An exposure–response analysis based on rifampin suggests CYP3A4 induction is driven by AUC: an in vitro investigation

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Abstract
1. Induction is an important mechanism contributing to drug–drug interactions. It is most commonly evaluated in the human hepatocyte assay over 48-h or 72-h incubation period. However, whether the overall exposure (i.e. Area Under the Curve (AUC) or Cmax) or maximum exposure (i.e. Cmax) of the inducer is responsible for the magnitude of subsequent induction has not been thoroughly investigated. Additionally, in vitro induction assays are typically treated as static systems, which could lead to inaccurate induction potency estimation. Hence, European Medicines Agency (EMA) guidance now specifies quantitation of drug levels in the incubation.
2. This work treated the typical in vitro evaluation of rifampin induction as an in vivo system by generating various target engagement profiles, measuring free rifampin concentration over 3 d of incubation and evaluating the impact of these factors on final induction response.
3. This rifampin-based analysis demonstrates that the induction process is driven by time-averaged target engagement (i.e. AUC-driven). Additionally, depletion of rifampin in the incubation medium over 3 d as well as non-specific/specific binding were observed.
4. These findings should help aid the discovery of clinical candidates with minimal induction liability and further expand our knowledge in the quantitative translatability of in vitro induction assays.

Keywords
CYP3A4, drug–drug interaction, exposure–response relationship, induction, pharmacokinetics pharmacodynamics (PKPD)

History
Received 6 July 2016
Revised 5 August 2016
Accepted 7 August 2016
Published online 31 August 2016

Introduction
Induction of expression of drug metabolizing enzymes is a well-established phenomenon in clinical pharmacology (Le Cluyse et al., 2010). Induction can lead to increases in metabolic clearance of drugs and subsequent decreases in exposure that can result in inefficacy. In therapeutic areas where the disease is life-threatening, loss of efficacy due to such a drug interaction has been characterized as a safety concern (Dickinson et al., 2001; Grub et al., 2001). While there are a few mechanisms of induction, one of the greatest concern in clinical pharmacology occurs via activation of the pregnane-X-receptor (PXR), since it can result in meaningful increases in expression of several drug metabolizing enzymes and transporters, most notably cytochrome P450 3A (CYP3A) (Sinz et al., 2006). In drug research, new compounds are routinely evaluated for their potential to be inducers using in vitro methods, since this is an undesirable property. In vitro systems to identify human inducers and characterize their pharmacology are varied, but the best established and most well-accepted in vitro model is cultured human hepatocytes.

Human hepatocyte induction protocols involve the continuous treatment of cultured cells with test compounds for 2–3 d and measurement of mRNA and enzyme activity of target proteins. This procedure is well established and data are acceptable by government drug regulatory authorities for use in decision-making regarding drug interaction studies, concomitant medications and product labeling (EMA, 2012; FDA, 2012). For the PXR, the most commonly measured endpoints are mRNA and activity for CYP3A4. Rifampin is the most well-studied PXR-mechanism inducer and it is commonly used as a positive control in induction assays, as increases in mRNA and enzyme activity can be in the ranges of 10–100-fold and 3–20-fold, respectively (Hariparsad et al., 2008; Martin et al., 2008). Induction is an agonistic response. In vitro measurements are designed to determine the EC50 (an indirect measure of affinity based on the nominal concentration of test compound) and the Emax (a measure of the degree of maximum agonism) (Smith et al., 2007). These parameters are used, along with other input parameters such as in vivo pharmacokinetic parameters of the inducer and the affected
drug and fraction of total clearance of the affected drug-mediated by inducible enzymes, to make estimates of the extent of drug–drug interactions with concomitant administration of an inducer and a drug for which the clearance is induced in humans. Several correlative models, static mathematical scaling models and dynamic physiologically-based pharmacokinetic models that aim to relate to indicate in vitro induction data to clinical pharmacokinetic outcome have been developed in this regard (Einolf et al., 2014; Fahmi & Ripp, 2010; Siccardi et al., 2013). A mechanistic PKPD model linking in vitro induction data to clinical induction response has also been reported (Yamashita et al., 2013).

