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Considerations from the IQ Induction Working Group in Response to Drug-Drug Interaction Guidances from Regulatory Agencies: Focus on Down-regulation, CYP2C Induction and CYP2B6 Positive Control

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Running title: Considerations on regulatory guidances for CYP induction from IQ

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Abbreviations:

Aryl hydrocarbon Receptor (AhR), Carboxyl Esterases (CEs), 6-(4-Chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO), Constitutive Androstane Receptor (CAR), Contract Research Organization (CRO), Cytochrome P450 (CYP), Drug-drug interactions (DDIs), drug metabolizing enzymes (DME), European Medicines Agency (EMA), Food and Drug Administration (FDA), glucocorticoid receptor (GR), Glutathione-S transferases (GSTs), Innovation and Quality (IQ) Consortium, *in vitro* to *in vivo* extrapolation (*IVIVE*), new chemical entity (NCE), P-glycoprotein (Pgp), multidrug resistance proteins (MRPs), new molecular entity (NME), Organic Anion Transporting Polypeptides (OATPs), Pharmaceutical and Medical Devices Agency (PMDA), Phenobarbital (PB), Pregnane-X Receptor (PXR), relative induction score (RIS), retinoid-X receptor (RXR), Sulfotransferases (SULTs), time dependent inhibition (TDI), Uridine 5'-diphospho-glucuronosyltransferases (UGTs), Induction Working Group (IWG)

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ABSTRACT

The European Medicines Agency (EMA), the Pharmaceutical and Medical Devices Agency (PMDA) and the Food and Drug Administration (FDA) have issued guidances for the conduct of drug-drug interaction studies. To examine the applicability of these regulatory recommendations specifically for induction, a group of scientists, under the auspices of the Drug Metabolism Leadership Group of the Innovation and Quality (IQ) Consortium, formed the Induction Working Group (IWG). A team of 19 scientists, from 16 of the 39 pharmaceutical companies, which are members of the IQ Consortium, and two Contract Research Organizations, reviewed the recommendations, focusing initially on the current EMA guideline. Questions were collated from IQ member companies as to which aspects of the guideline required further evaluation. The EMA was then approached to provide insights into their recommendations on the following; a) evaluation of down-regulation, b) *in vitro* assessment of CYP2C induction, c) the use of CITCO as the positive control for CYP2B6 induction by CAR, d) data interpretation (two-fold increase in mRNA as evidence of induction), and e) duration of incubation of hepatocytes with test article. The IWG conducted an anonymous survey among IQ member companies to query current practices, specifically focusing on the aforementioned key points. Responses were received from 19 companies. All data/information was blinded prior to being shared with the IWG. The results of the survey are presented together with consensus recommendations on down-regulation, CYP2C induction and CYP2B6 positive control. Results and recommendations related to data interpretation and induction time-course will be reported in subsequent manuscripts.

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INTRODUCTION

Enzyme induction can lead to decreased systemic exposure of the inducing drug (auto-induction) or of a co-administered drug that is metabolized by the induced enzyme, and can result in increased formation of active or toxic metabolites that change the pharmacological and toxicological outcomes in the induced state compared to the non-induced state (Guengerich et al., 1990; Lin, 2006). Since hepatocytes contain the full complement of transcription factors, metabolic enzymes and transporters as well as co-activators and co-repressors, isolated hepatocytes are now recognized as the most relevant and practical *in vitro* model for induction studies (Hewitt et al., 2007a; Hewitt et al., 2007c; Godoy et al., 2013). Therefore, the use of plated human hepatocytes is considered to be the "gold standard" *in vitro* assay for induction-risk assessment.

Given the common goal of regulatory authorities and industry sponsors to be able to predict the occurrence and extent of induction in humans from preclinical studies, the Pharmaceutical Research and Manufacturers of America (PhRMA) published a white paper with the intent of suggesting best practices for *in vitro* and *in vivo* pharmacokinetic DDI studies (Bjornsson et al., 2003). This was followed up with an additional white paper in which the Pharmaceutical Innovation Steering Committee, under the auspices of PhRMA, conducted a survey to understand the predictability of various *in vitro* experimental models for CYP induction that was used across the industry (Chu et al., 2009). Since the publication of this white paper and survey, the FDA, EMA and the PMDA have issued updated guidances; DDI Draft Guidance for Industry (FDA, 2012), Guideline on the Investigation of Drug Interactions (EMA, 2012),

and the Drug Interaction Guideline for Drug Development and Labeling Recommendations (MHLW, 2014).

The current EMA (EMA, 2012) and PMDA (MHLW, 2014) DDI guidelines specifically mention the potential for the *in vitro* induction studies to detect enzyme down-regulation and, if the effect of the new chemical entity (NCE) is concentration-dependent, recommend additional *in vitro* or *in vivo* studies to further investigate (EMA, 2012; MHLW, 2014). The key question being what constitutes a significant level of decrease in mRNA and protein *in vitro* and does this translate to *in vivo*? Similarly, the EMA guideline also recommends that pharmaceutical companies generate *in vitro* human hepatocyte CYP2C induction data if the compound activates the Pregnane-X Receptor (PXR) (EMA, 2012). It is recognized that induction of CYP2C enzymes can be important. For example, rifampicin is known to increase the clearance of S-warfarin (CYP2C9 substrate), a narrow therapeutic index drug (Bidstrup et al., 2004). However, challenges remain with generating reproducible *in vitro* CYP2C induction data, with a large enough induction signal across different lots of human donors, which can be used to assess the clinical risk of CYP2C-induction. Thus, could we consider alternative approaches to assess the CYP2C induction risk? Also, the EMA guideline on DDI (EMA, 2012) states that “strong inducers should be included as positive controls to verify functioning regulation pathways via PXR, Constitutive Androstane Receptor (CAR) and the Aryl hydrocarbon Receptor (AhR)”. CITCO, at concentrations ≤100 nM, is the positive control recommended by the EMA for CAR induction in *in vitro* experiments. This is in contrast to the FDA guidance (FDA, 2012) which recommends

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phenobarbital (PB) at a concentration between 500 – 1,000 µM as a positive control for induction of CYP2B6. Could either option be suitable to verify functioning CAR activity?

In order to address the aforementioned discussion points, the IWG approached IQ member companies as to which specific aspects of the recent DDI guidelines required further clarification and evaluation. The IWG then approached the EMA with questions developed from these responses requesting insights into the recommendations of the EMA and these questions were published online (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002963.pdf). As a follow-up, the IWG conducted an anonymous survey within IQ to gather information about current practices for the conduct of human hepatocyte induction studies and how companies interpret the derived data. Responses to the survey were received from 19 companies, which included 17 large companies (>10,000 employees) and two medium-sized companies (1,000–10,000 employees). Information gathered from the survey is presented in this article together with data driven recommendations on suggested changes to current Regulatory Guidances. Given the complexity and detailed assessment necessary for the data interpretation and the time-course objectives, we plan to publish this work in follow-up manuscripts.

