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Use of Immortalized Human Hepatocytes to Predict the Magnitude of Clinical Drug-Drug Interactions Caused by CYP3A4 Induction[ξ]

Sharon L. Ripp, Jessica B. Mills, Odette A. Fahmi, Kristen A. Trevena, Jennifer L. Liras,

Tristan S. Maurer, and Sonia M. de Morais

Department of Pharmacokinetics, Dynamics & Metabolism (S.L.R., O.A.F., K.A.T.,

J.L.L., T.S.M., S.M.D.) and Department of Scientific Development (J.B.M.)

Pfizer Global Research & Development

Groton Labs, Groton, CT

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Corresponding Author:

Sonia M. de Morais, Ph.D.

Department of Pharmacokinetics, Dynamics, and Metabolism

Pfizer Global Research and Development

Groton, CT 06340

Phone: (860) 715-4338

Fax (860) 715-7857

e-mail: sonia.m.demorais@pfizer.com

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Nonstandard Abbreviations: AUC, area under the plasma concentration vs. time curve; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; DDI, drug-drug interactions; f_u, fraction of unbound drug in plasma; MDR1, multidrug resistance protein 1; PXR, pregnane X receptor; RIS, relative induction score.

ABSTRACT

Cytochrome P4503A4 (CYP3A4) is the principal drug-metabolizing enzyme in human liver. Drug-drug interactions (DDI) caused by induction of CYP3A4 can result in decreased exposure to coadministered drugs, with potential loss of efficacy. Immortalized hepatocytes (Fa2N-4 cells) have been proposed as a tool to identify CYP3A4 inducers. The purpose of the current studies was to characterize the effect of known inducers on CYP3A4 in Fa2N-4 cells, and to determine whether these in vitro data could reliably project the magnitude of DDI caused by induction. Twenty-four compounds were chosen for these studies, based on previously published data using primary human hepatocytes. Eighteen compounds had been shown to be positive for induction, and 6 compounds had been shown to be negative for induction. In Fa2N-4 cells, all 18 positive controls produced greater than 2-fold maximal CYP3A4 induction, and all 6 negative controls produced less than 1.5-fold maximal CYP3A4 induction. Subsequent studies were conducted to determine the relationship between in vitro induction data and in vivo induction response. The approach was to relate in vitro induction data (E_{max} and EC_{50} values) with efficacious free plasma concentrations to calculate a relative induction score (RIS). This score was then correlated with decreases in AUC values for coadministered CYP3A4 object drugs (midazolam or ethinylestradiol) from previously published clinical DDI studies. Excellent correlations (r^2 values > 0.92) were obtained, suggesting that Fa2N-4 cells can be used for identification of inducers as well as prediction of the magnitude of clinical DDI.

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Introduction

Cytochrome P4503A4 (CYP3A4) is the major drug-metabolizing enzyme in human liver and is responsible for the clearance of many commonly used drugs, including benzodiazepines, statins, calcium channel blockers, and HIV protease inhibitors. Certain drugs can modulate the level of CYP3A4 activity, thereby causing changes in clearance of coadministered drugs that are CYP3A4 substrates. Levels of CYP3A4 activity can be decreased by inhibition of enzyme activity, or increased by induction of new protein synthesis. Changes in CYP3A4 activity, either through inhibition or induction, can result in potentially serious drug-drug interactions (DDI). While assays for evaluating inhibition of CYP3A4 are routine and the relationship between in vitro data and in vivo effects relatively well understood (Bjornsson et al., 2003), assays for evaluating induction are less well characterized and the relationships between in vitro and in vivo data less clear.

Induction of CYP3A4 is thought to occur primarily through transcriptional activation of the gene. The 5'-regulatory region of the CYP3A4 gene contains elements that bind various transcription factors that can up- or down-regulate transcription. One such transcription factor is the pregnane X receptor (PXR). PXR is a ligand-activated transcription factor that is activated by a variety of drugs and endogenous compounds to increase transcription of CYP3A4 as well as other drug-metabolizing enzymes and transporters (Kliewer et al., 2002). Most drugs that induce levels of CYP3A4 are thought to do so primarily via PXR activation, e.g., rifampicin, phenytoin, hyperforin, clotrimazole (Lehmann et al., 1998).