However, to this point the complexity of the in vitro induction system may be underappreciated as it is comprised of intact multi-compartmentalized cells and complex protein-containing medium, and the typical induction experiment is carried out in a multiple dosing manners over a few days. The induction response involves a cascade of binding, subcellular translocation, gene transcription and protein synthesis steps. This is in stark contrast to simple enzyme inhibition or binding experiments that use simpler non-cellular systems in buffers and are conducted over shorter time periods. Therefore, while nominal ligand concentrations added to a simple system are mostly representative of the concentration that the enzyme ‘sees’ during a short incubation, in a multi-day induction experiment in living cells, the concentration of the test compound available to engage the target receptor may be very different, due to compartmentalization or consumption, or both. Our main objective was to examine the exposure–response relationship of the in vitro induction system: essentially to treat it as a miniature in vivo system in that the phenomena such as binding and consumption of test compound would be taken into consideration when establishing the concentration–response relationship. Rifampin was used as the prototype inducer, based on the existing wealth of knowledge of the performance of this compound in induction assays and ability to obtain a full dynamic response (i.e. EC_{50} and E_{max}). The binding and pharmacokinetics of rifampin were measured in the induction assay and an assessment of extracellular total and unbound concentrations was made. A set of rifampin dosing protocols was utilized to construct its exposure–response relationship and delineate whether the in vitro induction response is more related to magnitude versus duration of exposure (i.e. “C_{max} versus C_{ave} effect”). The findings should be of use in interpreting in vitro induction experiments and potentially for utilizing the data for predictions of drug–drug interactions.

**Experimental procedures**

**Chemicals**

Dimethyl sulfoxide (DMSO), rifampin, testosterone and 6β-Hydroxytestosterone were obtained from Sigma-Aldrich (St. Louis, MO). Cryopreserved human hepatocytes, Lot Hu8123 (from a female donor), was obtained from Life Technologies (Carlsbad, CA). In VitroGRO™ HT thawing media, In VitroGRO™ plating media, In VitroGRO™ incubation media and Torpedo Antibiotic Mix were obtained from BioreclamationIVT (Baltimore, MD). Matrigel was obtained from BD Biosciences (Agawam, MA).

**In vitro studies**

**Study design**

Dedicated wells with plated hepatocytes were treated with rifampin at 1, 2, 3, 4, 6, 12 or 24 μM. For each concentration, the hepatocytes were exposed to rifampin for the following durations: 1, 2, 4, 6, 8, 14 or 24 h per day for 3 d (Figure 1). At the end of each incubation period within the day, all treatment media was removed for rifampin concentration analysis and the wells were refilled with fresh plain incubation media (vehicle control) for the rest of the day. At the end of the treatment on day 3, fold induction based on both enzyme activity and mRNA levels were measured as described below. Incubations were done in triplicate and DMSO was used as the vehicle control.

**Hepatocyte condition**

Cryopreserved human hepatocytes (Lot Hu8123) were recovered in thawing media and resuspended in plating media. The cells were seeded at approximately 7.5 × 10^3 viable cells per ml of cell density on collagen I precoated 96-well plates and placed in a humidified culture chamber (37 ± 1°C, at 95% ± 5% relative humidity and 5% ± 1% carbon dioxide [CO₂]). After an attachment period of at least 4 h, media and dead cells were removed from each well. The medium was then replaced with incubation medium containing Torpedo Antibiotic Mix and Matrigel (250 μg/ml). Cultures were then incubated for 24 h before treating with rifampin and vehicle control (0.1% DMSO).

**Fold induction based on mRNA expression**

Hepatocytes were lysed in RNeasy Lysis (RLT) buffer with β-mercaptoethanol (1:100) after 72 h of incubation with treatment media. Total RNA was isolated and purified using Rneasy Mini Kit (Qiagen Inc., Hilden, Germany). cDNA was synthesized from RNA with RT Master Mix (Applied Biosystems, Foster City, CA). Following this, RT-PCR was carried out according to the Applied Biosystems protocol using TaqMan® Fast Advanced Master Mix and TaqMan® Gene Expression probe for CYP3A4 (HS0060456_m1) and GAPDH as the endogenous probe (HS9999905_m1). CYP3A4 expression levels were normalized to endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to calculate the fold induction using the ΔΔCT method.

