Predictive Utility of In Vitro Rifampin Induction Data Generated in Fresh and Cryopreserved Human Hepatocytes, Fa2N-4, and HepaRG Cells

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ABSTRACT:

Rifampin is a potent inducer of CYP3A4 in vitro and precipitates numerous drug-drug interactions (DDIs) when coadministered with CYP3A4 substrates. In the current study, we have critically assessed reported rifampin in vitro CYP3A4 induction data in Fa2N-4, HepaRG, and cryopreserved or primary human hepatocytes, using either CYP3A4 mRNA or probe substrate metabolism as induction endpoints. An in vivo database of intravenously administered victim drugs (assuming hepatic induction only) was collated ($n = 18$) to assess the predictive utility of these in vitro systems and to optimize rifampin in vivo $E_{\text{max}}$. In addition, the effect of substrate hepatic extraction ratio on prediction accuracy was investigated using prediction boundaries proposed recently (Drug Metab Dispos 39:170–173). Incorporation of hepatic extraction ratio in the prediction model resulted in accurate prediction of 89% of intravenous induction DDIs ($n = 18$), regardless of the in vitro system or induction endpoint (mRNA or CYP3A4 activity). Effects of in vitro parameters from different cellular systems, and optimized in vivo $E_{\text{max}}$ on the prediction of 21 oral DDIs were assessed. Use of mRNA data resulted in pronounced overprediction across all systems, with 86 to 100% of DDIs outside the acceptable prediction limits; in contrast, CYP3A4 activity predicted up to 62% of the oral DDIs within limits. Although prediction accuracy of oral DDIs was improved when using intravenous optimized rifampin $E_{\text{max}} > 35\%$ of DDIs were incorrectly assigned, suggesting potential differential $E_{\text{max}}$ between intestine and liver. Implications of the findings and recommendations for prediction of rifampin DDIs are discussed.

Introduction

There is a considerable interest in predicting induction of in vivo CYP3A4-mediated drug metabolism from in vitro data (Huang et al., 2008; Chu et al., 2009). The concentration-dependent change in either CYP3A4 expression (mRNA or protein) or function (enzyme-specific substrate metabolism) can be modeled to estimate potency ($EC_{50}$) and magnitude ($E_{\text{max}}$) of induction in vitro. Primary and cryopreserved human hepatocytes and the immortalized liver cell lines Fa2N-4 and HepaRG are four in vitro systems commonly used to estimate $EC_{50}$ and $E_{\text{max}}$ (Fahmi et al., 2008a; Hariparsad et al., 2008; McGinnity et al., 2009). The data from these in vitro systems have been shown to be predictive of in vivo drug metabolism; however, each system has its own characteristics that may influence the induction parameter obtained (Vermeir et al., 2005).

Several approaches have been proposed for the prediction of in vivo induction drug-drug interactions (DDIs) based on $EC_{50}$ and $E_{\text{max}}$ estimates. Initially, quantitative predictions from in vitro data were based on calculation of an induction score or induction ratio (Kato et al., 2005; Ripp et al., 2006). More recently, prediction models incorporating either solely induction or induction in combination with inhibition mechanisms have been applied (Fahmi et al., 2008b; Shou et al., 2008; Galetin et al., 2010; Kirby et al., 2011). These prediction models use a static average or maximal inducer plasma concentration assumed to reflect steady-state enzyme activity before substrate administration. Recently, dynamic models based on an inducer concentration-time profile to account for the change in enzyme expression have been proposed (Almond et al., 2009; Fahmi et al., 2009). Comparison of prediction accuracy between static and dynamic models (both allowing multiple interaction mechanisms) resulted in $< 5\%$ difference between the models based on the analysis performed with midazolam DDIs (Fahmi et al., 2009).

Rifampin, a potent inducer of CYP3A4 in vitro and in vivo, is commonly used as a positive control or calibrator to evaluate the

ABBREVIATIONS: $E_{\text{max}}$, maximum induced fold change; $E_{\text{int}}$, hepatic extraction ratio; $AUC_{\text{iv}}/AUC_{\text{iv}}$,

$\text{ind}$, ratio of the intravenous victim drug $AUC$ in the absence and presence of rifampin; $\text{DDI}$, drug-drug interaction.
relative induction potential of other inducers (Burk et al., 2005; Paris et al., 2009; Kirby et al., 2011). This calibration is based on comparison of the EC50 and Emax of the test inducer to that of rifampin in the same in vitro system; thus, this approach is very sensitive to the accuracy of in vitro rifampin induction parameter estimates. Rifampin EC50 has been assumed to be similar in vitro and in vivo; however, rifampin Emax may be affected by mechanisms not present in vitro. Because in vivo estimates of this parameter are based on very limited data, attempts have been made to optimize in vitro Emax to in vivo by fitting a prediction model to in vitro and in vivo induction data (Fahmi et al., 2008a). In practice, in vitro EC50 and, in particular, Emax estimates vary greatly between in vitro systems (Hariparsad et al., 2008; Martin et al., 2008; Shou et al., 2008), and this variability in rifampin in vitro parameters represents a major challenge to the utility of rifampin as a calibrator for novel inducers.