There are several in vitro tools for assessment of CYP3A4 induction. To this

point, primary human hepatocyte cultures have been considered the gold standard for in

vitro induction assessment (Madan et al., 2003). Primary hepatocytes are typically

treated with test compound for two or three days, then CYP3A4 levels compared to

those in vehicle-treated cells. CYP3A4 expression can be evaluated by measuring

enzymatic activity with a selective probe substrate, or by measuring CYP3A4 mRNA.

Primary hepatocytes are considered a reliable way to assess induction, however, ability

to attain data can be limited by quality and availability of human hepatocytes. Also,

there is considerable interindividual variability in induction responses in primary

hepatocytes, with some preparations showing very little response to inducers, and

others showing a high degree of response (Madan et al., 2003). A weak inducer could

be missed if hepatocytes from "low responders" are used. Also, this system is not

amenable to higher throughput applications, and as such does not meet the needs of

early drug discovery. In contrast, PXR-based reporter assays have provided a means

for high throughput induction assessment. These assays typically involve cultured cell

lines that have been transfected with both an expression vector for PXR and a reporter

gene construct containing a responsive element for PXR (Goodwin et al., 1999).

Induction potential is then measured by the ability of a test compound to activate

transcription of the reporter gene. These systems generally have a wide dynamic range

and are good indicators of a test compound's ability to activate PXR. However, there

are several drawbacks to the use of PXR-based reporter assays. They are typically run

in tumorigenic cell lines, which might or might not have all the coregulatory factors that

are required for full transcriptional activation. Also, reporter gene assays do not measure native gene expression or native enzyme activity.

Recently, Mills et al. described the initial characterization of a non-tumorigenic immortalized human hepatocyte line, Fa2N-4, and demonstrated the inducibility of CYP3A4 in response to prototypical inducers (Mills et al., 2004). The Fa2N-4 cell line is derived from immortalization of primary human hepatocytes and maintains expression and inducibility of various drug-metabolizing enzymes and transporters, as well as morphological characteristics of primary hepatocytes (Xenotech, 2003). However, in contrast to primary hepatocytes, the immortalized hepatocytes are easily maintained and propagated in culture, are amenable to higher throughput applications, and provide a system with less intrinsic variability. In addition, Fa2N-4 cells do not require transfection of PXR or reporter genes, and induction assessment can be done using either mRNA levels or native enzyme activity. In addition to CYP3A4, other PXR-regulated genes (CYP2C9, MDR1) are induced in response to prototypical inducers (Mills et al., 2004).

The purpose of these studies is to further characterize CYP3A4 induction in Fa2N-4 cells, using a series of positive and negative controls. In addition, a method is proposed whereby potency and efficacy of induction in Fa2N-4 cells can be used to predict the magnitude of induction observed in clinical studies.

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Materials and Methods

Chemicals

β-Naphthoflavone, carbamazepine, dexamethasone, methotrexate, methylprednisolone, mifepristone, nifedipine, phenobarbital, phenytoin, pioglitazone, probenecid, reserpine, rifampicin, roxithromycin, and sulfinpyrazone were purchased from Sigma-Aldrich (St. Louis, MO). Clarithromycin, clotrimazole, efavirenz, rifabutin, rifapentine, ritonavir, and rosiglitazone were purchased from Sequoia Research Products (Oxford, UK). Troglitazone was purchased from TRC Research Chemicals (Toronto, Canada). Avasimibe and Compound A (6-methoxy-1-methyl-1-trifluoromethyl-isochroman-7-ylmethyl)-(2-phenyl-piperidin-3-yl)-amine) were produced at Pfizer Global Research and Development (Groton, CT).

Cell Culture

The Fa2N-4 cell line of immortalized hepatocytes were generated in collaboration with MultiCell Technologies (Warwick, RI), as previously described (Mills et al., 2004). Cells were grown in Biocoat Type I collagen-coated flasks (BD Biosciences, Bedford, MA) in a humidified 37° C incubator (5% CO_2) in serum-free Multi-Function Enhancing (MFE™) medium (Xenotech LLC, Lenexa, KS).