**Fold induction based on enzyme activity**

After treatment of cells for 3 d, the media containing vehicle controls and rifampin was removed and the cells were rinsed three times with phosphate-buffered saline (PBS). Fresh incubation media (200 μl) containing 200 μM testosterone (CYP3A4 substrate) was added to each well and the plate was returned to the incubator for 30 min. At the end of each incubation period, reactions were stopped by freezing the supernatant at −20°C and analyzed as described below for 6β-hydroxytestosterone formation by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Determination of rifampin metabolism in hepatocyte cultures**

Medium samples from induction assays containing rifampin or vehicle control were analyzed by liquid chromatography-
mass spectrometry (LC-MS). Four volumes of CH$_3$CN were added to each sample followed by vortex mixing and spinning in a centrifuge (1700 g for 5 min). The supernatant was transferred and subjected to evaporation in a vacuum centrifuge. To the dried residue was added 0.1 ml of 1% HCOOH. The sample (0.01 ml injected) was analyzed by HPLC-UV/Vis-MS on a Thermo Orbitrap Elite high-resolution mass spectrometer containing an Accela UHPLC pump.
and photodiode array detector. Separation was effected using a Waters Acquity HSS T3 C18 column (2.1 × 100; 1.8 μ) in 0.1% HCOOH containing 20% CH₃CN at a flow rate of 0.35 ml/min. This condition was held for 0.5 min, followed by a linear gradient to 95% CH₃CN at 8 min, maintaining this composition for 1.5 min, followed by re-equilibration at initial conditions for 1.5 min. The UV/Vis signal was monitored at 200–600 nm. The mass spectrometer was operated in the positive ion mode, with source and instrument settings adjusted to optimize the signal for rifampin.

**Induction media protein binding assay**

Induction media were spiked with rifampin to a final concentration of 2 μM. Two hundred and twenty microliter of the solution was added to the donor wells and 350 μl PBS (PBS without calcium or magnesium, Lonza, Walkersville, MD) was added to the receiver wells of the rapid equilibrium dialysis (RED) device (Thermo Scientific, Rockford, IL) quadruplicates were run for the test compound. Before and after incubation, an aliquot of 15 μl of the induction media spiked with 2 μM rifampin was added to a 96-well deep plate containing 45 μl of PBS and 200 μl of cold acetonitrile (ACN) containing terfenadine (5 ng/ml) and tolbutamide (0.5 ng/ml) as internal standard (IS). These samples were used for recovery and stability evaluation. The RED sample blocks were covered with Breathe Easy™ gas permeable membranes (Sigma-Aldrich) and placed on an orbital shaker (450 rpm, VWR Scientific Products, Radnor, PA) in a humidified (75% RH) incubator at 37 °C with 5% CO₂/95% O₂ for 4 h. At the end of the incubation, 15 μl of media samples from the donor wells were taken and added to a 96-well deep plate containing 45 μl of PBS and 200 μl of cold ACN with IS. Aliquots of 45 μl dialyzed PBS were taken from the receiver wells and added to 15 μl of blank media and 200 μl of cold ACN with IS in a 96-well deep plate. The plates were sealed (Nunc aluminum sealing tape, Thermo Scientific) and mixed with a vortex mixer (Multi-Tube Vortexer, VWR scientific products) for 1 min, then centrifuged (Eppendorf, Hauppauge, NY) at 3000 rpm, room temperature for 5 min. The supernatant was transferred to a new deep well plate, sealed and subsequently analyzed using LC-MS/MS. Sertraline was used as a quality control sample.

Fraction unbound \( f_u \) was calculated using Equation (1). Masses in receiver and donor wells were determined from the area counts or concentrations obtained from LC-MS/MS analysis corrected to account for sampling volumes. Recovery and stability were calculated using Equations (2) and (3), respectively:

\[
f_u = \frac{\text{Receiver mass}}{\text{Donor mass}} \quad (1)
\]

\[
\% \text{ Recovery} = \frac{\text{Donor mass} + \text{Receiver mass}}{\text{Donor mass at time zero}} \times 100\% \quad (2)
\]

\[
\text{Stability as remaining} = \frac{\text{Mass at last time point}}{\text{Mass at zero time point}} \times 100\%. \quad (3)
\]

