induction. The accumulation of considerable data from *in vitro* hepatocyte induction studies, and the *in vitro* and *in vivo* correlations, albeit semi-quantitative at this time, has eliminated the need for unnecessary clinical drug-drug interaction studies for compounds that are not inducers *in vitro*. The acceptance of the *in vitro* data by clinical groups in pharmaceutical companies as well as regulatory agencies as a reliable predictor of clinical drug-drug interactions due to CYP induction has been a notable development.

Experimentally, emergence of highly sensitive and rapidly deployable quantitative assays for measurement of mRNA (e.g., Taqman) and isoform-specific probe catalytic activities (e.g., LC/MS/MS) has made a tremendous impact on the conduct of these assays, which previously have been time-consuming and cumbersome. In addition, cryopreservation and immortalization of hepatocytes has enhanced the flexibility of conducting these assays. The burden has now shifted from the experimental portion to data interpretation and extrapolation to the *in vivo* situation. As described in the IVIVC section, notable progress has been made in this area recently. It should be clearly stated that a simple experimental approach, for example NME tested at concentrations spanning clinical concentrations, with comparison to a positive control at

a single concentration, would be sufficient for decision making purposes, and such data will provide enough information to help in the design a clinical program. If a company feels that a more quantitative prediction is necessary, a more elaborate set of *in vitro* studies may be performed and an *in vitro – in vivo* correlation may be attempted, but such strategies are not needed as a routine practice.

One of the important considerations in interpreting *in vitro* data is to take into account the context of the clinical situation. As discussed in the IVIVC section, there is generally an acceptable correlation between *in vitro* and *in vivo* observations for CYP3A4 induction. This is to some extent due to the wealth of information available for this enzyme. However, for CYP1A2 and CYP2B6, that is not the case. As noted elsewhere in this document, the example of omeprazole demonstrates the importance of understanding the relationship between *in vitro* derived induction information in the context of systemic exposure of the NME.

This paper does not address induction of minor CYP isozymes such as CYP2E1, Phase II enzymes such as uridine glucuronosyltransferases, and influx/efflux transporters. On a case-by-case basis, it might be prudent to assess the inducibility of these enzymes and/or transporters if it is suspected that a clinically relevant DDI will occur between the NME and a coadministered medication that is primarily cleared via these pathways.

Acknowledgements

Authors would like to thank Mr. M. Garvin and his team at PhRMA for compiling the anonymous survey responses. Input from the PhRMA Drug Metabolism Technical Group, and Drs. W. Brian, B. Carr, O. Fahmi, V. Fischer, S. Hall, N. Hariparsad, J. Keirns, K. Kochansky, M. Mitchell, A. D. Rodrigues, J. Stone, M. Torrent and S. Wrighton is gratefully acknowledged.

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DMD Fast Forward. Published on April 23, 2009 as DOI: 10.1124/dmd.109.027029
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Legends for Figures

Figure 1: Comparison of Correlation (calibration curve) Approaches to predict clinical induction of CYP3A

(**A**) Relative induction score (RIS) approach. (**B**) The AUC/F_2 approach. (**C**) The $C_{max,u}$ /EC_{50} approach. Data is from (Ripp et al., 2006). Compound abbreviations: CBM, carbamazepine; CP A, 6-methoxy-1-methyl-trifluoromethyl-isochroman-7-ylmethyl)-(2-phenyl-piperidin-3-yl)-amine; NIF, nifedipine; PIO, pioglitazone; RIF, rifampicin; RSG, rosiglitazone.

Figure 2. Decision Tree for Clinical Induction Studies with a New Molecular Entity

[a] An empirical assessment or prediction based on mathematical or correlation-based approaches described in the IVIVC section.

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Table 1. A representative set of clinical drug-drug interactions due to cytochrome P450 induction

CYP	Inducer (substrate)	Clinical effect	Reference
1A2	Omeprazole (caffeine)	39% increase in exhalation of ^{14}C-CO_2 in CYP2C19 poor and intermediate metabolizers compared to 12% increase in extensive metabolizers.	Rost et al., 1992
2B6	Rifampicin (bupropion)	Apparent clearance increased from 2.6 L*h/kg to 7.9 L*h/kg	Loboz et al., 2006
2C9	Rifampicin (celecoxib)	AUC decreased by 64%	Jayasagar et al., 2003
2E1	Alcohol (chlorzoxazone)	Total plasma clearance 73% higher in alcoholics	Girre et al., 1994
3A4	Efavirenz (simvastatin)	Effect on Simvastatin-acid AUC decreased 58% and HMG-CoA activity decreased 60%	Gerber et al., 2005
3A4 and 2C8	Rifampicin (repaglinide)	AUC decreased by 57%; Blood glucose decremental AUC(0-3 h) reduced from 0.94 to -0.23 mmol/L	Niemi et al., 2000
Multiple 1A2 2C9 2C19	Lopinavir/Ritonavir (caffeine) (warfarin) (omeprazole)	urinary metabolite ratios increased by 43% AUC decreased by 1.4-fold Omeprazole/5-hydroxy-omeprazole ratio decreased 2.7-fold	Yeh et al., 2006