The apparent link between baseline CYP3A4 activity and maximum rifampin response in vitro is an additional factor complicating the estimation of an in vivo rifampin Emax (LeCluyse et al., 2000; Madan et al., 2003). Despite pronounced variability in baseline activity between individual hepatocyte donors, the maximal induced activity achieved in vitro is relatively consistent. Whether this is also true in vivo is difficult to ascertain because of limited availability of in vivo rifampin Emax data. Baseline and induced 24-h urinary 6β-hydroxycortisol/cortisol ratios, a measure of CYP3A4 activity, were compared in 12 subjects (Ged et al., 1989). In this study, two subjects with the lowest baseline ratio displayed the greatest fold change after rifampin administration, supporting the relationship seen in vitro; however, larger datasets are required to appropriately define this relationship.

In the current study, available literature values for in vitro rifampin EC50 and Emax were critically assessed. The prediction accuracy of induction parameters from four in vitro systems was compared using an in vivo dataset of 18 intravenous DDIs between rifampin and CYP3A4 substrates. In all cases, the static induction DDI model (Shou et al., 2008; Fahmi et al., 2009) was used to assess predictive utility of either CYP3A4 mRNA or activity data from each in vitro system. In addition, the impact of the substrate hepatic extraction ratio (Kirby and Unadkat, 2010) on prediction outcome was investigated. Prediction accuracy was evaluated using the prediction boundaries proposed recently (Guest et al., 2011). In addition, the use of induction data from both in vitro and in vivo studies to estimate in vivo rifampin Emax was explored, assuming EC50 values were comparable between in vitro and in vivo. The application of optimized in vivo Emax on prediction of 21 oral DDIs was investigated.

Materials and Methods

In Vitro Induction Dataset. To minimize the effect of variability in reported in vitro EC50 and Emax on DDI predictions, selection criteria were applied to ensure consistency in the in vitro experimental design and allow comparison across studies. Rifampin EC50 and Emax values generated from human-derived cell lines were solely included in the in vitro dataset. In vitro induction data were excluded from the dataset if parameters were estimated from substrate cocktail incubations. Rifampin EC50 estimates were excluded if Emax was not reached in the concentration-dependent experiment. Only rifampin incubations run between 48 and 72 h (with media replacement every 24 h) were included in the dataset, based on the recent recommendations (Chu et al., 2009). Rifampin EC50 and Emax values estimated in Fa2N-4, HepaRG, and human hepatocytes (both primary and cryopreserved) were collated from filtered literature results and included in the in vitro dataset. In vitro induction data from other cell lines included in the literature search (LS174T, CV-1, and HepG2) did not meet the selection criteria. Induction parameter estimates were based either on expression (CYP3A4 mRNA) or specific function (midazolam 1′-hydroxylation or testosterone 6β-hydroxylation) in each in vitro system. In cases in which induction parameters were not explicitly stated in the text, EC50 and Emax were estimated from digitized figure data using Plot Digitizer 2.4.1. Rifampin EC50 and Emax parameters were estimated by fitting a sigmoid Emax model in Graph 5.0.10 (Erithacus Software Limited, Surrey, UK). The Hill coefficient, which determines the shape of the response versus concentration curve, was rarely reported in the induction studies. To use as many reported EC50 and Emax values as possible, the Hill coefficient was not included in our prediction model, analogous to other studies that have focused on prediction of induction DDIs (Fahmi et al., 2006b). Weighted mean EC50 and Emax (based on CYP3A4 mRNA or activity induction) were calculated for each in vitro system investigated; individually reported parameter estimates were weighted by the number of measures contributing to the estimate. Weighted mean was used as a more systematic representation of the average value across multiple in vitro experiments, in particular considering the variation in the data reported in the literature (individual or average). In comparison, other metrics, such as arithmetic mean or median values, were less sensitive to variability of in vitro experiment design. The weighted mean in vitro data were used to compare the predictive utility of Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes.

In Vivo Induction Dataset. Our dataset of in vivo DDIs between rifampin and CYP3A4 substrates was compiled by expanding upon a previously published dataset (Shou et al., 2008). The resulting in vivo induction dataset was composed of DDIs between rifampin and either intravenous or oral CYP3A4 substrates. Selection criteria were also applied to the in vivo data to reduce variability and inconsistency. Case study reports and studies in patient populations were excluded from the in vivo dataset. In addition, DDIs were excluded if additional interacting medications were allowed during the induction study. The in vivo dataset was limited to multiple-dose rifampin studies comparing the effect of a single dose of substrate before and after the induction period. In most in vivo interaction studies, oral rifampin was administered once daily, typically 600 mg/day, for 4 to 18 days. Maximum rifampin induction is not likely to occur until after five daily 600-mg doses (Ohnhaus et al., 1989). Finally, DDIs were only included in our dataset if intestinal availability (Fm) and fraction of substrate metabolized by CYP3A4 (fmCYP) had been validated (Galetin et al., 2008; Houston and Galetin, 2008; Gertz et al., 2010). In vivo induction DDIs were classified as either weak (1.25–2-fold change in AUC), moderate (2–5-fold), or strong (>5-fold), analogous to criteria identified for inhibition (Huang et al., 2008). These in vivo studies have been used to assess the predictive accuracy of our collated in vitro induction dataset and to estimate rifampin Emax in vivo.