**Bioanalytical**

**6β-Hydroxytestosterone**

Assays were performed on an API 4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) with Shimadzu HPLC pumps (Columbia, MD) and a CTC PAL autosampler system (LEAP Technologies, Carrboro, NC). The LC mobile phases were: (A) HPLC grade water containing 0.1% formic acid and (B) ACN containing 0.1% formic acid. A solvent gradient was applied from 5% (B) to 95% (A) over 1.0 min and held at 95% (B) for 0.6 min at a flow rate of 0.5 ml/min was used to elute the analyte from the column (Kinetex C18, 30 × 2 mm, 2.6 μm, Phenomenex, Torrance, CA). The \( m/z \) transition of 6β-hydroxytestosterone was from 305 to 269. The dynamic range of the assay is 0.05–25 um. The IS was 100 ng/ml (E)-3-(4-((2S,3S,4S,5R)-5-1-(3-chloro-2,6-difluorobenzyloxyimino)ethyl)-3,4-dihydroxytetrahydro furan-2-yl)-3-hydroxyphenyl)-2-methyl-N(3aS,4R,5R,6S,7R,7aR)-4,6,7-trihydroxyhexahydrobenzo[d][1,3]dioxidol-5-yl) acrylamide (PF-05218881) in ACN. The \( m/z \) transition of internal standard was from 687 to 320. Analyst™ 1.5.1 software (Applied Biosystems) was applied to data collection processing and analysis.

**Rifampin**

At predetermined time points, incubation medium was collected and rifampin was quantitated by LC-MS/MS using API 4000 instrument (AB Sciex) with Shimadzu HPLC pumps and a CTC PAL autosampler system (LEAP Technologies). A standard curve with a dynamic range of 0.16–20 μM was applied for quantitation of rifampin remaining in the incubation medium. The \( m/z \) transition was from 823 to 151. The LC mobile phases were: (A) HPLC grade water containing 0.1% formic acid and (B) ACN containing 0.1% formic acid. A solvent gradient was applied from 5% (B) to 95% (A) over 1.0 min and held at 95% (B) for 0.6 min at a flow rate of 0.5 ml/min was used to elute the analytes from the column (Kinetex C18, 30 × 2 mm, 2.6 μm, Phenomenex). Analyst™ 1.5.1 software (Applied Biosystems) was applied to data collection processing and analysis.

**Data analysis**

In order to capture rifampin concentration change over time after initially added to the wells, the measured rifampin concentrations at the end of each incubation period (i.e., 1, 2, 4, 6, 8, 14 or 24 h each day) from different wells were plotted for each dose (i.e. nominal concentration added to each well). Rifampin AUC over 3 d of incubation was subsequently determined based on the linear trapezoidal rule. The measured \( C_{ave} \) was calculated as AUC/72 h to represent the average rifampin exposure over 3 d. The measured \( C_{max} \) was determined as the highest rifampin concentration over 3 d. The measured concentrations were then multiplied by the fraction unbound in induction media to derive unbound concentrations. Subsequently, these two pharmacokinetic endpoints were used as independent variables and fitted to fold.
induction based on enzyme activity measured after 72 h using the $E_{\text{max}}$ model (Equation (4)):

$$FI = \frac{E_{\text{max}} \cdot C_{\text{RIF}}}{C_{\text{RIF}} + EC_{50}}$$  \hspace{1cm} (4)

where $FI$ represents fold induction based on enzyme activity or mRNA; $C_{\text{RIF}}$ represents either average or maximum unbound rifampin concentration; $E_{\text{max}}$ represents maximum fold induction and $EC_{50}$ represents rifampin concentration required for half-maximal induction. Induction responses were log transformed before model fitting, the difference between the observed values and the model-fitted values was described by a residual error model represented by Equation (5):

$$\ln(FI) = \ln\left(\tilde{FI}\right) + \varepsilon$$  \hspace{1cm} (5)

where $FI$ and $\tilde{FI}$ represent the observed and model-predicted fold induction, respectively, and $\varepsilon$ is the lognormal additive (i.e. proportional) error.

The analyses were conducted using non-linear mixed-effects modeling as implemented in the NONMEM (NONMEM software system, version 7.2, Globomax LLC, Hanover, MD). The analyses were conducted using the first-order conditional estimation with the interaction (FOCE INTERACTION) method. Parameter estimate confidence intervals were determined by parametric bootstrapping ($n = 1000$). Graphical data display was performed using R 3.1.0.

