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_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_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_max >35% of DDIs were incorrectly assigned, suggesting potential differential E_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 (EC50) and magnitude (E_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 EC50 and E_max (Fahmi et al., 2008a; Hariparsad et al., 2008; McGinnity et al., 2009).php

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ABBREVIATIONS: E_max, maximum induced fold change; E_in, hepatic extraction ratio; AUCiv/AUCiv\textsuperscript{ref}, ratio of the intravenous victim drug AUC in the absence and presence of rifampin; 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 EC\textsubscript{50} and E\textsubscript{max} 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 EC\textsubscript{50} has been assumed to be similar in vitro and in vivo; however, rifampin E\textsubscript{max} 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 E\textsubscript{max} to in vivo by fitting a prediction model to in vitro and in vivo induction data (Fahmi et al., 2008a). In practice, in vitro EC\textsubscript{50} and, in particular, E\textsubscript{max} 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 E\textsubscript{max} (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 E\textsubscript{max} data. Baseline and induced 24-h urinary 6\beta-hydroxy cortisol/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 EC\textsubscript{50} and E\textsubscript{max} 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 E\textsubscript{max} was explored, assuming EC\textsubscript{50} values were comparable between in vitro and in vivo. The application of optimized in vivo E\textsubscript{max} 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 EC\textsubscript{50} and E\textsubscript{max} on DDI predictions, selection criteria were applied to ensure consistency in the in vitro experimental design and allow comparison across studies. Rifampin EC\textsubscript{50} and E\textsubscript{max} 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 EC\textsubscript{50} estimates were excluded if E\textsubscript{max} 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 EC\textsubscript{50} and E\textsubscript{max} 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 6\beta-hydroxylation) in each in vitro system. In cases in which induction parameters were not explicitly stated in the text, EC\textsubscript{50} and E\textsubscript{max} were estimated from digitally extracted figure data using Plot Digitizer 2.4.1. Rifampin EC\textsubscript{50} and E\textsubscript{max} parameters were estimated by fitting a sigmoid E\textsubscript{max} model in Grafit 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 EC\textsubscript{50} and E\textsubscript{max} 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., 2008b). Weighted mean EC\textsubscript{50} and E\textsubscript{max} (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 data 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 DDI 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 (F\text{int}) and fraction of substrate metabolized by CYP3A4 (fmCYP\text{int}) 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 E\textsubscript{max} 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.

\[
\frac{\text{AUC}_\text{D}}{\text{AUC}_{\text{CYP}3\text{A}}^\text{mtd}} = \frac{\text{fmCYP}_{3\text{A}1}}{1 + \frac{E_{\text{max}} \times [I]}{E_{\text{C50}} \times f_u + [I] \times f_u}} + (1 - \text{fmCYP}_{3\text{A}1})
\]

(1)

where AUC\text{D} and AUC\text{CYP3A}\text{mtd} 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{mtd}). 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\text{int}) were taken from the literature (Houston and Galetin, 2008; Shou et al., 2008).

\[
[I]_{\text{m}} = \frac{D}{C_{\text{CYP3A}}} F
\]

(2)

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 (CL_{ori}/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) (Willkins 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 (f_u) 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 cell concentration for those studies in which cell number and incubation volume were reported was equal to the cell concentration used by Shou et al. (0.8 × 10^6 cells/ml). Hence, the current analysis was applied under the assumption that the free fraction of rifampin is comparable across the in vitro systems investigated.

To predict oral DDIs, eq. 1 was expanded to account for induction in the intestine (eq. 3),

\[
\frac{\text{AUC}_{\text{oral,ind}}}{\text{AUC}_{\text{oral}}} = \left[ F_c + (1 - F_c) \times \left( 1 + \frac{E_{\text{max}} \times [H]}{E_{\text{50}} + [H]} \right) \right] \\
\times \left[ \text{fmCYP3A4} \times \left( 1 + \frac{E_{\text{max}} \times [I]_{av}}{E_{\text{50}} \times f_u \times f_h + \frac{[I]_{av}}{f_u} + \frac{[I]_{av}}{f_h}} \right) + (1 - \text{fmCYP3A4}) \right]
\]  (3)

where \( F_c \) represents the fraction of the dose escaping intestinal first-pass metabolism. The \( F_c \) values of the 12 victim drugs investigated were estimated from intravenous and oral data (Galetin et al., 2008; Shou et al., 2008) (alfentanil, verapamil).

In addition, we assessed the effect of incorporating the substrate hepatic extraction ratio \( (E_b) \) 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.

\[
\frac{\text{AUC}_{\text{v}}} {\text{AUC}_{\text{v}}^{\text{ref}}} = \frac{\text{fmCYP3A4} \times \left( 1 + \frac{E_{\text{max}} \times [H] \times f_u \times f_h \times f_h}{EC_{50} \times f_u \times f_h + [I]_{av} \times f_u} \right) + (1 - \text{fmCYP3A4}) + E_b}{1 - E_b} + (1 - f_{\text{lep}})
\]  (5)

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 \( f_{\text{lep}} = 1 \). 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 system was assessed using both equations by comparing the ratio of predicted \( \text{AUC}_{\text{v}}^{\text{oral,ind}} \) to observed \( \text{AUC}_{\text{v}}^{\text{oral,ind}} \). Prediction accuracy was quantified as percentage of studies predicted within acceptable limits calculated by eq. 6, as proposed recently (Guest et al., 2011).