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METHODS

The results presented in this article were obtained by conducting a survey of pharmaceutical companies that are members of the IQ Consortium. Survey questions were provided by members of the IWG (https://iqconsortium.org/initiatives/working-groups/induction/). The questionnaires were formulated using Microsoft Excel (2010). Questions included single/multiple choice, and open ended or free text. The final survey was aimed at gathering information on five different areas; a) evaluation of down-regulation, b) *in vitro* assessment of CYP2C induction, c) use of CITCO as the positive control for CYP2B6 induction by CAR, d) data interpretation (two-fold increase in mRNA as evidence of induction), and e) duration of incubation of hepatocytes with test article. Data/information were collected for both non-proprietary and proprietary compounds. In some instances, certain questions had a small degree of overlapping information but were collated in the area that provided the best context for the question. Protocol questions focused on how studies were conducted at different organizations which provided information on data variability across companies. In addition, induction data were collected for individual batches of hepatocytes such that variability across batches could also be assessed and to assess CYP2B6 induction by CITCO and phenobarbital. In these cases, all data were generated using standard internal company methods. Data were also collected to ascertain the manner in which *in vitro* hepatocyte induction data were interpreted by different companies. Given that establishing an *in vitro* to *in vivo* extrapolation (IVIVE) was critical, in addition to collecting *in vitro* data, clinical data were also collected and collated. Finally, the team also sought to understand the types of questions that were received from regulators related to

induction. This part of the survey was conducted using an online questionnaire and survey software (SurveyMonkey; www.surveymonkey.com).

The survey was conducted anonymously via the IQ Secretariat and sent to representatives of IQ Consortium member companies. Each representative was responsible for providing responses that were reflective of the company as a whole since only one response was collected from each company. The IWG received the blinded responses from the IQ Secretariat and could not identify specific responses from specific companies. Survey limitations include the following; a) the survey responses were blinded by the IQ Secretariat, which is a requirement for conducting surveys within the IQ consortium. Therefore it is not possible to follow-up with respondents to clarify information/data entries, etc.; b) the survey was sent to the Drug Metabolism Leadership Group representative of each member company with the request to have an internal expert complete the survey with appropriate input from others to represent the company's perspective and not that of the individual reporter. It was not possible to ensure that this was conducted as requested; c) IQ members may not have responded to all questions in the survey. As such, it is not possible to assess whether this introduced any bias in the survey responses; d) the IQ Induction Working Group tried to provide clear questions. As with all blinded surveys, it is not possible to assess whether the respondent interpreted the question differently; e) the survey was intended as a touch point to gain some insights into practices. The outcome of the survey is being used as one part of a strategy to develop a data based response to specific aspects of regulatory DDI guidances. The survey responses are not considered a definitive index of induction practices within the pharmaceutical industry. The

collection of responses occurred during the third quarter of 2015. Data analysis and graphs were generated using Microsoft Excel (2010) or GraphPad Prism 6.0.

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RESULTS

Evaluation of Down-Regulation

Responses from the survey indicated that the majority of companies use monolayer cultures (12 of 16 responses), a 96 well plate format (11 of 16 responses) and 48 hour exposure (12 of 16 responses) to test compounds (Figure 1A). The survey confirmed that decreases in mRNA and/or enzyme activity mediated by new chemical entities (NCEs) are observed (Down regulation and negative control survey; Part 2. Observations of down regulation, (https://iqconsortium.org/initiatives/working-groups/induction/)). Of the 17 respondents, 16 indicated that they have observed decreases in enzyme activity or mRNA levels during routine induction studies. In many cases companies use multiple formats, cytotoxicity methods and have observed decreases in multiple enzymes so the total is not always equal to the number of respondents (17). In nearly half of all companies, 10-20% of compounds screened for induction potential showed decreases in mRNA levels that were concentration dependent, unrelated to cytotoxicity and occurred across the enzymes evaluated (typically CYP1A2, CYP2B6 and CYP3A4), while a few companies also evaluated other enzymes and noted commensurate decreases (CYP2C8, CYP2C9 and UGT1A1) (Figure 1B). Thirteen companies observed decreases in mRNA <0.3-fold, of which 7 companies showed changes <0.1 (Figure 1C). In general, changes in mRNA levels appeared to be more sensitive than changes in enzyme activity. In most cases (7 of 11 responses) there was no clear correlation with structures, therapeutic area, or compound class when decreases in mRNA or activity were observed. Decreases in enzyme activity observed during routine *in vitro* induction studies can often be attributed

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to time-dependent inhibition (TDI) occurring over the incubation time-course (Fahmi et al., 2010), as noted by 7 companies in the survey. In addition, when decreases in mRNA or enzyme activity are observed only at higher concentrations of an NCE, cytotoxicity is often a plausible mechanism which should be evaluated. Eight companies noted this as the mechanism. In cases where decreases in mRNA and/or enzyme activity are concentration dependent and not related to TDI or cytotoxicity, mechanistic studies could potentially aid in identifying the cause(s). Mechanisms which have been shown to mediate down-regulation of enzymes *in vitro* and/or *in vivo* could serve as a basis for designing follow-up studies (Dvorak et al., 2005; Lu et al., 2012; Lim et al., 2012; Healan-Greenberg et al., 2008).

Of the 17 responding companies, 9 had no established cut-off criteria for down-regulation, while the remaining companies used very similar cut-off criteria which included both >50% decrease and concentration dependence. Only 3 companies performed follow up mechanistic studies. Two of these studies involved a pre-clinical rat study where compound was administered and *ex vivo* mRNA levels and microsomal enzyme activity were measured. Due to variability in response, one study was deemed inconclusive. In the other study, down-regulation of drug metabolizing enzymes (DMEs) in rat hepatocytes had occurred at much higher concentrations of the NCE than was observed in human hepatocytes. In an *ex vivo* rat study, doses were selected which resulted in total systemic concentrations greater than the nominal *in vitro* incubation concentrations at which down-regulation was observed. In the prepared extracts, from treated rat livers, no changes in microsomal enzyme activity or mRNA levels were observed. Hence it was concluded that, in the rat, the *in vitro* down-regulation did not

translate to an *in vivo* effect. No companies currently use positive control compounds which have been shown to lead to down-regulation of enzyme activity and mRNA *in vitro*. Indeed, the lack of small molecule positive controls, where the underlying mechanism of down-regulation is fully understood, significantly limits the ability to validate models for assessing down-regulation. Overall, 3 companies chose to conduct a clinical interaction study based on the NCE-mediated down-regulation observed *in vitro*. No companies showed clinical significance of these *in vitro* observations. There were examples from 2 of the 16 companies where an attempt was made to relate the *in vitro* derived parameters (fit similarly to induction data) to expected clinical concentration. In one case no clinical effect was anticipated and only a moderate inhibition was anticipated in the other.