Prediction of Rifampin Interaction In Vivo. For our initial analysis, a mechanistic static induction DDI model (eq. 1) was used to predict the magnitude of rifampin induction of CYP3A4-mediated metabolism of intravenous-administered drugs, assuming in vivo DDI represents interaction in the liver only (Galetin et al., 2010). This approach is similar to that used by Shou et al. (2008). Details of the clinical studies are shown in Table 1, and the corresponding references are listed in the supplemental material.

\[
\frac{\text{AUC}_\text{n}}{\text{AUC}_{\text{CYP}}} = \text{fmCYP}_{\text{CYP}} \times \left(1 + \frac{\text{Emax} \times [I]_{\text{in}} \times f_d}{\text{EC50} \times f_d + [I]_{\text{in}} \times f_d} \right) + (1 - \text{fmCYP})
\]

where \(\text{AUC}_\text{n}\) and \(\text{AUC}_{\text{CYP}}\) represent the area under the plasma concentration-time curve of intravenous-administered substrates before and after multiple oral doses of rifampin, respectively. The average systemic concentration of rifampin after multiple oral doses was used as a surrogate inducer concentration (\(I_{\text{in}}\)). In addition to average systemic concentration, maximum concentration at steady state has been used in DDI prediction as the worst-case scenario (Shou et al., 2008). In the interest of clarity, we have chosen to limit our analysis to a single estimated inducer concentration. Inducer concentration was calculated for each in vivo study according to eq. 2 below (Ito et al., 2004). Values for fraction of overall metabolism attributed to CYP3A4 (fmCYP) were taken from the literature (Houston and Galetin, 2008; Shou et al., 2008).

\[
[I]_{\text{in}} = \frac{D \tau_r}{\text{CL}_{\text{sys}} \text{F}}
\]

where \(D\) represents the oral dose of rifampin administered in the interaction study, and \(r\) represents the dosing interval. For the purposes of parameter
estimation, rifampin oral clearance (CLoral/F) was assumed to be 19.2 l/h based on a nonlinear mixed-effects population pharmacokinetic model fitted to data from 261 pulmonary tuberculosis patients (95% confidence interval, 18.4–20.0 l/h) (Wilkins et al., 2008). This value is comparable to the estimate obtained by a noncompartmental analysis of rifampin pharmacokinetics in 28 healthy women (18.9 ± 5.6 l/h) (LeBel et al., 1998).

The free fraction of rifampin in human hepatocytes (fu,h) was taken from the literature (Shou et al., 2008). Unfortunately, the in vitro free fraction was not measured in most induction experiments. However, the median cellular concentration for those studies in which cell number and incubation volume were measured in most induction experiments. However, the median cell concentration used in our predictions was experimentally determined by equilibrium dialysis, as described in the supplemental material.

In addition, we assessed the effect of incorporating the substrate hepatic extraction ratio (Ehep) on the prediction of DDIs between rifampin and intravenous CYP3A4 substrates, as proposed recently (Kirby and Unadkat, 2010). Considering our focus on the prediction of CYP3A4 induction DDIs, the Kirby and Unadkat (2010) equation has been rearranged to eq. 5 shown below.

\[
\frac{\text{AUC}_{\text{inhibitor}}}{\text{AUC}_{\text{control}}} = \frac{1 - f_{\text{hep}}}{1 - E_{\text{hep}}} \left( \frac{f_{\text{hep}} \times \text{EC50} \times f_{\text{u,h}}}{\text{EC50} \times f_{\text{u,h}} + f_{\text{inhibitor}}} \right) + \frac{1 - f_{\text{hep}}}{1 - E_{\text{hep}}} + E_{\text{hep}}
\]

The assumption in the current analysis is that the liver is solely responsible for the systemic elimination of the CYP3A4 substrates in our in vivo dataset, therefore fhep = 1. The hepatic extraction ratio for the victim drugs in the dataset was calculated based on reported intravenous clearance data (Gertz et al., 2010). The prediction accuracy of eqs. 1 and 5 was compared using a collated dataset of 18 intravenous DDIs. The predictive utility of each in vitro experimentally determined by equilibrium dialysis, as described in the supplemental material.