An alternative approach to compare the model fit between $C_{\text{max}}$ and $C_{\text{ave}}$ was also performed. In this approach, instead of pooling data from different incubation intervals, data from each incubation interval were modeled independently where $C_{\text{RIF}}$ ($C_{\text{max}}$ or $C_{\text{ave}}$) was fitted to $FI$ based on either activity or mRNA according to Equation (4). $E_{\text{max}}$ values were fixed to previous estimates based on pooled dataset because limited data from each incubation interval is not sufficient to independently determine both $E_{\text{max}}$ and $EC_{50}$.

**Results**

**Fold induction based on mRNA**

After 3 d treatment, up to a 400-fold mRNA increase was observed across different designs (Figure 2). Generally, higher initial rifampin concentration resulted in more significant mRNA increase. Similarly, longer incubation duration led to more significant mRNA increase with the exception of the continuous 24 h/day incubation. mRNA increase measured after 24 h/day are consistently lower than that from 14 h exposure/day suggesting decreasing mRNA levels despite the presence of rifampin during Day 3. The same trend where mRNA level peaked on day 2 was also observed from a previous study (not reported here) from the same lot of human hepatocyte (Hu8123). Similar finding that mRNA level peaks at ~40h post rifampin exposure has been reported previously (Yamashita et al., 2013). This could be due to the reported negative feedback mechanism for PXR, where ligand-activation of PXR suppresses transcription of the PXR gene and attenuates PXR protein expression (Bailey et al., 2011). In order to remove confounding effect of diminishing mRNA levels in subsequent direct response modeling, 24 h/day duration mRNA data was excluded from further analysis.

**Fold induction based on activity**

Upon 3 d of treatment, a range of 2–35-fold CYP3A4 activity increase was observed across different designs (Figure 3). In general, higher initial rifampin concentration resulted in more significant activity increase. Similarly, longer incubation duration results in more significant activity increase with the exception of 1 h exposure/day. The activity fold induction measured after 1 h/day incubation appeared to be higher than that after 2 h/day incubation. This unusual finding was not reproduced in a subsequent repeat study. Consequently, induction result from 1 h/day incubation was excluded from later analyses.

**Nominal versus measured rifampin concentration**

Measured rifampin concentration at the first time point (1 h) is consistently lower than nominal concentration potentially...
due to non-specific binding to the incubation wells and hepatocytes. The difference between measured and nominal concentration at 1 h decreases with increasing number of incubation days suggesting the non-specific binding process is saturable (Figure 4). As a result, maximum rifampin concentration is always observed on Day 3. Within each incubation day, rifampin concentration continues to decrease to approximately half of the starting concentration by 24 h. Non-specific binding and compound depletion contributed to approximately two-fold reduction in observed average concentration when compared with nominal concentration.

Rifampin metabolism

Analysis of induction incubations for rifampin metabolites showed the presence of a single new peak which is absent from either the vehicle control or the time zero sample, in addition to rifampin itself. The peak possessed a protonated molecular ion at 781.4019 (C_{41}H_{57}N_{4}O_{11}; 0.07 ppm) and an in-source fragment ion at m/z 749.3758 indicating the loss of methanol (Supplemental File). The metabolite was identified as desacetylrifampin.

Fraction unbound

Rifampin is moderately bound in induction media (fraction unbound in media was determined to be 0.57 ± 0.13).