Induction Experiment

Cells were maintained in collagen-coated flasks until two-to-three days post-confluency. Medium was removed, cells trypsinized, and resuspended in MFE™

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medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), at a concentration of approximately 750,000 cells per ml. One hundred microliters of cell solution was added to each well of 96-well collagen-coated plates (75,000 cells/well). After cell attachment, (approximately 3 hours), serum-containing medium was replaced with serum-free MFE™ medium. Cells were incubated for 48 hours before treating with test compounds. This adaptation period was required in order for the cells to exhibit robust induction response. Stock solutions of test compounds (1000X) were prepared in DMSO and diluted 1000-fold with serum-free medium for cell treatment (0.1% DMSO final). After 24 hours of treatment with test compounds, or vehicle control, medium was removed and fresh medium containing test compounds was added and cells incubated for an additional 24 hours. At the end of the incubation period, medium was removed and cells were washed twice with saline. Cells were lysed and mRNA was harvested as described below.

Rifampicin (1-50 μM) was tested with each plate of compounds as a positive control. The experiment was considered acceptable if the maximal induction response with rifampicin was greater than 8-fold. This degree of response is needed in order to ensure adequate dynamic range to detect weak inducers. Test compounds were initially assayed at 1, 10, 30, and 50 μM. If no evidence of induction was observed at these concentrations, the compound was considered negative for induction. Some compounds showed a positive response with at least one of these initial concentrations, but a full dose-response curve was not achieved. In these cases, the range was extended to either higher or lower concentrations until either a maximal fold induction was achieved, or further extension was limited by cell toxicity.

Cell Toxicity Assessment

Parallel sets of plated cells were treated and cell viability was measured using a WST1 Cell Proliferation Assay (Roche Diagnostics, Indianapolis, IN), which measures mitochondrial dehydrogenase activity as a marker for metabolically active cells. Briefly, after the 48-hour treatment period, WST1 reagent was added to cell culture medium (1 part reagent to 10 parts cell culture medium), mixed gently, and incubated at 37°C for 20 min. Plates were shaken vigorously on an orbital shaker for 1 min, and absorbance was measured at 440 nm. Wells containing culture medium, but no cells, were used as background (measured at 660 nm). Treatments were deemed toxic if there was a significant decrease in absorbance compared to vehicle-treatment.

Quantification of CYP3A4 mRNA

Total RNA was extracted from cells using the RNeasy 96 kit according to instructions provided by the manufacturer (Qiagen, Valencia, CA). RNA (100 ng) was analyzed using the Invader RNA assay reagent kits according to instructions provided by the manufacturer (Third Wave Technologies, Madison, WI), and as previously described (Mills et al., 2004).

Statistics and Curve-Fitting

Experiments were carried out in triplicate and each compound was tested on at least 2 different days. All statistical analyses were carried out using Microsoft Excel. All curve fitting was carried out using SigmaPlot 8.0 (Systat Software, Inc., Chicago, IL). To

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determine EC_{50} and E_{max} values, concentration-response data were fit to a 3-parameter

sigmoid (Hill) function, according to the following equation: $y=(E_{max}*x^{\gamma})/(EC_{50}^{\gamma}+x^{\gamma})$.

Results

Characterization of Fa2N-4 cells using positive and negative controls. In order to

assess the utility of the Fa2N-4 cell line to correctly identify inducers of CYP3A4, cells

were treated with a variety of compounds that had previously been identified as positive

or negative for CYP3A4 induction. The criterion for inclusion was that data from primary

human hepatocyte experiments conclusively identified a compound as either positive or

negative for induction of CYP3A4 using either enzyme activity or mRNA as an endpoint.