Thus, the limit of prediction acceptability changes with the magnitude of the observed change in AUC; the upper and lower limits are more constricted 2-fold range as the magnitude of the interaction becomes larger. The limits were adjusted to account for the estimated baseline interindividual variability in substrate exposure. The estimated variability of the observed data (δ) was

### Table 1

<table>
<thead>
<tr>
<th>Victim Drug</th>
<th>fmCYP3A4</th>
<th>AUC Fold Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfentanil</td>
<td>0.87</td>
<td>21.9</td>
<td>Kharasch et al., 2004</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>0.80</td>
<td>8.5</td>
<td>Schmider et al., 1999</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.77</td>
<td>5.1</td>
<td>Backman et al., 2005</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>0.71</td>
<td>3.7</td>
<td>Hebert et al., 1992</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>7.2</td>
<td>Adams et al., 2005</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>8.0</td>
<td>Chung et al., 2006</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>14.5</td>
<td>Gorski et al., 2003</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>17.7</td>
<td>Gurley et al., 2006</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>19.0</td>
<td>Kharasch et al., 2004</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.94</td>
<td>14.6</td>
<td>Floyd et al., 2003</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>0.71</td>
<td>12.2</td>
<td>Holtecker et al., 1996</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.76</td>
<td>6.0</td>
<td>Twum-Barima et al., 1981</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.76</td>
<td>8.8</td>
<td>Dammier et al., 1999</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.49</td>
<td>4.9</td>
<td>Bidsstrup et al., 2004</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.49</td>
<td>2.4</td>
<td>Nuern et al., 2000</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.49</td>
<td>1.5</td>
<td>Hatop et al., 2003</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>0.99</td>
<td>3.1</td>
<td>Hebert et al., 1999</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0.92</td>
<td>20.0</td>
<td>Villikka et al., 1997</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.30</td>
<td>15.4</td>
<td>Barbarash et al., 1988</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>0.60</td>
<td>3.6</td>
<td>Villikka et al., 1997</td>
</tr>
</tbody>
</table>

\(^a^\) Hepatic extraction ratio calculated from reported intravenous clearance data (Gertz et al., 2010).

\(^b^\) Fraction metabolized by CYP3A4. Values were collected from the literature (Houston and Galetin, 2008; Shou et al., 2008) (alfentanil, verapamil).
based on the reported variability of 20% in midazolam exposure ($\delta = 1.25$) (Guest et al., 2011).

**Estimation of Rifampin $E_{\text{max}}$ In Vivo.** In vivo $E_{\text{max}}$ was estimated by fitting eq. 1 or eq. 5 to the intravenous substrate data. The $EC_{50}$ values used in the model were estimated from either CYP3A4 mRNA or activity data as induction endpoints. In the current study, MATLAB version 7.10 (The MathWorks, Inc., Natick, MA) was used to minimize the difference between predicted and observed AUCiv/AUCind in terms of geometric mean fold error (GMFE) (eq. 7). The error was minimized to optimize our estimate of in vivo rifampin $E_{\text{max}}$.

$$\text{GMFE} = \frac{\sum_{n} \log \left( \frac{\text{predicted AUCiv/AUCind}}{\text{observed AUCiv/AUCind}} \right)}{n}$$

The sample size ($n$) is the number of intravenous substrate AUC ratios (AUCiv/AUCind) in the dataset used to estimate $E_{\text{max}}$. All parameters in either prediction model, with the exception of $E_{\text{max}}$, were fixed to in vitro and in vivo values from our dataset. This optimization used rifampin $EC_{50}$ values estimated from mRNA or activity data in Fa2N-4, HepaRG, or human hepatocytes (cryopreserved or primary cells). For these predictions, in vivo $EC_{50}$ was assumed to be equal to the weighted mean in vitro estimate. The percentage of studies predicted within acceptable limits defined by eq. 6 was used as a criterion to assess prediction accuracy of both in vitro and estimated in vivo $E_{\text{max}}$. In addition, the effect of using optimized $E_{\text{max}}$ (from intravenous substrate DDI data) on prediction outcome for oral DDIs ($n = 21$; Table 1) was assessed. Prediction accuracy for oral DDIs was assessed analogous to the intravenous DDIs, as detailed above.

**Results**

**In Vitro Parameters.** Weighted mean rifampin $EC_{50}$ estimates were calculated from literature data for Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes based on both induction of CYP3A4 mRNA (Fig. 1A) and CYP3A4 activity (Fig. 1B). The weighted mean $EC_{50}$ values estimated from mRNA induction were 3.7, 0.8, 1.0, and 3.4 $\mu$M in Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes, respectively. A 5-fold difference in weighted mean mRNA $EC_{50}$ was observed across the systems, with Fa2N-4 and HepaRG showing the highest and the lowest values, respectively (Fig. 1A). The mRNA $EC_{50}$ estimates in primary human hepatocytes covered a greater range of values than any other in vitro system (details shown in Table 1 and supplemental data). Induction measured by CYP3A4 activity yielded $EC_{50}$ estimates similar to those based on CYP3A4 mRNA. Weighted mean rifampin $EC_{50}$ estimates based on CYP3A4 activity were 4.0, 4.3, 0.4, and 0.8 $\mu$M in Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes, respectively. Rifampin was a more potent inducer of CYP3A4 activity in both primary and cryopreserved hepatocytes ($EC_{50} < 1 \mu$M), compared with immortalized cell lines Fa2N-4 or HepaRG (Fig. 1B).

This analysis shows that rifampin $EC_{50}$ estimates from HepaRG and primary human hepatocytes are the most sensitive to induction metric used; up to a 5-fold difference in estimates was observed between CYP3A4 mRNA and activity $EC_{50}$ values.