In addition to responding to the questionnaire, IQ member companies were asked to provide example questions received from regulators which were related to induction-based DDIs. Fourteen questions were submitted which were directly related to induction. Of these 14 questions, 4 were specifically targeted to gain a better understanding of NCE-mediated decreases in mRNA levels and enzyme activities observed during *in vitro* induction studies with human hepatocytes. Regulators' questions probed both for mechanistic insights and extrapolation to other enzymes that potentially could be down-regulated. Similar to TDI, down-regulation could result in exposure increases in patients. One regulatory question related to a small molecule which demonstrated decreases in mRNA levels and enzyme activity during the *in vitro* induction study and also showed *in vitro* TDI of some of the same enzymes. In that study, time-dependent decreases in activity for many of the enzymes evaluated were

observed in a cocktail clinical DDI study and the applicant was asked to discuss whether down-regulation could be excluded as an additional mechanism; if it could not, which other enzymes might be affected.

In Vitro Assessment of CYP2C Induction

Ten companies responded to questions related to CYP2C induction. Similar to data in the literature (Yajima et al., 2014), all companies reported variable CYP2C9 induction responses across different lots of hepatocytes, with induction values for CYP2C9 that were < 4-fold higher than vehicle control. Also, consistent with the literature, robust induction of CYP3A4 mRNA (12- to 47-fold) was observed in the same batches of hepatocytes (Figure 2) (Yajima et al., 2014). In order to negate the impact of protocol differences on the observed CYP2C mRNA inductive response between human hepatocyte donors, data were obtained from the same member company in 10 different batches of hepatocytes (Figure 3). Again, while the CYP3A4 mRNA induction response to rifampicin was robust (8- to 80-fold), the extent of CYP2C8 and CYP2C9 mRNA induction ranged between 2- to 6–fold and 1.5- to 4–fold, respectively. While the CYP2C8 and CYP2C9 mRNA induction responses were low, there was a good correlation with the observed functional changes in enzyme activity. The survey results demonstrate a low magnitude of induction of CYP2C8 and CYP2C9 mRNA.

In contrast, data from the survey showed that increases in CYP2C19 activities were highly variable and were quite dramatic in some donors, up to 17-fold (Figure 3). In addition to data obtained with rifampicin, the IWG also compiled *in vitro* induction data for other compounds known to induce CYP2C isoforms (Supplementary Table 1).

Given that the pathways involved in the regulation of CYP2C and CYP3A4 are similar, it was not surprising to see positive correlations for induction between CYP3A4 and CYP2C mRNA, with r^2 values ranging from 0.6 to 0.99. Representative correlation plots for CYP2C and CYP3A4 enzymes, following induction by rifampicin, are shown in Figure 4. This positive correlation can also be observed for bosentan, phenytoin and carbamazepine which cause varying extents of AUC decline of CYP2C9 probe substrates and CYP3A4 probe substrates in the clinic (Table 3). Data in Table 3 summarize the percentage reduction of AUC of an *in vivo* CYP2C9 probe, S-warfarin, in response to various xenobiotics and the *in vitro* induction of CYP2C and CYP3A evoked by the same compounds. Rifampicin caused the greatest decrease in the AUC of S-warfarin and also induced CYP3A4 more than the other compounds.

Positive Control for CYP2B6 Induction by CAR

Data were provided by 3 member companies from 26 human hepatocyte donors showing CYP2B6 mRNA expression data following treatment with 100 nM CITCO and with ≥ 750 µM PB (figure 5). Both treatments demonstrated a >2-fold increase in CYP2B6 mRNA compared to vehicle control in all donors. The smallest increases observed in CYP2B6 mRNA with CITCO or PB treatment were 2.25-fold and 7.04-fold, respectively. In 23 of the 26 donors the increase in CYP2B6 mRNA was greater after PB treatment compared to CITCO. The ratio of induction of CYP2B6 by PB compared to CITCO ranged from 0.87 to 4.08.

DISCUSSION

Transcriptional down-regulation of CYPs by chemicals was identified as an area requiring further exploration over ten years ago (Riddick et al., 2004). Despite this, published observations of drug-induced down-regulation by small molecules *in vitro* are scarce (Healan-Greenberg et al., 2008; Krausova et al., 2011; Yang et al., 2014; Zamek-Gliszczynski et al., 2014; Sager et al., 2017). While potential mechanisms for down-regulation have been identified (as detailed below); none have established a clinical effect for the major inducible enzymes (CYP1A2, CYP2B6, CYP2C or CYP3A), from these *in vitro* observations. Indeed, when member companies were questioned as to whether the *in vitro* observations of down-regulation led to clinical changes in specific probe substrates, no examples were provided. The EMA indicated that they had one example from a sponsor where down regulation observed in human hepatocytes resulted in a clinical finding. Because of the confidentiality in submissions, additional details on this compound are not available at this time. A recent publication concluded that the IVIVE for bupropion mediated inhibition of CYP2D6 could be quantitatively predicted with the inclusion of down-regulation (Sager et al., 2017), however the mechanisms behind the CYP2D6 down-regulation were not identified. There is also conflicting literature available with respect to the inducibility of CYP2D6. While CYP2D6 is generally thought to not be inducible (Ingelman-Sundberg, 2005; Teh and Bertilsson, 2012; Haertter, 2013), a recent publication shows that it can be induced in hepatocyte cultures in the absence of dexamethasone (Farooq et al., 2016). More work is needed to confirm the *in vitro* observations from these two studies and to explore the mechanisms for regulation of CYP2D6. No data on CYP2D6 induction or down-

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regulation was collected from the IQ group members during this survey because evaluation of CYP2D6 induction is not conducted (EMA, 2012; FDA, 2012; MHLW, 2014).