Compounds used as negative controls were β-naphthoflavone, methotrexate,

methylprednisolone, clarithromycin, roxithromycin, and probenecid (Pichard et al., 1992;

Ledirac et al., 2000; Luo et al., 2002; Madan et al., 2003). Compounds used as positive

controls were mifepristone, troglitazone, carbamazepine, dexamethasone, efavirenz,

rosiglitazone, clotrimazole, avasimibe, ritonavir, phenytoin, pioglitazone, phenobarbital,

reserpine, rifabutin, sulfinpyrazone, nifedipine, rifapentine, and rifampicin (Kocarek et

al., 1995; Li et al., 1997; Drocourt et al., 2001; Luo et al., 2002; Madan et al., 2003; Sahi

et al., 2003a; Sahi et al., 2003b; Hariparsad et al., 2004). Fa2N-4 cells were treated for

2 days with varying concentrations of test compounds and CYP3A4 mRNA was

measured. CYP3A4 mRNA levels were normalized to the vehicle control, and

expressed as fold-induction. Maximal fold-induction values for the 24 test compounds

and the vehicle control are shown in Fig. 1. Note that the concentrations required for

maximal fold-induction varied from 0.1 μM for mifepristone to 1000 μM for

phenobarbital. Maximal fold-induction produced with compounds identified as negative

controls was less than 1.5-fold, and the maximal fold-induction produced with positive

controls was greater than 2-fold. No false positives or false negatives were observed.

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Concentration-response for inducers in Fa2N-4 cells. Since induction of CYP3A4 is

a result of receptor-mediated processes, it should be concentration-dependent and

saturable. Concentration-response relationships for the current set of compounds fell

into one of three categories. Nine compounds - carbamazepine, dexamethasone,

nifedipine, phenobarbital, phenytoin, pioglitazone, rifampicin, rosiglitazone, and

sulfinpyrazone - showed standard dose-response curves with fold-induction increasing

with dose to a point of saturation. These concentration-response data were fitted to a

sigmoidal curve and EC_{50} and E_{max} parameters determined. (Fig. 2A; Table 2). Nine

compounds showed atypical dose-response curves, in that fold-induction increased with

dose to a point, then decreased at higher concentrations (Fig. 2B). In five of these

cases (avasimibe, clotrimazole, efavirenz, mifepristone, and troglitazone), the bell-

shaped dose-response could be attributed to toxicity (Fig. 2B, right graphs). However, in

the other four cases, the atypical dose-response was not associated with toxicity, as

assessed by the mitochondrial dehydrogenase assay (reserpine, rifabutin, rifapentine,

and ritonavir)(Fig. 2B, left graphs). The reason for this bell-shaped dose response is not

known, but has been observed in other studies of induction (Reinach et al., 1999;

Raucy, 2003; Xenotech, 2003).

Assay reproducibility. In order to assess the inter-assay variability in CYP3A4

induction in Fa2N-4 cells; concentration-response experiments with rifampicin were

included each time the assay was run. Intra-assay variability (variability among replicate

wells) was generally less than 25% (Table 1). Inter-assay variability in maximal fold-

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induction and EC$_{50}$ values was also assessed (Table 1). Using mRNA as an endpoint, maximal fold-induction values ranged from 9.7- to 19-fold with an average of 13-fold and standard deviation of 3.7-fold (coefficient of variation of 28%). EC$_{50}$ values ranged from 1.3 μM to 3.1 μM, with an average of 1.9 μM and standard deviation of 0.70 (coefficient of variation of 37%). Concentration response curves for the additional 23 test compounds were carried out at least twice. Intra-assay variability was generally less than 25%. Inter-assay variability ranged from 3% to 53%, with a mean ($\pm$ S.D.) inter-assay variability of 30 $\pm$ 16% (data not shown).

The stability of induction response with passage number has been shown in previous publications, and results indicated that fold-induction is stable at least up to passage number 47 (Xenotech, 2003; Mills et al., 2004). In the current studies, the passage number of the cells used was between passages 22 and 35.