Weighted mean $E_{\text{max}}$ estimates were also calculated from four in vitro systems and expressed as fold increases (Fig. 2). Overall, rifampin $E_{\text{max}}$ estimates based on CYP3A4 mRNA (Fig. 2A) were greater than estimates based on CYP3A4 activity (Fig. 2B). Weighted mean rifampin $E_{\text{max}}$ estimates based on mRNA were 28-, 83-, 34-, and 72-fold in Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes, respectively. Rifampin $E_{\text{max}}$ estimates based on CYP3A4 activity were 11-, 43-, 16-, and 9-fold, respectively. The most pronounced difference between mRNA- and activity-based $E_{\text{max}}$ estimates was observed in primary human hepatocytes (8-fold greater when based on mRNA induction). CYP3A4 mRNA $E_{\text{max}}$ ranged 3-fold across in vitro systems between the highest (HepaRG) and lowest (Fa2N-4) weighted mean $E_{\text{max}}$ Values (Fig. 2A). A similar range across systems (4-fold) was observed between weighted mean $E_{\text{max}}$ estimates based
The relative ranking of rifampin $E_{\text{max}}$ across in vitro systems was dependent on the induction metric used. For example, CYP3A4 mRNA $E_{\text{max}}$ was 2-fold greater in primary human hepatocytes compared with cryopreserved human hepatocytes. In contrast, greater induction of CYP3A4 activity was reported in cryopreserved human hepatocytes compared with primary human hepatocytes (Fig. 2).

**Rifampin In Vivo Dataset.** Induction DDIs between rifampin and CYP3A4 substrates were compiled from literature data (Table 1) using criteria outlined earlier under CYP3A4 substrates were compiled from literature data (Table 1) using criteria outlined earlier under Materials and Methods. This in vivo dataset contained 18 DDIs between rifampin and seven intravenously administered CYP3A4victim drugs. The intravenous DDIs were used for our initial analysis of the predictive utility of Fa2N-4, HepaRG, cryopreserved, and primary human hepatocyte EC50 and $E_{\text{max}}$. Rifampin interactions with midazolam represented up to half of the rifampin interactions with midazolam represented up to half of the DDIs in the intravenous dataset. The majority of intravenous induction DDIs were classified as moderate (56%) or weak (36%). On average, 600 mg of rifampin was orally administered once daily for 1 week. However, rifampin was administered for only 5 days in some studies. CYP3A4 played an important role in the elimination of each substrate: $f_{\text{in}}$ CYP3A4 values were greater than 0.60 for all victim drugs in the intravenous DDI studies (Houston and Galetin, 2008), and $E_{H}$ values ranged from 0.03 (tacrolimus) to 0.64 (verapamil), with a median $E_{H}$ of 0.32 (alfentanil) (Gertz et al., 2010).

In addition to intravenous DDIs ($n = 18$), our in vivo dataset contained 21 interactions between rifampin and 12 well characterized orally administered CYP3A4 victim drugs. All seven CYP3A4 substrates in the intravenous dataset were also represented in the oral dataset, along with five additional substrates (Table 1). Oral DDIs were primarily characterized as strong (71%) but also included several moderate (24%) and one weak interaction. The rifampin oral dose and administration periods were similar to the design described for the intravenous studies. In the oral dataset, the substrate $f_{\text{in}}$ CYP3A4 ranged from 0.49 (repaglinide) to 0.99 (tacrolimus) (Kroemer et al., 1993; Houston and Galetin, 2008). The oral DDI data were used to further evaluate the predictive utility of the collated in vitro induction dataset and $E_{\text{max}}$ optimized from intravenous data.

**Prediction of Intravenous Drug-Drug Interactions.** Initial assessment of the predictive utility of Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes was performed by predicting the change in AUC ratio precipitated by rifampin for each of 18 intravenous induction DDIs (Table 2). Predictions were based on the rifampin free fraction in plasma measured here by equilibrium dialysis (0.17 ± 0.04) (details in the supplemental material). The change in substrate AUC was predicted using induction parameter estimates based on either CYP3A4 mRNA or CYP3A4 activity data and either eq. 1 (the static induction model without incorporation of victim drug $E_{H}$) or eq. 5 (the static model that accounts for substrate $E_{H}$). All in vivo DDIs were overpredicted when predictions were based on mRNA data without accounting for $E_{H}$ (Fig. 3, A–C); for some systems, no studies were predicted within the assigned limits (Table 2). The largest extent of overpredictions was seen using HepaRG data (Fig. 3B), whereas Fa2N-4 mRNA data predicted more intravenous substrate AUC ratios within the accepted range than any other in vitro system when $E_{H}$ was not included in the prediction (Table 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>In Vitro System</th>
<th>CYP3A4 mRNA Data</th>
<th>CYP3A4 Activity Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equation 1</td>
<td>Equation 5</td>
</tr>
<tr>
<td>Fa2N-4</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>HepaRG</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>Cryopreserved hepatocytes</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>0</td>
<td>89</td>
</tr>
</tbody>
</table>

* Based on static induction model with no $E_{H}$ incorporated.