Exposure–response analysis

When both averages unbound rifampin concentration (i.e. C_{ave}) and maximum unbound rifampin concentration (i.e. C_{max}) was plotted against the corresponding fold induction results for all studies, it is clear that the magnitude of induction is more responsive to changes in C_{ave} than in C_{max} (Figures 5 and 6). The direct response E_{max} model sufficiently characterized the relationship between C_{ave} and the final induction response based on both enzyme activity and mRNA (Figures 5a and 6a). All model parameters were identifiable and are listed in Table 1. The E_{max} for mRNA and activity-based induction was estimated to be 399 (95%CI: 333–474) and 36 (95%CI: 34–38), respectively. The unbound C_{ave} EC_{50} for mRNA and activity-based induction was estimated to be 1.0 um (95%CI: 0.7–1.3) and 0.4 um (95%CI: 0.4–0.5), respectively. Such a relationship was not evident between C_{max} and induction where the same C_{max} corresponds to multiple levels of induction (Figures 5b and 6b). Although the model parameters were still identifiable with mRNA E_{max} of 415 (95%CI: 292–661), activity E_{max} of 34 (95%CI: 28–41), mRNA C_{max} EC_{50} of 4.8 um (95%CI: 2.9–8.6) and activity C_{max} EC_{50} of 1.9 um (95%CI: 1.3–2.7), the confidence intervals are much higher than those from a C_{ave}-based model. More importantly, the much higher residual error was associated with the C_{max}-based model than the C_{ave}-based model (0.64 versus 0.38 for mRNA and 0.44 versus 0.16 for activity) indicating that C_{ave} is more predictive of induction response than C_{max}. In the alternative modeling approach where individual models were generated based on each incubation duration, when using C_{ave} as the dependent variable, the EC_{50} values from different incubation durations are relatively consistent ranging from 0.31 to
0.44 μm for activity and 0.29–0.88 um for mRNA (Figures 5c and 6c). This range is much wider (0.51–4.2 μm for activity and 0.80–11 um for mRNA) when using C_{max} as the dependent variable (Figures 5d and 6d) suggesting C_{max} alone is not predictive of induction response. These results both visually and quantitatively suggest that the induction response is mainly driven by rifampin C_{ave} instead of C_{max}.

Discussion

In the design of new drugs, it is highly desirable to avoid compounds that cause induction in the clinic, and in vitro assays using cultured human hepatocytes have become an accepted standard for understanding the potential for induction (EMA, 2012; FDA, 2012). However, the determination of
the salient parameters EC$_{50}$ and E$_{max}$ in these assays is made by using the nominal concentrations of test compound added to the culture incubations, and aspects of a free fraction, compound consumption during the incubation, and dosing interval have not been considered. To the latter two aspects, knowledge of whether induction arises via a threshold effect versus time-averaged activation of the receptor can be important. In a threshold effect, the inducer would merely need to meet/exceed a concentration that activates the induction cascade, which would continue to operate even after the inducer concentrations drop below the threshold concentration. Alternatively, induction could be related to overall exposure to the inducer irrespective of whether the system is exposed to a high concentration for a short period or a low concentration over a long period. In this study, we sought to address this question by employing cultured human hepatocytes and an experimental design of dosing of the system with the prototypical inducer rifampin using different dosing regimens (i.e. different dose levels and dose intervals that would yield similar overall exposures but different concentration versus time relationships). These data were used to develop an exposure–response relationship for rifampin induction in cultured human hepatocytes. By producing different rifampin exposure profiles in vitro and quantitatively investigating their impact on induction response, we clearly demonstrated that overall exposure throughout the incubation represented by C$_{ave}$ is more predictive of induction than maximum exposure (i.e. the induction effect is the result of time averaged activation of PXR (i.e. AUC-driven effect) at currently tested concentration range) (Figure 5). Also, other phenomena occurring in the in vitro system, that is non-specific binding and clearance of the inducer, were also considered. This approach is essentially the same as that commonly applied to understand in vivo pharmacology: pharmacodynamic effects over different durations, the pharmacokinetics of the test compound in the system and binding in the system are all accounted for in generating the model and estimating salient parameters EC$_{50}$ and E$_{max}$. This finding has practical implications for drug discovery. For example, development of a controlled release formulation or dividing the dose over the same dosing period to lower the C$_{max}$ of drug candidates as a way to mitigate induction liability would not likely be effective since the overall AUC is not changed.