Modeling the relationship between induction of CYP3A4 in Fa2N-4 cells and clinical induction. In order to establish a correlation between data generated in vitro and that observed in clinical studies, the potency and efficacy of inducers in Fa2N-4 cells was related to exposures achieved in vivo. Inducers were scored based on the relationship of the EC$_{50}$ and E$_{max}$ values in Fa2N-4 cells and the efficacious concentration observed in vivo, according to the following equation:

$$\text{Relative Induction Score} = \frac{C_{eff,\,free} * E_{max}}{C_{eff,\,free} + EC_{50}}$$

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The value $C_{eff,free}$ is the published plasma concentration of inducer achieved after a standard therapeutic dose, corrected for protein binding. This concentration was chosen to approximate the concentration of drug available for binding to PXR and effecting induction of CYP3A4. The RIS for various inducers using the above equation are shown in Table 2. The published clinical changes in either midazolam or ethinylestradiol AUC upon coadministration with inducers are also shown. Note that the relationship between RIS and clinical DDI was evaluated only for inducers. Compounds that are capable of simultaneous inhibition and induction of CYP3A4 at clinically relevant concentrations (e.g., ritonavir and efavirenz) were excluded from this analysis.

The correlation between RIS and clinical DDI was assessed by plotting the RIS versus the percent decrease in AUC of a coadministered CYP3A substrate, data that was obtained from published clinical DDI trials. Figure 3 shows the correlation plots for the widely used CYP3A probe substrate, midazolam (Fig. 3A) and ethinylestradiol (Fig. 3B). The relationship between RIS and percent decrease in midazolam AUC fits a Hill function with an E_{max} of 97%. A positive correlation is also apparent between RIS and percent decrease in ethinylestradiol AUC, and achieves an E_{max} of 66%. Excellent correlations between the RIS and clinical DDI were obtained using either midazolam or ethinylestradiol clinical data ($r^2 \geq 0.92$).

Discussion

Since induction of CYP3A4 can result in significant DDI, it is important to assess a new drug candidate's ability to cause CYP3A4 induction. In the current work, we demonstrate that the Fa2N-4 line of immortalized hepatocytes can be used not only to identify inducers, but are a robust and reproducible enough system to be used to predict extent of clinical induction.

Induction of CYP3A4, CYP2C9, and MDR1 had previously been demonstrated in Fa2N-4 cells in response to rifampicin, phenobarbital, or dexamethasone (Mills et al., 2004). The current work indicates that these immortalized hepatocytes can appropriately identify a larger diversity of inducers, including those compounds that are relatively weak inducers in vitro. Since the 18 positive controls tested had maximal fold-induction values of greater than 2, and the 6 negative controls had maximal fold-induction values of less than 1.5, it seems appropriate that when screening compounds with unknown induction potential to consider a compound in vitro positive if it produces a maximal fold-induction of greater than or equal to 2. Using this criterion, even the weak in vitro inducers such as carbamazepine and dexamethasone would be correctly identified.

In order to assess the utility of the Fa2N-4 immortalized hepatocytes for developing an in vitro-in vivo correlation for induction, we first examined the robustness and reproducibility of the data. The inter-assay variability observed with Fa2N-4 cells (21-37%, depending on the parameter) was considerably less than that reported for primary human hepatocyte cultures. For example, Madan et al. assessed induction response across primary hepatocytes from 62 individuals using enzyme activity as an

endpoint. Fold-induction for 50 μM rifampicin ranged from 1.5- to 145-fold, with an

average of 9.7-fold and standard deviation of 19-fold (coefficient of variation of 200%)

(Madan et al., 2003; Xenotech, 2003). The relatively smaller variability in response in

Fa2N-4 cells suggested that this system could provide a means to develop in vitro-in

vivo correlations, and clinical predictions.

El-Sankary et al. demonstrated the utility of using in vitro potency and efficacy to

rank order inducers (El-Sankary et al., 2001). These investigators generated E_{max} and

EC_{50} values for several inducers, using HepG2 cells transfected with glucocorticoid

receptor, PXR, and a CYP3A4 reporter gene. Ranking of compounds was achieved by

dividing maximal fold-induction by EC_{50}. We extended this approach and demonstrated

that by incorporating free plasma concentrations into a simple E_{max} model to generate

the RIS, one could attain an excellent in vitro-in vivo correlation for CYP3A4 induction.

Ideally, the RIS equation would incorporate the intracellular free concentration of drug in

hepatocytes, which should reflect the amount of drug available for binding to PXR.