In vitro induction studies are conducted using plated human hepatocytes either with (sandwich culture) or without matrix overlay (monolayer). While hepatocytes are considered to be the gold standard for performing *in vitro* CYP induction studies, there are losses in enzyme activity for standard 2D (monolayer) and sandwich cultured primary human hepatocytes over culture time resulting in a lower basal CYP activity compared to freshly isolated suspension hepatocytes (LeCluyse et al., 2000; Hamilton et al., 2001; Pichard-Garcia et al., 2002; Rodriguez-Antona et al., 2002; Aitken et al., 2006; Hewitt et al., 2007b; Fardel and Le Vee, 2009; Huang et al., 2010; Lee et al., 2010). The exact mechanisms for the enzyme loss are not clear, but recent papers highlight an important role of micro RNAs (miRNAs) as drivers of hepatic dedifferentiation and demonstrate a complete change in the proteome of standard 2D cultures (Bell et al., 2016; Lauschke et al., 2016). Would long-term hepatocyte models offer advantages to evaluate down-regulation? Models have been developed which aim to improve the longevity and maintenance of activity of drug metabolizing enzymes and include co-culture models (Khetani and Bhatia, 2008; Krause et al., 2009), organotypic models (LeCluyse et al., 2012), 3D spheroid (Bell et al., 2016) and flow systems (Soldatow et al., 2013). However these models have not been as rigorously tested for induction as standard cultured hepatocyte models. Therefore, routine induction studies that satisfy current regulatory requirements are performed using sandwich cultured or monolayer hepatocytes. While reduced enzyme expression levels and activity offer a

robust response window for induction, adequate basal levels are needed in order to characterize metabolism with and without down-regulation (Evers et al., 2013). Differences in culture conditions can also impact the basal levels of drug metabolizing enzymes and the ability of the culture to respond to cytotoxicity. An example was provided whereby the same unidentified company, using different culturing conditions at different sites, could not reproduce observed *in vitro* down-regulation. The exact reason for this difference was not determined. However, differences between sites in media formulations, plating format, donor used and culturing techniques may have contributed. Additionally, the half-life of the end point being measured (mRNA or protein (enzyme activity) compared to the incubation time course need to be considered when interpreting *in vitro* data. While the half-life for mRNA of a given enzyme is shorter than the half-life for enzyme (Ramsden et al., 2015; Dixit et al., 2016) there can be differential turn-over observed between enzymes. As an example, differences in potency assessments for IL-6 mediated down-regulation of CYP3A4 mRNA have been observed between 48 and 72h incubation times (Evers et al., 2013).

There is also a potential for cytotoxicity to occur, especially with the high *in vitro* concentrations used during an induction study. High compound test concentrations aim to cover potential gut concentrations and achieve levels sufficient enough to describe E_{max} when induction is observed. While cytotoxicity is typically assessed during induction studies, most researchers use morphological assessment and end-point assays such as ATP, LDH, MTT, Presto Blue, WST-1 or monitoring of a house-keeping gene by TaqMan (Figure 1B). These assays are capable of detecting overt cytotoxicity. In many cases more sensitive assays would be required to rule out a link between early

cytotoxic events and down-regulation of enzymes. Setting stringent criteria for assessment of cytotoxicity (e.g. 20-25% cutoff compared with control) could help to avoid assigning significance to down-regulation which is the result of cytotoxicity. Depending on the mode of hepatocyte toxicity, other assays may be more or less sensitive and this toxicity could also be compound dependent. There are potentially more sensitive approaches available to assess cytotoxicity such as high content imaging or systems such as xCelligence (Gerets et al., 2012; Grimm et al., 2015; Joshi and Lee, 2015; Li et al., 2015). Overall, the potential for cytotoxicity to contribute to down-regulation should be considered as part of the complete picture toward developing an understanding of mechanisms behind these observations.

Alterations of the levels of CYP isoforms can occur through modulation of steroid receptors such as PXR, CAR, and AhR, with PXR and CAR partly regulated by the glucocorticoid receptor (GR) (Honkakoski and Negishi, 2000). These signal transduction pathways can be affected at any step of the cascade, leading to CYP down-regulation, e.g. interference with binding of nuclear receptor retinoid-X receptor (RXR) with PXR, and disturbances with the cytoskeletal structure in the cell. A well-known example of small molecule mediated down-regulation, in which these two mechanisms could be operative, is that of colchicine which has been shown to broadly reduce expression of CYPs, both in the basal state as well as in the induced state (Dvorak et al., 2003; Dvorak et al., 2005). Colchicine is an anti-tubulin agent that inhibits the polymerization of microtubulin, and thus disrupts the dynamics of the microtubulin cellular skeletal structure, the structure that is considered necessary for the proper functioning of nuclear receptor signaling cascades (Lu et al., 2012). A

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mechanistic study was conducted to understand potential causes of the broad reduction of CYPs and led to the hypothesis that GR is more readily affected by cytoskeletal disruption (Dvorak et al., 2005). Mechanistic explorations, in parallel with CYP modulation, could involve assessments of P-glycoprotein (Pgp), multidrug resistance proteins (MRPs), uridine 5'-diphospho-glucuronosyltransferases (UGTs), glutathione-S-transferases (GSTs), sulfotransferases (SULTs), organic anion transporting polypeptides (OATPs), carboxyl esterases (CEs), etc., which are co-regulated by these nuclear receptors (Urquhart et al., 2007; Chen et al., 2012). Specific knowledge gaps in this area include: (a) differential down-regulation in the liver and gut; (b) combined down-regulation of closely related enzymes, such as CYP3A5 along with CYP3A4; and (c) species differences in CYP down-regulation. GR and HNFα are important for basal expression of multiple CYPs and play a supportive role in classical induction (Pascussi et al., 2001). Their role in combined down-regulation needs to be further explored. It can become even more challenging when mixed mechanisms of DDI are occurring. For instance, as highlighted by one of the regulatory questions, it would be very difficult to differentiate down-regulation of mRNA observed *in vitro* from TDI or to identify which mechanism or combination of mechanisms and their relative contributions lead to observed clinical changes. As highlighted above, more research in the area of down-regulation is needed to evaluate whether mechanisms of down-regulation can affect multiple enzymes analogously to coordinate induction of multiple enzymes (e.g. through PXR). This could be relevant since TDI may be isoform specific whereas down-regulation could potentially affect multiple co-regulated enzymes. In this case, when the mechanism of *in vitro* down-regulation occurs at the nuclear receptor, consideration of

co-regulated enzymes, not affected by TDI, or modeling of effects based on clinical concentrations and *in vitro* parameters, EC_{50} and $E_{min,}$ for the down-regulation or K_I and k_{inact} for TDI, might offer some insights.

Examples of small molecules that disrupt interactions between nuclear receptors and co-activators involve sesamin in sesame oil (Lim et al., 2012), camptothecin (Chen et al., 2010), ketoconazole (Takeshita et al., 2002) and ET743 (Synold et al., 2001). Small molecules can serve as direct antagonists, inhibitors or repressors of nuclear receptors. The PXR antagonist, A-792611 (Healan-Greenberg et al., 2008), causes CYP down-regulation. Other examples of molecules that down-regulate *in vitro* are LY2090314, sulforaphane, fluoxetine, norepinephrine, epinephrine and riluzole (Zhou et al., 2007; Zamek-Gliszczynski et al., 2014; Badolo et al., 2015; Shang et al., 2016). Metformin has been shown to specifically enhance the phosphorylation of CAR, thus blocking nuclear translocation and activation (Yang et al., 2014). None of these example drugs resulted in clinical effects, either because down-regulation did not manifest with a change in the exposure of clinical substrates or co-medications or, like colchicine, because the clinical concentration was much lower than the concentrations where down-regulation was observed *in vitro*.