* Hepatic extraction ratios of the substrates listed in Table 1 were incorporated in the prediction model, as defined in eq. 5.
A representative example of the impact of $E_H$ on the prediction outcome using mRNA induction data from cryopreserved hepatocytes is illustrated in Fig. 3D; trends observed were consistent across all four in vitro systems investigated. Incorporation of substrate $E_{H}$ into the prediction model using mRNA $EC_{50}$ and $E_{max}$ resulted in an overall increase in prediction accuracy for all in vitro systems (Table 2); 89% of intravenous substrate interactions were predicted within acceptable limits as defined by eq. 6. The median values of the predicted/observed changes in the AUC were comparable across all four in vitro systems when $E_{H}$ was included in the model. Although the use of this model (eq. 5) represented a noticeable improvement over ignoring $E_{H}$ (eq. 1), mRNA $EC_{50}$ and $E_{max}$ in all systems overpredicted the AUC ratio of the two compounds with the lowest $E_{H}$ in our in vivo dataset: tacrolimus ($E_{H} = 0.03$) and cyclosporine ($E_{H} = 0.19$). When tacrolimus and cyclosporine were excluded from the intravenous DDI dataset, 100% of all induction predictions incorporating $E_{H}$ were within the acceptable limits (eq. 6) based on mRNA $EC_{50}$ and $E_{max}$ as induction endpoints.

Predictions based on CYP3A4 activity data (Fig. 3) showed the same trend as mRNA for all four in vitro systems when $E_{H}$ was ignored (Fig. 3, A–C); however, the extent of overprediction was less pronounced compared with mRNA data. Of the four in vitro systems investigated, CYP3A4 activity $EC_{50}$ and $E_{max}$ in cryopreserved human hepatocytes generated the largest overpredictions. Analogous to mRNA analysis, the predicted/observed ratios were reduced for all in vitro systems when substrate $E_{H}$ was incorporated in the prediction of rifampin DDIs with intravenous substrates (Fig. 3D). The prediction accuracy of CYP3A4 activity parameters was similar for Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes when $E_{H}$ was incorporated in the prediction (Table 2) and in good agreement with mRNA-based predictions. HepaRG and human hepatocyte data predicted 100% of the in vivo interactions within the calculated limits if tacrolimus and cyclosporine were excluded from the intravenous dataset; however, the accuracy of predictions based on Fa2N-4 parameters was not affected. All predictions discussed above were based on the average unbound systemic rifampin concentration. Use of estimated total rifampin $C_{max,xs}$ (10 μM) (Shou et al., 2008) as the worst-case scenario had minimal effect on prediction accuracy when $E_{H}$ was incorporated in the DDI model.

**Estimation of Rifampin In Vivo $E_{max}$**. Estimation of in vivo $E_{max}$ was performed by fitting a static induction prediction model, with or without incorporating substrate $E_{H}$ (eqs. 5 and 1), to the dataset of 18 intravenous DDIs. Optimized $E_{max}$ obtained by fitting eq. 1 ranged from 3- to 9-fold or from 2- to 10-fold for estimates based on CYP3A4 mRNA or activity $EC_{50}$ values as input, respectively (as summarized in Table 3). Optimized $E_{max}$ was based on the same weighted mean in vitro $EC_{50}$ estimates initially used to compare the predictive utility of each in vitro system (Fig. 3). The estimated in vivo $E_{max}$ of rifampin were, on average, 5-fold greater across all in vitro systems, when $E_{H}$ was incorporated in the model. Rifampin $E_{max}$ estimates were 2.4-fold higher when the model with $E_{H}$ incorporated was used after excluding the tacrolimus and cyclosporine DDIs. Of the in vitro systems compared, in vivo rifampin $E_{max}$ estimates from primary human hepatocyte or HepaRG were more sensitive to the selection of the induction metric, reflecting the dependence of $EC_{50}$ estimates on whether induction was measured with mRNA or CYP3A4 activity.

**Prediction of Oral Drug-Drug Interactions**. Comparison of prediction success for 21 oral DDIs obtained using the weighted mean in vitro $EC_{50}$ and $E_{max}$ in Fa2N-4, HepaRG, cryopreserved, and primary human hepatocytes is shown in Fig. 4; in all cases, the prediction model accounting for both hepatic and intestinal induction (eq. 3) was applied. Predictions based on mRNA were poor, because only Fa2N-4 data predicted 14% of oral DDIs within acceptable limits defined by eq. 6 (Table 4); other systems predicted none. In contrast, predictions based on CYP3A4 activity data performed better compared with mRNA, particularly for Fa2N-4 and primary human hepatocytes in which 62% of the oral DDIs were predicted within limits (Table 4; Fig. 4).