It should be noted that the primary goal of the current study is to identify the contributing factor (C$_{ave}$ or C$_{max}$) for in vitro induction response, not for the establishment of induction in vitro in vivo correlation (IVIVC) as this would require study of a large set of compounds. Nonetheless, this current analysis with rifampin as a model inducer revealed three processes that might help improve induction IVIVC if correctly accounted for: 1) non-specific binding to wells/ hepatocytes, 2) specific binding to media protein (commonly used induction media contains 0.2% bovine serum albumin (BSA)) and 3) compound depletion. Ignoring these processes by using nominal inducer concentration could lead to overprediction of free drug concentration and subsequent under prediction of induction potency (EC$_{50}$), hence the recent EMA requirement of measuring drug levels in the incubation (EMA, 2012). Our results also suggest that measuring only the concentrations prior to media change (i.e. C$_{min}$) would overestimate the induction liability. The magnitude of the impact is dependent on the property of the tested compound. While the specific binding to the medium protein and non-specific binding to the apparatus was not excessively high for rifampin, this phenomenon could become an issue for more lipophilic compounds where actual free concentrations are much lower than nominal concentrations (Fahmi et al., unpublished observations). Although CYP enzymes from plated hepatocytes are not expected to be as active as those from hepatocytes in suspension, rifampin depletion was clearly observed over 24 h. The observed desacetyl metabolite in treated samples confirmed that the rifampin loss is due to metabolism. This depletion effect could be more severe for compounds with greater metabolic intrinsic clearance. In the current rifampin example, ignoring these factors by using nominal concentration leads to an approximately three-fold overestimate of EC$_{50}$. Rifampin is neither highly bound (with fraction unbound in the media of 0.57) nor rapidly cleared (human clearance ~5.7 l/h) (Loos et al., 1985). It is conceivable that more significant under prediction of induction potential could result in a compound that is either highly bound and/or rapidly cleared when the only nominal concentration is used. Alternatively, over prediction of induction potential could result if only the concentration prior to media change is used. Verifying this hypothesis with additional compounds could help improve our ability to predict in vivo induction potential in humans.

Ultimately, it is the hepatocyte intracellular drug concentration that is most relevant for induction potency estimation. However, human induction potential most often is predicted based on free plasma concentration since tissue concentration from clinical studies is rarely available. Thus, the assumption in using free media concentration-based induction potency would be that K$_{p,uu}$ in an in vitro induction assay is similar to K$_{p,uu}$ between free plasma and hepatocytes in humans. This assumption has been verified in rodent where the magnitude of active uptake in vitro strongly correlated with that of in vivo for several drugs (Yamano et al., 1999, 2000). Another confounding factor that was not considered in current analysis is the potential contribution from the metabolite. It is conceivable.

Table 1. Pharmacodynamic parameter estimates from induction exposure–response analyses.

| Dependent variable | Independent variable | Parameter | Estimate | 95%CI |
|--------------------|----------------------|-----------|----------|-------|
| mRNA              | C$_{ave}$            | E$_{max}$ | 399      | 333–474 |
|                   | EC$_{50}$ (um)       | 1.0       | 0.7–1.3  |
|                   | Residual error       | 0.38      | 0.33–0.43|
|                   | cResidual error      | 0.38      | 0.33–0.43|
| Activity          | C$_{ave}$            | E$_{max}$ | 415      | 292–661 |
|                   | EC$_{50}$ (um)       | 4.8       | 2.9–8.6  |
|                   | Residual error       | 0.64      | 0.57–0.73|
|                   | cResidual error      | 0.64      | 0.57–0.73|
|                   | C$_{max}$            | E$_{max}$ | 36       | 34–38   |
|                   | EC$_{50}$ (um)       | 0.4       | 0.4–0.5  |
|                   | Residual error       | 0.16      | 0.14–0.17|
|                   | cResidual error      | 0.16      | 0.14–0.17|
|                   | C$_{ave}$            | E$_{max}$ | 34       | 28–41   |
|                   | EC$_{50}$ (um)       | 1.9       | 1.3–2.7  |
|                   | Residual error       | 0.44      | 0.39–0.49|

*Unbound average rifampin concentration;  
*Unbound maximum rifampin concentration;  
*Lognormal additive residual error; CI: confidence interval.
that IVIVC could be poor for a compound with a metabolite that also is an inducer because the metabolite has not been accounted for in the in vitro experiment, and furthermore, the metabolite profile in cultured hepatocytes might not be the same as that generated in vivo. As stated above, exploration of these aspects of relating in vitro concentration-effect relationships to in vivo induction requires examination of a large set of drugs with varying extents of binding, metabolic lability, membrane permeability and metabolites that also induce. These observations with rifampin lay the groundwork upon which such an endeavor can be undertaken.

In summary, a model-based approach was applied to the standard in vitro induction system after generating a diverse range of rifampin exposure profiles. exposure–response analysis based on observed free media rifampin concentration and final fold induction convincingly demonstrated that induction is driven by average rather than maximum exposure. The current study also identified compound depletion, specific and non-specific binding as phenomena that could lead to under-estimation of induction potential. While the data support the continued use of the standard 3-d treatment protocol in human hepatocyte induction studies, these findings have the potential to improve the usefulness of in vitro induction assays to predict clinical outcomes as well as aid drug research teams in designing the best in vitro induction studies to enable drug candidate selection. With current quantitative understanding of rifampin induction in hand, further work to determine this type of relationship for other inducers (PXR and other mechanisms) is underway.