\[
\delta = 2 \times \left( \frac{\text{AUC}_{\text{v}}}{\text{AUC}_{\text{v}}^{\text{oral,ind}}} - 1 \right) \frac{1}{\text{AUC}_{\text{v}}^{\text{oral,ind}}}
\]  (6)

Thus, the limit of prediction acceptability changes with the magnitude of the observed change in AUC; the upper and lower limits are more constricted when inducer effect is low and increase gradually to the generally accepted 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 (\( \delta \)) was experimentally determined by equilibrium dialysis, as described in the supplemental material.
based on the reported variability of 20% in midazolam exposure (δ = 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 AUC\(_{\text{iv}/\text{iv}}\)/AUC\(_{\text{ind}}\) in terms of geometric mean fold error (GMFE) (eq. 7). The error was minimized to optimize our estimate of in vivo rifampin \( E_{\text{max}} \).

\[
\text{GMFE} = 10^{\left(\frac{1}{n}\sum \log_{10}\left(\frac{\text{predicted AUC}_{\text{iv}/\text{iv}}}{\text{observed AUC}_{\text{ind}}}\right)\right)}
\]

(7)

The sample size (n) is the number of intravenous substrate AUC ratios (AUC\(_{\text{iv}}\)/AUC\(_{\text{ind}}\)) in the dataset used to estimate rifampin \( E_{\text{max}} \). 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 primary 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 described 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 \( E_{\text{max}} \) based on mRNA was 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
on CYP3A4 activity data. 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 human hepatocytes compared with cryopreserved human hepatocytes.

**Prediction of Intrahepatic 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 free fraction in plasma $E_H$) 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 1b</td>
<td>Equation 5b</td>
</tr>
<tr>
<td>Fa2N-4</td>
<td>11</td>
<td>72</td>
</tr>
<tr>
<td>HepaRG</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Cryopreserved hepatocytes</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

b Based on static induction model with no $E_H$ incorporated.

c Based on eq. 5.

d Based on static induction model with no $E_H$ incorporated.

e Based on eq. 5.

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 $fm_{\text{CYP3A4}}$ 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.
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$_{max}$ (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, respectively), 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 (Fahimi 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 (Fahimi et al., 2008a; Haririparsad 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 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 affect midazolam oral clearance based on previous findings (Floyd et al., 2003). In contrast, repaglinide administered either 1 or 24 h after final rifampin administration displayed more than 3-fold difference in the extent of DDI (1.5-versus 4.9-fold change in repaglinide AUC, respectively) despite the same dose of victim drug (4 mg) and similar rifampin dose and duration (>5 days, 600 mg/day) (Birdstrup et al., 2004). The complexity of repaglinide disposition (hepatic uptake via OATP1B1 and differential contribution of CYP3A4 and CYP2C8 metabolism) in conjunction with multiple interaction mechanisms associated with rifampin (Zheng et al., 2009) may explain this dose-staggering effect; the most pronounced induction was observed when repaglinide was administered 24 h after rifampin.

To compare the predictive utility of induction data from various in vitro systems, we have chosen to use the mechanistic static prediction model, which assumes a constant concentration of inducer throughout the time course of substrate exposure, a simplification relative to incorporation of the concentration-time profile of inducer (Almond et al., 2009). This approach is appropriate for our assessment of in vitro systems that considers induction in isolation of other interaction mechanisms, such as competitive or time-dependent inhibition. The concentration-time profile of rifampin is not directly linked to the time-dependent expression of CYP3A4 because enzyme level is determined by degradation half-life. Hepatic CYP3A4 degradation half-life has been estimated to range from 24 to 72 h, up to 6 to 8 days in some individuals (Galetin et al., 2006; Yang et al., 2008; Reitman et al., 2011), in contrast to intestinal half-life of approximately 24 h (Gertz et al., 2008; Yang et al., 2008). Regardless of reported variability, CYP3A4 half-life is longer than rifampin elimination half-life (~2 h (Furesz et al., 1967)), and functional expression of CYP3A4 should be relatively constant over the period of rifampin exposure.

### 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 Vitro $E_{\text{max}}$ mRNA</th>
<th>Activity</th>
<th>In Vivo $E_{\text{max}}$ (eq. 1$^a$) mRNA</th>
<th>Activity</th>
<th>In Vivo $E_{\text{max}}$ (eq. 5$^a$) mRNA</th>
<th>Activity</th>
<th>In Vivo $E_{\text{max}}$ (eq. 3$^a$) 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>

$^a$ Based on static induction model with no $K_{I}$ incorporated.

$^b$ Hepatic extraction ratios of the substrates listed in Table 1 were incorporated in the prediction model, as defined in eq. 5.

$^c$ Based on static induction model for oral drug-drug interactions.
In contrast, the prediction model incorporating insensitive to propagation of up to 30% variability in this parameter. unbound fraction of rifampin in plasma, the model was relatively reported previously (Houston and Galetin, 2008). Considering the high (Galetin et al., 2010).

The static prediction model was very sensitive to $E_{\text{max}}$ in conjunction with $f_{m_{\text{CYP3A4}}}$, 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 can 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 $F_{\text{a,o}}$, $k_{\text{a}}$, or $Q_{\text{int}}$ 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 (Gaeser 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 $F_{\text{CI}}$, 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 well 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 DI 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 victims with complex disposition associated with interplay between transporters and metabolism, as illustrated in the case of repaglinide. To accurately predict oral induction DDIs, additional studies are required to investigate relative differences between in vivo rifampin $E_{\text{max}}$ 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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