Certainly a key challenge for *in vitro* observations of decreases in mRNA or enzyme activity (not due to inhibition) remains the translation of the *in vitro* findings to the clinic. Multiple rising dose studies in healthy volunteers are often the first opportunity to evaluate down-regulation clinically but only if the pathway which is down-regulated represents a major route of clearance. With the small number of individuals and the large variability that can occur with inducible human CYPs, these studies are

typically not powered to provide definitive outcomes. However, it may still be possible to leverage this information as a means of developing an understanding of the predictability of such down-regulation. Clinical plasma concentrations could provide a basis for identifying possible CYP down-regulation if the CYP affected is a major clearance pathway for the compound itself and where these changes can be related to concentration dependence in the hepatocyte studies. As noted above, this was the case with colchicine, where no DDIs were observed with relevant drugs, probably due to low systemic concentrations in humans (Colcrys, Package Insert).

The challenges of describing down regulation in cultured hepatocytes are also reflected in the typically small signal for induction of CYP2C isoforms. A limited number of publications describe induction of human CYP2C8, CYP2C9, and CYP2C19 using either fresh or cryopreserved hepatocytes (Gerbal-Chaloin et al., 2001; Raucy et al., 2002; Fahmi et al., 2010). Moreover, the reports on induction of CYP2C isoforms are contradictory. For example, induction of CYP2C9 and CYP2C19 by rifampicin in human hepatocytes has been documented (Zilly et al., 1975; Gerbal-Chaloin et al., 2001) but others show no induction (Li et al., 1997; Runge et al., 2000; Dixit et al., 2015). Recently, Yajima et al. (Yajima et al., 2014) evaluated the induction of human CYP2C8, CYP2C9, and CYP2C19 mRNA using eight lots of cryopreserved human hepatocytes. Following 72 hr incubation with rifampicin, induction of CYP2C8 and CYP2C9 mRNA was variable, while induction of CYP2C19 mRNA was not observed in any of the eight hepatocyte lots tested. Our results are consistent with the observation by Yajima et al. (Yajima et al., 2014). Additionally, recent evidence also suggests that miRNA may impact the basal expression of CYP2C8 and CYP2C19 in human hepatocytes (Yu et al.,

2015; Makia and Goldstein, 2016). *In vitro* studies have also demonstrated that protein-protein interactions occur between CYP2C9 and CYP3A4 which result in lower CYP2C9 activity with increasing levels of CYP3A4 (Subramanian et al., 2010; Ramsden et al., 2014). In the human HepatoPac model, induction of CYP2C9 activity by rifampicin was greater when CYP3A4 levels were decreased by siRNA knockdown. Thus the apparent induction of CYP2C9 was dependent on the ratio of CYP2C9:CYP3A4 protein expression levels (Ramsden et al., 2014). These factors may be responsible for the low magnitude and variable induction observed in our survey between the various CYP2C isoform mRNA induction responses. Additional mechanistic studies are needed to further understand the lack of robust induction and the variable response of CYP2C isoforms as determined by mRNA.

IVIVE approaches have been developed for induction of CYP3A to predict the potential of a compound to cause a clinically relevant DDI if co-administered with a compound that is primarily metabolized by CYP3A4 (Einolf et al., 2014). Given that CYP3A4 shares transcriptional regulation factors with CYP2C, it may be possible to ascertain the risk of CYP2C induction based upon the risk assessment for CYP3A4 induction using static and dynamic models (Einolf et al., 2014).

PB and CITCO are both known CAR activators that have been shown to work through different mechanisms. Kawamoto et al. demonstrated that PB induced CYP2B genes by causing CAR to translocate from the cytoplasm to the nucleus where it binds to the phenobarbital-responsive enhancer module and increases transcription of downstream genes (Kawamoto et al., 1999). Activation of CAR does not require direct binding of PB (Yang et al., 2014). This is in contrast to CITCO which causes nuclear

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translocation by directly binding to the receptor (Xu et al., 2004). These observations have led to two proposed mechanisms of CAR activation, “direct activation” which requires ligand binding and “indirect activation” which does not.

CYP2B6 induction can occur through activation of either CAR or PXR (Wang and Negishi, 2003). The ligand binding profile of PXR is known to include a diverse array of drug-like molecules while the number of chemicals that activate CAR appears to be much less structurally diverse and shows considerable overlap with PXR activators (Mackowiak and Wang, 2016). To date CITCO is the only chemical that has been shown to be highly selective for CAR over PXR. In transient transfection assays, CITCO displayed a >100-fold selectivity for CAR over PXR, with EC_{50} values of 25 nM for CAR and ~3 μM for PXR (Maglich et al., 2003). In contrast, PB has been shown to activate both CAR and PXR (Kawamoto et al., 1999; Sinz et al., 2006) although the degree of receptor selectivity has not been quantified.

In primary human hepatocytes it has been consistently shown that 100 nM CITCO induces CYP2B6, a concentration that is unlikely to activate PXR due to the selectivity described above. Increases in CYP2B6 mRNA, after treatment with 100 nM CITCO, were shown in 13 separate hepatocyte preparations (Maglich et al., 2003; DeKeyser et al., 2009; Sahi et al., 2009; Yang et al., 2010; Koh et al., 2012). Eleven of the 13 donors showed a >2-fold increase in mRNA. Based on the data provided it could not be determined if the remaining 2 donors met this threshold. Additional data collected for this survey adds another 26 donors demonstrating a >2-fold increase in CYP2B6 mRNA by 100 nM CITCO. Thus, of the 39 total donors evaluated, 37 showed a response to 100 nM CITCO, defined as an increase in CYP2B6 mRNA >2-fold above

vehicle control, a failure rate of approximately 5%. These data strongly support that CAR remains functional in primary hepatocytes as used in typical induction experiments and that hepatocytes are an adequate *in vitro* model for the assessment of CYP2B6 induction through CAR. In contrast, immortalized hepatocyte cell lines, such as HepG2 and Fa2N4, lack CAR functionality (Kawamoto et al., 1999; Hariparsad et al., 2008). The data collected for this survey also demonstrated approximately equal or greater CYP2B6 induction by PB compared to CITCO across 26 donors. This additional increase in CYP2B6 may represent the contribution of PXR in addition to the CAR component. If true, PB should better represent the *in vitro* response of mixed activators of CAR and PXR.