Subsequently, we compared the predictive utility of our optimized in vivo rifampin $E_{max}$ estimates using a dataset of 21 oral DDIs. Prediction accuracy was improved when in vivo rifampin $E_{max}$ was estimated by fitting eq. 1 (no $E_{H}$) to the intravenous DDI data, regardless of in vitro system or induction endpoint (Table 4), but still failed to predict up to 67% of the oral DDIs correctly. This trend is illustrated for the case in which HepaRG in vitro $EC_{50}$ was used for $E_{max}$ optimization (Table 4; Fig. 5). Use of optimized rifampin $E_{max}$ estimated by fitting eq. 5 ($E_{H}$ incorporated) to the intravenous DDI data resulted in poor prediction of oral studies, ranging from 0 to 29% of the oral interactions predicted within acceptable limits (Table 4). However, even $E_{max}$ optimization based on the oral DDI dataset could not fully recover the magnitude of DDIs observed, because a maximum 62% of the oral interactions were predicted within acceptable limits (Fig. 5). This result is in contrast to success seen when similar validation of the $E_{max}$ optimization method was performed using the intravenous DDI dataset.

**Discussion**

Rifampin is a potent inducer of CYP3A4 in vitro and in vivo and is therefore responsible for numerous DDIs associated with CYP3A4 substrates. Recently, a number of in vitro systems have been used to predict the magnitude of rifampin induction in vivo (Fahmi et al., 2008b; Shou et al., 2008; Galetin et al., 2010). Whereas hepatocytes are considered the “gold standard,” both HepaRG and Fa2N-4 have been proposed as appropriate surrogates for assessment of CYP3A4 induction (Fahmi et al., 2008a; Hariparsad et al., 2008; McGinnity et al., 2009). However, in vitro induction data vary greatly between in vitro systems (Figs. 1 and 2; supplemental data), due partially to interlaboratory differences in experimental design. The main objective of the current study was to compare predictive utility of four commonly used in vitro systems: primary and cryopreserved human hepatocytes and hepatocyte-derived cell lines Fa2N-4 and HepaRG. Specific criteria were applied to select published in vitro data to minimize experimental inconsistencies and the impact of study design on parameter estimates. In addition, the effects of substrate $E_{H}$ on prediction accuracy and $E_{max}$ optimization were investigated using a dataset of intravenous DDIs ($n = 18$). The hypothesis that optimization of intravenous DDIs would improve prediction accuracy of 21 oral DDIs was subsequently tested.
Although variability due to clinical study design was controlled by excluding ambiguous studies from our dataset (e.g., case studies, patient population), the magnitude of DDIs varied for the most prevalent victim drug in the oral dataset, midazolam (7.2–19-fold change in midazolam AUC was observed across seven studies). No apparent correlation between magnitude of DDI and timing of midazolam administration, relative to the final dose of rifampin, was established. This was in agreement with recently reported findings showing no effect of dose staggering on midazolam oral clearance (Kirby et al., 2011). Midazolam absorption was not expected to contribute to ob-

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**TABLE 4**

Comparison of different in vitro systems, induction points, and \( E_{\text{max}} \) optimization methods for the prediction of 21 oral rifampin drug-drug interactions

<table>
<thead>
<tr>
<th>System</th>
<th>In Vivo ( E_{\text{max}} ) mRNA</th>
<th>Activity</th>
<th>In Vivo ( E_{\text{max}} ) (eq. 1) mRNA</th>
<th>Activity</th>
<th>In Vivo ( E_{\text{max}} ) (eq. 5) mRNA</th>
<th>Activity</th>
<th>In Vivo ( E_{\text{max}} ) (eq. 3) mRNA</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fa2N-4</td>
<td>86</td>
<td>38</td>
<td>52</td>
<td>48</td>
<td>100</td>
<td>100</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>HepaRG</td>
<td>100</td>
<td>95</td>
<td>67</td>
<td>43</td>
<td>86</td>
<td>100</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>Cryopreserved hepatocytes</td>
<td>100</td>
<td>95</td>
<td>71</td>
<td>67</td>
<td>86</td>
<td>71</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>100</td>
<td>38</td>
<td>52</td>
<td>67</td>
<td>100</td>
<td>86</td>
<td>43</td>
<td>38</td>
</tr>
</tbody>
</table>

* Based on static induction model with no \( E_{\text{IC50}} \) incorporated.
* Hepatic extraction ratios of the substrates listed in Table 1 were incorporated in the prediction model, as defined in eq. 5.
* Based on static induction model for oral drug-drug interactions.
The static prediction model was very sensitive to $E_{\text{max}}$ in conjunction with $f_{\text{mCYP3A4}}$, consistent with inhibitory DDI predictions reported previously (Houston and Galetin, 2008). Considering the high unbound fraction of rifampin in plasma, the model was relatively insensitive to propagation of up to 30% variability in this parameter. In contrast, the prediction model incorporating $E_{\text{H}}$ was largely insensitive to all parameters other than $E_{\text{H}}$ itself. Although the use of this model significantly reduced the extent of overpredictions for intravenous DDIs observed across all in vitro systems (regardless of induction endpoint used; Table 3), it had a marginal effect on the prediction accuracy of cyclosporine and tacrolimus DDIs. This contrasts with the expectation that accounting for low $E_{\text{H}}$ in the model could reduce the extent of DDI overprediction seen for these drugs (considering their $E_{\text{H}}$ of $<0.2$). In the case of oral DDIs, the predicted AUC ratio was primarily sensitive to $E_{\text{max}}$, whereas changes in rifampin $F_w$, $k_{e}$, or $Q_{\text{ent}}$ had minimal effect on prediction outcome. Variability in $E_{\text{max}}$ was amplified by the multiplicative effect of induction in the intestine and therefore closely related to substrate $F_{\text{gi}}$, as outlined previously (Galetin et al., 2010).