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3A4	(midazolam)	Apparent clearance decreased by 12-fold	

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Table 2. Recommended *in vivo* substrates for CYP induction studies

CYP	**Probe Substrate to evaluate NME as an Inducer**	
CYP isoform	**Substrate**[a]	**Recommended dose and route**
1A2[b]	**Theophylline**, caffeine	200 mg oral
2B6	**Bupropion**,[b] efavirenz	150 mg oral
2C8	**Rosiglitazone**, repaglinide	8 mg oral
2C9	**Warfarin**, tolbutamide	2 to 5 mg oral
2C19	**Omeprazole**, esomeprazole, lansoprazole, pantoprazole	20 mg oral
2E1[b]	**Chlorzoxazone**	500 mg oral
3A4/3A5	**Midazolam,** buspirone, felodipine, lovastatin, eletriptan, sildenafil, simvastatin, triazolam	10 mg oral

[a] Substrates in bold are preferred substrates. [b] Assessment of hydroxy-bupropion exposure.

Figure 1A

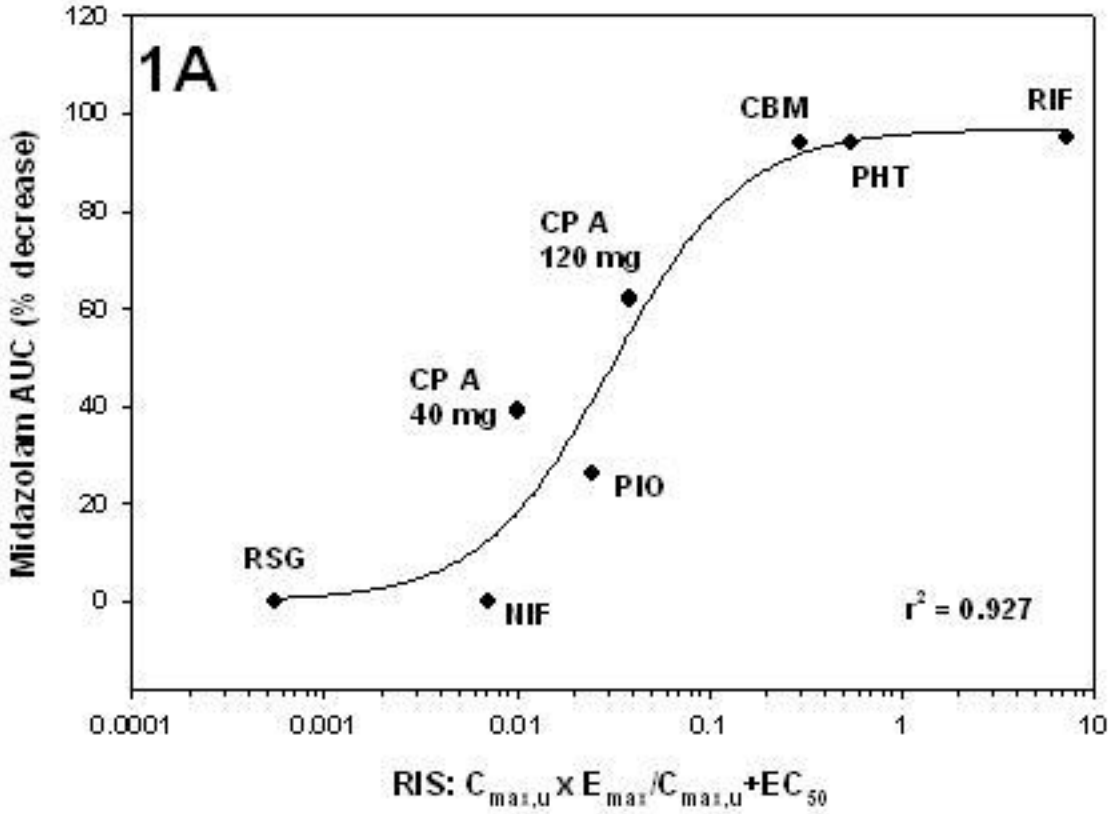

Figure 1B

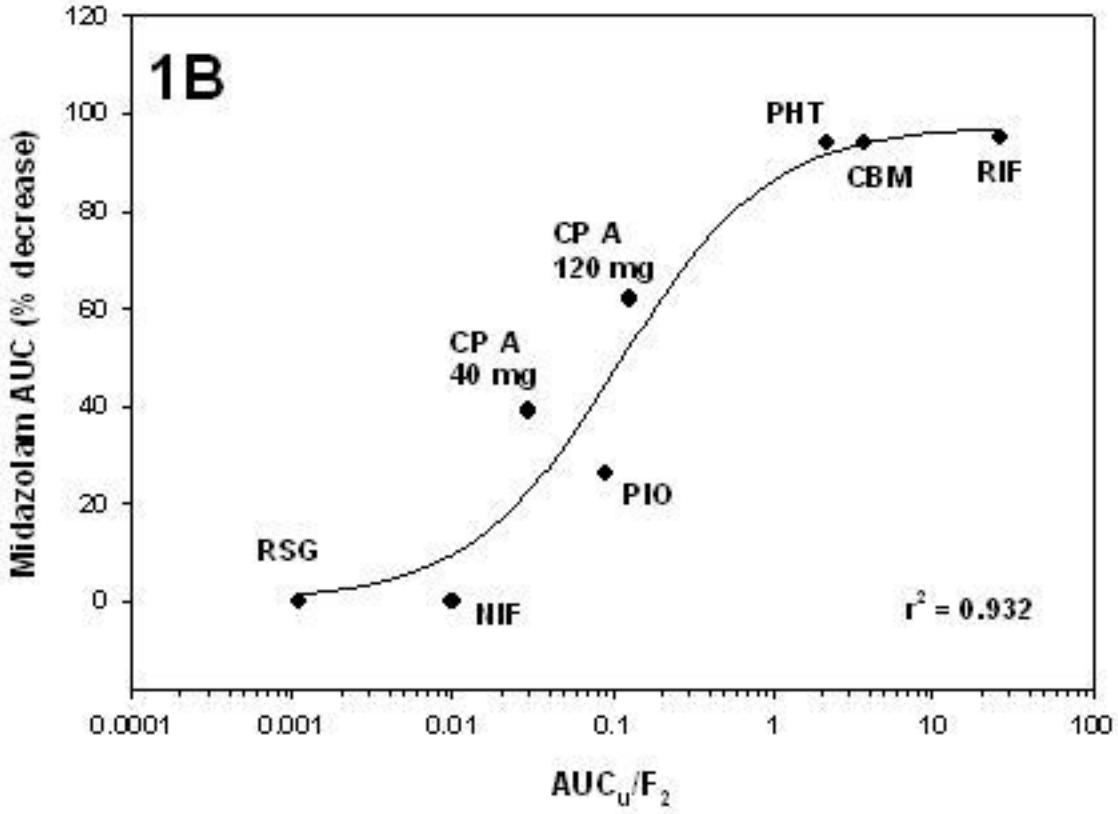

Figure 1C

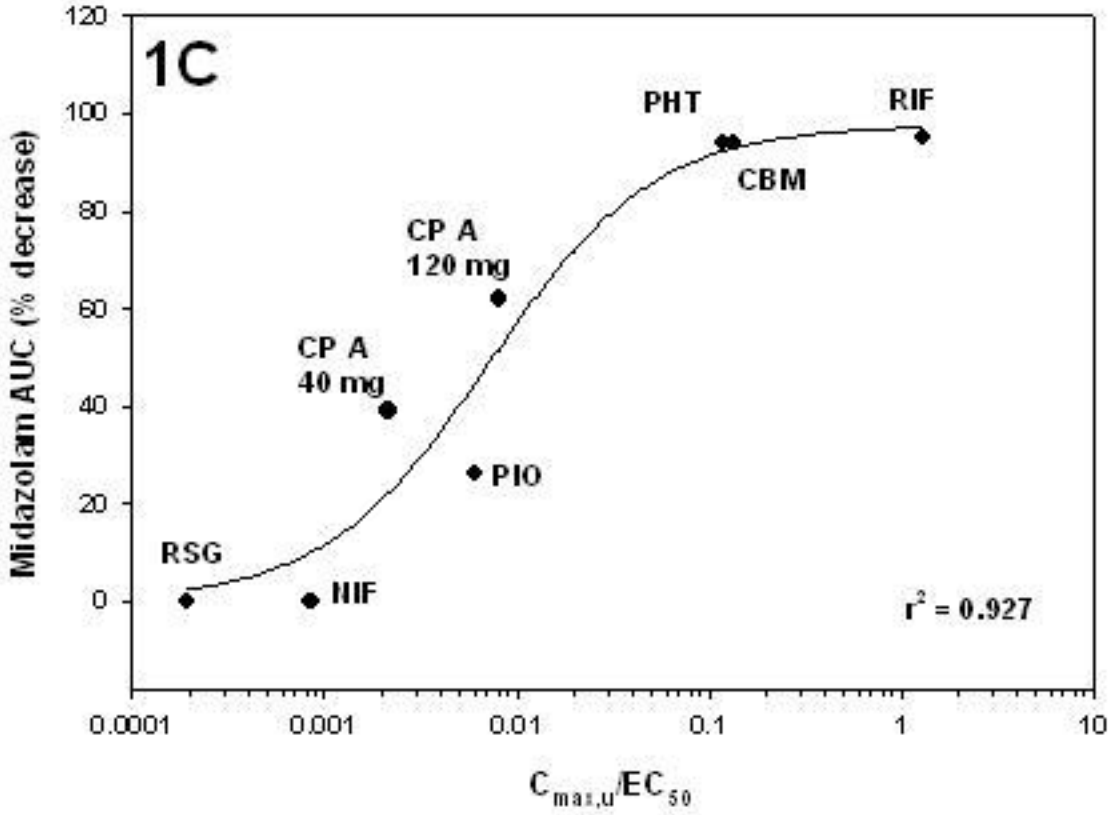

Fig 2