The utility of these hepatocyte models increases greatly if *IVIVE* can be established, allowing for prediction of a clinical response from the *in vitro* data. In order for an *in vitro* model to be informative of an *in vivo* effect, it is necessary for the model to maintain the functional mechanisms through which that effect is manifested. Based on data presented here it appears that primary human hepatocytes are adequate in this regard. Challenges have been noted in developing *IVIVE* for CYP2B6 induction (Fahmi et al., 2016) and it is currently unknown what effect, if any, the different mechanisms of CAR activation have on clinical outcome. As such, it is the opinion of the IWG that both CITCO and PB are viable positive controls for *in vitro* studies of CYP2B6 by CAR.

Summary and Recommendations:

- When a decrease in mRNA or activity is observed *in vitro*, there should be careful consideration of whether the decrease is concentration dependent and whether the compound is an inhibitor or an inactivator of the enzyme. Consideration should also be given to whether the decrease in mRNA or activity is linked to cytotoxicity or the mechanism of action.
- Correspondence with the EMA indicated that the Agency did have experience with down-regulation observed in human hepatocytes which was confirmed *in vivo* for one compound from a sponsor.
- Establishing an *IVIVE* in the absence of compounds which cause clinically relevant decreases in enzyme activity as a result of down regulation of enzymes, is currently not possible. As such, *in vitro* observations of down-regulation have to be considered with caution.
- Given the variable and low dynamic range of response in human hepatocytes, accurately predicting the potential for DDIs due to induction of CYP2C isoforms is challenging.
- Improved *in vitro* models with better dynamic range and less variability for the assessment of CYP2C induction are needed. Based upon co-regulatory pathways and positive correlation analyses, induction of CYP3A4 can be used as a sensitive marker of the potential for induction of CYP2C isoforms. Thus, a compound determined to have low risk to cause a CYP3A4-induction based DDI will have a very low risk to cause a CYP2C induction-based DDI.

- Data from the literature, and new data provided in this survey, demonstrate that primary human hepatocytes largely maintain a functional CAR regulatory pathway and are a reliable tool for assessing CYP2B6 induction by CAR. The risk of missing a CAR activator, due to potentially decreased CAR functionality, is further mitigated by the use of three individual human donors for *in vitro* CYP induction studies as recommended by regulatory agencies. As such, the potential to have a false negative for CAR induction in a human hepatocyte study is very small.
- Both CITCO and PB are activators of CAR but work through different mechanisms, direct or indirect activation, respectively. It is currently unknown what clinical differences may be attributable to the different modes of action.
- The IWG recommends the use of either 100 nM CITCO or ≥750 µM PB as the positive control for CYP2B6 induction in human hepatocytes.

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CONCLUDING REMARKS

CYP induction has gained wide acceptance as an important factor for consideration in drug development, particularly as it relates to DDI (Hewitt et al., 2007a; Chu et al., 2009). A number of factors have facilitated the emergence of human hepatocytes as the gold standard for the evaluation of CYP induction (Chu et al., 2009), including improvements in the cryopreservation of hepatocytes (Saliem et al., 2012) and the consequent availability of large batches of human hepatocytes which can be characterized prior to routine use. The availability of cryopreserved human hepatocytes, from the same donor, in sufficient abundance, has also enabled the development of calibration models (Fahmi et al., 2008) which provides greater confidence to regulators for risk-based decision making. The continued progress in the understanding of induction requires collaboration between regulatory agencies and pharmaceutical companies with the overall goal of designing and conducting the most appropriate, necessary, and insightful clinical induction studies. It needs to be ensured that the design of preclinical studies is optimal. This survey of IQ member companies on current practices is intended as a step towards this goal, which, together with recommendations, specifically as they relate to key questions such as down-regulation, the *in vitro* assessment of CYP2C induction, as well as the use of CITCO as the *in vitro* CYP2B6 positive control will hopefully lead to optimized studies.

The IWG intends to publish our survey results and recommendations related to the data interpretation and the time-course objectives in follow-up manuscripts. Our focus thus far has been to conduct an expanded analysis of clear positive and negative thresholds for *in vitro* induction. To this end, we set out to gather hepatocyte induction

and clinical DDI data for both prototypical inducers and proprietary compounds. In addition, the IWG is also in the process of collating and generating primary hepatocyte CYP mRNA and activity time-course data to gather more information regarding the magnitude of induction response over-time and to determine the robustness of the assay at shorter time-points, in comparison to the 72 hr incubation time recommended by the EMA (EMA, 2012).

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Figure Legends:

Figure 1: Results from an IQ member survey focused on observations of in vitro down-regulation/suppression. Panel A highlights the number of responses and the distribution for culture format, # of wells and length of exposure to test compound. Panel B highlights the number of responses and the distribution for the type of cytotoxicity method run during the induction study and the enzymes where decreased levels were observed. Panel C highlights the number of responses and the distribution for the magnitudes of decreases observed for mRNA and enzyme activity levels as well as the percentage of standard induction studies where observations of down-regulation/suppression are made.

Figure 2: CYP3A4 and CYP2C9 mRNA induction data obtained from the same donors following hepatocyte incubations with rifampicin. Data obtained from 8 respondents

Figure 3: Induction of CYP2C and CYP3A4 mRNA (fold of induction) and functional activity in 10 different cryopreserved human hepatocyte donors following incubation with rifampicin at 10 uM. Data obtained from 1 respondent.

Figure 4: Correlation of CYP3A4 and CYP2C mRNA levels following incubation of human hepatocytes with rifampicin. Data are from 3 respondents. Linear regression and correlation analysis described in the plot were performed using Graph Pad Prism (version 6.04)

Figure 5: Comparison of CYP2B6 induction between CITCO and phenobarbital in cultured human hepatocytes from 26 different donors.