Whereas the assumption of comparability of rifampin EC$_{50}$ between in vitro and in vivo seems reasonable, we expect in vivo $E_{\text{max}}$ to be modified by factors not present in vitro. For this reason, in vivo rifampin $E_{\text{max}}$ was estimated by optimizing the prediction success of 18 intravenous DDIs (Table 3). Fitting of the static induction prediction model (with or without substrate $E_{\text{H}}$ incorporated) to in vivo data was performed using in vitro EC$_{50}$ collated for various systems. Because other parameters in the model were fixed, any disparity observed in the value of estimated rifampin $E_{\text{max}}$ reflected differences in weighted mean EC$_{50}$. The use of estimated in vivo $E_{\text{max}}$ improved predictive accuracy of both mRNA and activity data for all in vitro systems, when the standard static induction model ($E_{\text{H}}$ not incorporated) was used. However, use of in vivo rifampin $E_{\text{max}}$ estimated by a model with $E_{\text{H}}$ incorporated did not lead to further improvement in prediction of intravenous DDIs. These findings suggest that in vitro induction data generated in any of the four systems investigated should be sufficient to accurately predict intravenous DDIs when the model accounts for substrate $E_{\text{H}}$.

Subsequently, in vivo optimized $E_{\text{max}}$ (estimated from intravenous DDIs) was used to predict oral DDIs. In contrast to the intravenous dataset, use of optimized $E_{\text{max}}$ resulted in poor prediction accuracy for oral DDIs. Depending on the method used, 38 to 100% of studies were outside prediction limits (Table 4). Rifampin in vivo $E_{\text{max}}$ optimized from intravenous data is based solely on hepatic induction, and improvement in prediction success for oral DDIs can be expected only if induction effect is the same in intestine and liver. Data on rifampin induction of CYP3A4 mRNA and protein in enterocytes are limited and based on a small group of healthy subjects, with no corresponding matched hepatic induction data (Glaeser et al., 2005). However, this study indicated differential and lower intestinal response to rifampin relative to liver. The assumption of intestinal $E_{\text{max}}$ of 3-fold resulted in improvement in oral DDI predictions when paired with estimated in vivo hepatic $E_{\text{max}}$. For example, the percentage predicted within assigned limits from primary hepatocyte mRNA data increased from zero to 62% of the oral dataset. In addition, 3-fold intestinal $E_{\text{max}}$ predicts a 60 to 70% decrease in cyclosporine and tacrolimus $F_{\text{gi}}$, and is in better agreement with the observed 50 to 57% change in intestinal extraction estimated in the same individuals after intravenous and oral administration in the presence of rifampin (Galetin et al., 2010). Hence, there is circumstantial evidence for the hypothesis that rifampin induces intestinal CYP3A4 to a reduced extent compared with the liver.

In conclusion, rifampin DDIs with intravenous CYP3A4 substrates were better predicted by in vitro induction data generated in Fa2N-4, HepaRG, and both cryopreserved and primary human hepatocytes. Although EC$_{50}$ and $E_{\text{max}}$ determined by CYP3A4 mRNA or activity display large variability, the prediction was insensitive to differences in the parameters when $E_{\text{H}}$ was included in the model. On the basis of this analysis, either CYP3A4 mRNA or activity may be used to predict intravenous induction DDIs when $E_{\text{H}}$ is
accounted for within the prediction model. There was no improvement in prediction accuracy when estimated in vivo $E_{\text{max}}$ was used rather than in vitro $E_{\text{max}}$. Although oral DDI prediction accuracy was improved by intravenous optimized rifampin $E_{\text{max}}$, substantial proportion of DDIs failed to be predicted within acceptable limits.

In view of regulatory recommendations for induction studies (Huang et al., 2008; Chu et al., 2009), it is important to obtain a rigorous approach to analyzing DDIs involving rifampin. Considering multiple interaction mechanisms and differential effects on OATP1B1 and metabolic enzymes, the application of a dynamic, physiologically based pharmacokinetic model for the assessment of rifampin DDIs would be prudent, in particular for DDIs involving drugs with complex disposition associated with interplay between transporters and metabolism, as illustrated in the case of rapamycin. To accurately predict oral induction DDIs, additional studies are required to investigate relative differences between in vivo $E_{\text{max}}$ in liver and the intestine.

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Authorship Contributions

Participated in research design: Templeton, Houston, and Galetin. Performed data analysis: Templeton. Wrote or contributed to the writing of the manuscript: Templeton, Houston, and Galetin.

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