Acknowledgements
We thank Larry Tremaine and Dennis Smith for critical review of the manuscript. We also thank Larry Tremaine for supporting this study.

Declaration of interest
The authors report no declarations of interest.

References
Bailey I, Gibson GG, Plant K, et al. (2011). A PXR-mediated negative feedback loop attenuates the expression of CYP3A in response to the PXR agonist pregnenalone-16α-carbonitrile. PLoS One 6:e16703.
Dickinson BD, Altman RD, Nielsen NH, Sterling ML. (2001). Drug interactions between oral contraceptives and antibiotics. Obstet Gynecol 98:853–60.

Einolf HJ, Chen L, Fahmi OA, et al. (2014). Evaluation of various static and dynamic modeling methods to predict clinical CYP3A induction using in vitro CYP3A4 mRNA induction data. Clin Pharmacol Ther 95:179–88.

EMA. (2012). Guideline on the investigation of drug interactions. Pp 21–25. Available from: http://www.ema.europa.eu/docs/en_GB/document_ library/Scientific_guideline/2012/07/WC500129606.pdf.

Fahmi OA, Ripp SL. (2010). Evaluation of models for predicting drug–drug interactions due to induction. Expert Opin Drug Metab Toxicol 6:1399–416.

FDA. (2012). Drug interaction studies - study design, data analysis, implications for dosing, and labeling recommendations. Draft Guidance. Pp 2130. Available from: http://www.fda.gov/downloads/drugs/guidanceregulatoryinformation/guidances/ucm292362.pdf.

Grub S, Bryson H, Goggin T, et al. (2001). The interaction of saquinavir (soft gelatin capsule) with ketoconazole, erythromycin and rifampicin: comparison of the effect in healthy volunteers and in HIV-infected patients. Eur J Clin Pharmacol 57:115–21.

Hariparsad N, Carr BA, Evers R, Chu X. (2008). Comparison of immortalized Fa2N-4 cells and human hepatocytes as in vitro models for cytochrome P450 induction. Drug Metab Dispos 36:1046–55.

Le Cluyse EL, Sinz MW, Hewitt N, et al. (2010). Cytochrome P450 induction In: Lu C, Li A, eds. Enzyme inhibition in drug discovery and development. Hoboken, NJ: John Wiley & Sons, Inc, 265–314.

Loos U, Musch E, Jensen JC, et al. (1985). Pharmacokinetics of oral and intravenous rifampicin during chronic administration. Klin Wochenschr 63:1205–11.

Martin P, Riley R, Back DJ, Owen A. (2008). Comparison of the induction profile for drug disposition proteins by typical nuclear receptor activators in human hepatic and intestinal cells. Br J Pharmacol 153:805–19.

Siccardi M, Marzolini C, Seden K, et al. (2013). Prediction of drug-drug interactions between various antidepressants and efavirenz or boosted protease inhibitors using a physiologically based pharmacokinetic modelling approach. Clin Pharmacokinet 52:583–92.

Sinz M, Kim S, Zhu Z, et al. (2006). Evaluation of 170 xenobiotics as trans activators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. Curr Drug Metab 7:375–88.

Smith DA, Dickins M, Fahmi OA, et al. (2007). The time to move cytochrome p450 induction into mainstream pharmacology is long overdue. Drug Metab Dispos 35:697–8.

Yamano K, Yamamoto K, Kotaki H, et al. (1999). Correlation between in vivo and in vitro hepatic uptake of metabolic inhibitors of cytochrome P-450 in rats. Drug Metab Dispos 27:1225–31.

Yamano K, Yamamoto K, Kotaki H, et al. (2000). Quantitative prediction of metabolic inhibition of midazolam by erythromycin, diltiazem, and verapamil in rats: implication of concentrative uptake of inhibitors into liver. J Pharmaco Exp Ther 292:1118–26.

Yamashita F, Sasa Y, Yoshida S, et al. (2013). Modeling of rifampicin-induced CYP3A4 activation dynamics for the prediction of clinical drug-drug interactions from in vitro data. PLoS One 8:e70330.

Supplementary material available online