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Table 1: Drug-drug Interactions with Perpetrator Compounds that Induce CYP2C

Victim	Perpetrator	%Change AUC	Victim Dose (Oral)	Perpetrator Dose (Oral)	References
Tolbutamide CYP2C9 *in vivo* Probe	Aprepitant	-30.8	500 mg	125/80 mg (3 days)	(Shadle et al., 2004)
Tolbutamide CYP2C9 *in vivo* Probe	Rifampicin	-62.5	500 mg	600 mg (14 days)	(Adams et al., 2005)
Tolbutamide CYP2C9 *in vivo* Probe	Ritonavir	-50.5	500 mg	200-400 mg (average of 14 days)	(Kirby et al., 2011)
(S)-warfarin CYP2C9 *in vivo* Probe	Bosentan	-29.3	26 mg	500 mg (10 days)	(Weber et al., 1999)
(S)-warfarin CYP2C9 *in vivo* Probe	Dalcetrapib	-14	5 mg of racemic warfarin	900 mg (7 days)	(Derks et al., 2009)
(S)-warfarin CYP2C9 *in vivo* Probe	Rifampicin	-74.4	0.75 mg/kg	300 mg (14 days)	(Heimark et al., 1987)
(S)-warfarin CYP2C9 *in vivo* Probe	Ritonavir	-24.3	10 mg	100 mg (14 days)	(Morcos et al., 2013)
Repaglinide CYP2C8 *in vivo* probe	Rifampicin	-79.6	4 mg	600 mg (7 days)	(Bidstrup et al., 2004)
Repaglinide CYP2C8 *in vivo* probe	Flucloxacillin	-46.8	4 mg	500 mg (7 days)	(Du et al., 2013)
Omeprazole CYP2C19 *in vivo* Probe	arbamazepine	-39.7	20 mg	400-600 mg/day (3 weeks)	(Bertilsson et al., 1997)
Omeprazole CYP2C19 *in vivo* Probe	Efavirenz	-46.4	20 mg	600 mg (17 days)	(Michaud et al., 2012)
Omeprazole CYP2C19 *in vivo* Probe	enzalutamide	-70.5	20 mg	160 mg (85 days)	(Gibbons et al., 2015)
OmeprazoleCYP2C19 *in vivo* Probe	rifampicin	-93	10 mg	600 mg (7 days)	(Derungs et al., 2016)

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Table 2: Compounds Reported to Perpetrate CYP2C Induction-Based Drug-Drug Interactions also Perpetrate CYP3A4 Induction-Based Drug-drug Interactions

Victim	Perpetrator (Oral)	% Change AUC	Victim Dose	Perpetrator Dose	References
Simvastatin CYP3A *in vivo* Probe	Bosentan	-34.4	40 mg (5.5 days)	125 mg (5.5 days)	(Dingemanse et al., 2003)
Simvastatin CYP3A *in vivo* Probe	Carbamazepine	-74.5	80 mg	300 mg (14 days)	(Ucar et al., 2004)
Simvastatin CYP3A *in vivo* Probe	Rifampicin	-91	40 mg	600 mg (9 days)	(Chung et al., 2006)
Simvastatin CYP3A *in vivo* Probe	Rifampicin	-86.1	40 mg	600 mg (5 days)	(Kyrklund et al., 2000)
Simvastatin CYP3A *in vivo* Probe	Troglitazone	-37.7	40 mg (10 days)	400 mg (24 days)	(Prueksaritanont et al., 2001)
Sildenafil CYP3A *in vivo* Probe	Bosentan	-69	100 mg	62.5-125 mg (8 weeks)	(Paul et al., 2005)
Midazolam CYP3A *in vivo* Probe	Rifampicin	-98.4	7.5 mg	600 mg (6 days)	(Link et al., 2008)

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Table 3: Comparison of Mean (n=3) Fold of Induction of CYP2C9 and CYP3A4 mRNA *In Vitro* in Human Hepatocytes with Reduction in S-Warfarin or Phenytoin AUC *In Vivo*

Compound (10 uM)	**2C9**	**3A4**	**2C9 % AUC ↓**
Rifampicin	3 (0.7)	15 (6)	74.4[a]
Bosentan	3 (0.5)	13 (3)	29.3[a]
Ritonavir	2.6 (0.2)	10 (3)	24.3[a]
Dalcetrapib[b]	2.4 (0.3)	7.4 (2.5)	14[a]
Vigabitran	1.3 (0.2)	1.4 (0.1)	3.7[c]

[a] Decrease in S-warfarin AUC; 100 uM; [c] Phenytoin AUC

Change in AUC data obtained from the University of Washington Drug-Drug Interaction Database (https://www.druginteractioninfo.org/)

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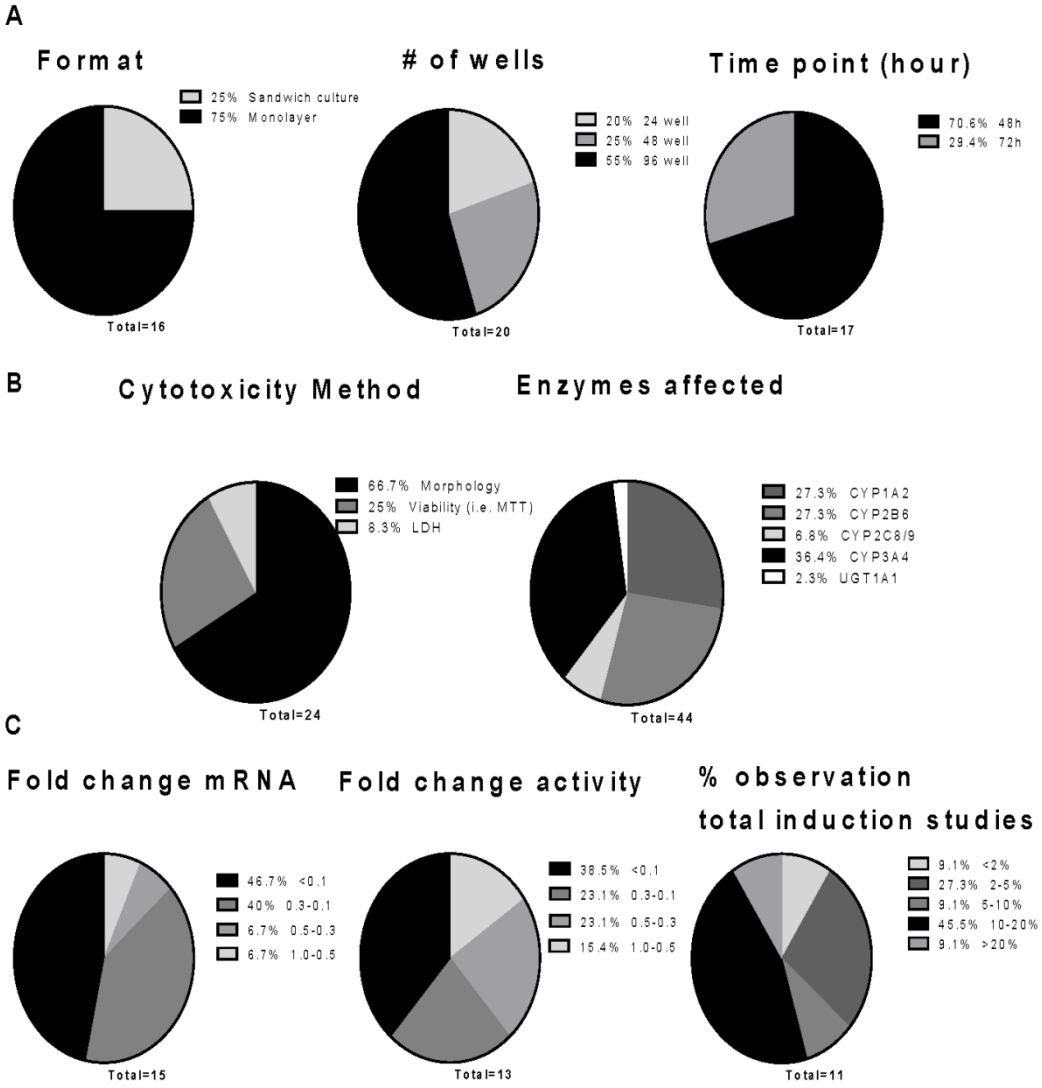

Figure 1

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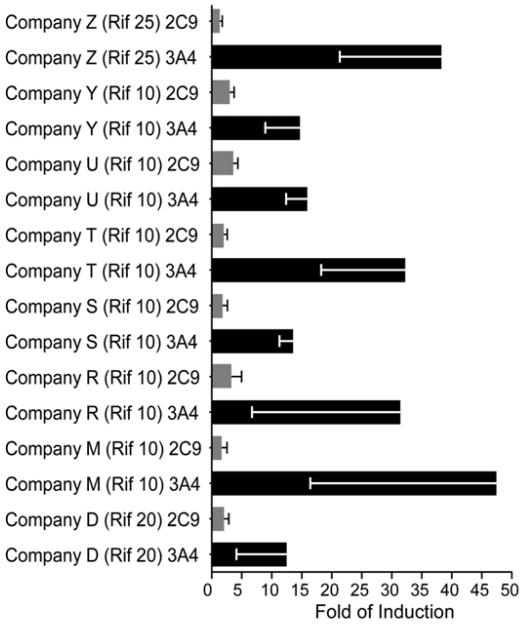

Figure 2

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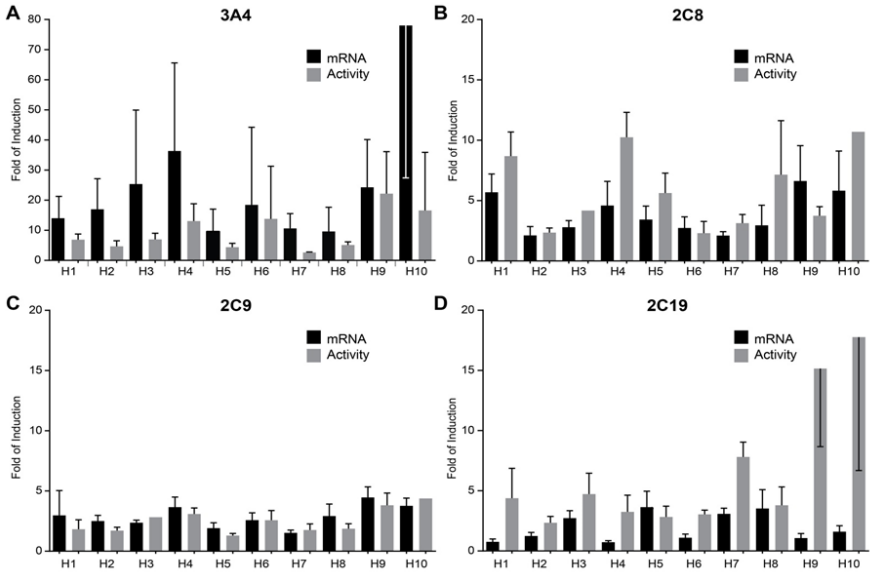

Figure 3

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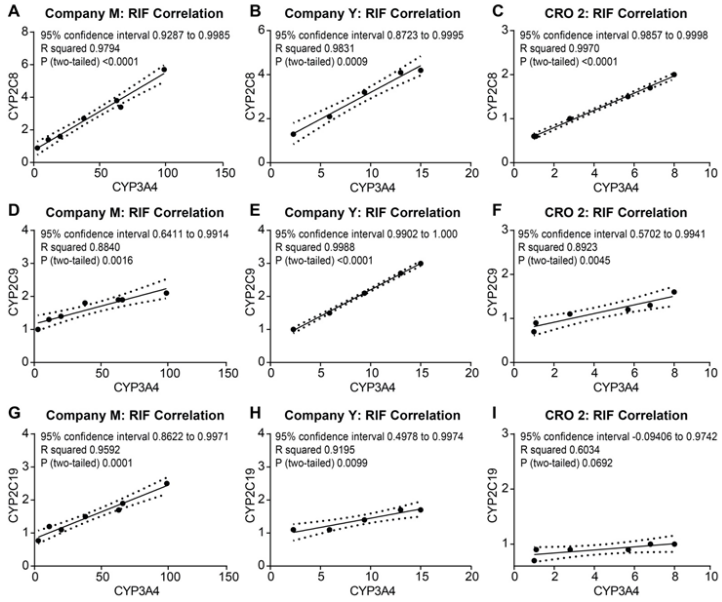

Figure 4

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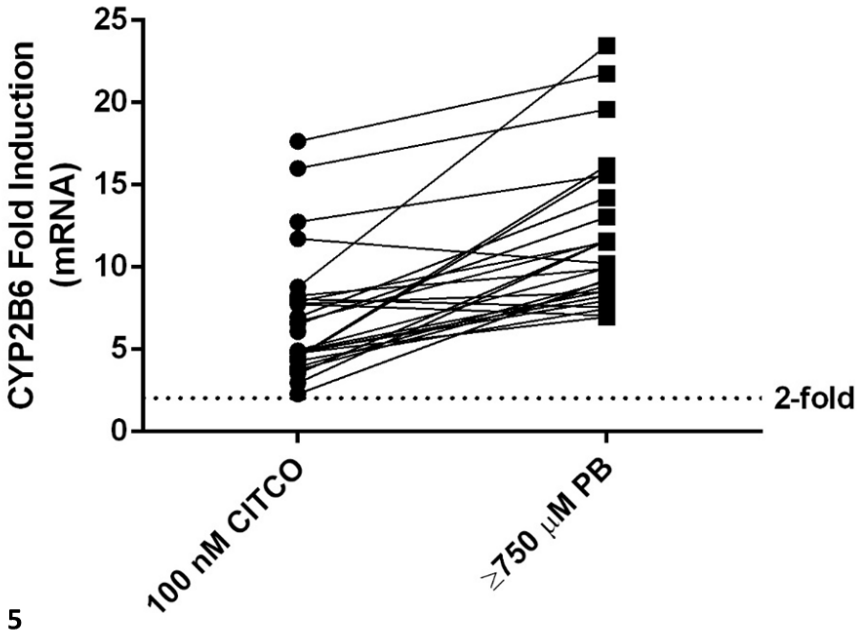

Figure 5