However, concentration of drug within hepatocytes is not readily measurable, and as a

surrogate, the published free plasma concentration at C_{max} was used. Using free C_{max},

the in vitro-in vivo correlation, using either midazolam or ethinylestradiol as probe

substrate, was excellent ($r^2 \geq 0.92$). The in vitro-in vivo correlation was also attempted

using total plasma concentration, but the correlation coefficient was low ($r^2 < 0.55$; not

shown). Note that treatment of Fa2N-4 cells with test compounds was done under

serum-free conditions, and therefore, it is not surprising that free plasma concentrations

produced a better correlation. The incorporation of free plasma concentrations is critical

to appropriately identifying induction risk. For example, nifedipine and pioglitazone are

relatively potent and efficacious inducers in vitro, and might be predicted to be strong

inducers in vivo. However, by factoring in the low plasma concentrations and free

fractions of these two drugs, the actual RIS is very low and indeed these compounds

show very little to no clinical induction (Horsmans et al., 1991; Sachse et al., 1998).

Conversely, carbamazepine is a very weak inducer in vitro, with a high EC_{50} and low

E_{max}. However, when the high plasma concentration and free fractions of

carbamazepine are considered, the RIS is at least an order of magnitude higher than

pioglitazone and nifedipine, and this correlates with the clinical DDI observed with this

compound (Crawford et al., 1990; Backman et al., 1996b).

It is interesting to note that the EC_{50} values from the in vitro experiments were in

many cases higher than the published plasma concentrations for a given drug, yet in

several cases the drug still produced clinically measurable induction. This suggests that

in order to appropriately assess clinical induction risk, the ratio of the plasma

concentration to EC_{50} is not enough. This is in contrast to the risk assessment for

inhibition, which typically can be predicted from the ratio of the in vivo inhibitor

concentrations and the in vitro K_i for inhibition. It appears that for induction, it is

important to include not only the in vivo concentration and in vitro EC_{50}, but also the

maximal fold-induction achieved in vitro. The RIS equation incorporates these three

parameters.

The relationship between RIS and percent decrease in AUC of coadministered

drug can be used as a calibration curve for clinical induction, and hence it is critical

when evaluating induction response for new compounds that the assay conditions are

consistent with those in which this calibration curve was generated. In other words,

under experimental conditions whereby rifampicin exhibits a greater fold-induction, then

a test compound would also tend to have a greater fold-induction. Therefore, it is

recommended that in order to use the in vitro-in vivo correlation shown here, the

rifampicin response in the same experiment should be within the range of rifampicin

responses observed in the calibration experiments, that is E_{max} between 9- and19-fold

and EC_{50} between 1.3 and 3.1 µM. Alternatively, an abbreviated calibration curve could

be generated for each experiment. For example, for each set of test compounds,

rosiglitazone or nifedipine could be included as a low standard, pioglitazone or

avasimibe as a middle standard, and rifampicin as a high standard, and a RIS/DDI

relationship generated in each run.

One caveat with predicting clinical induction from in vitro data is the potential

effect of metabolites. If metabolites are also inducers, and if exposure to these

metabolites is significant, the RIS could under-predict clinical induction. In fact, we have

encountered an example of a compound that had a major circulating metabolite that

was also an inducer. If the RIS were calculated with the parent compound, no clinical

DDI would have been predicted. However, when the RIS of the metabolite was added

in, the data correlated well with the actual induction observed in clinical DDI studies

(unpublished observations).

Another caveat to the use of the RIS concerns compounds that are both inducers

and inhibitors of CYP3A4 at clinically relevant concentrations (e.g., ritonavir and

efavirenz). These compounds would not be accurately predicted by factoring in only the

induction component. Note that some compounds might be competitive inhibitors of

CYP3A4, but have K_i values that are significantly higher than therapeutic

concentrations. In these cases, inhibition would not be expected to be clinically relevant, and the RIS could be used. Pioglitazone and troglitazone are examples of such compounds. The K_i values for CYP3A4 inhibition are approximately 12 μM and 1 μM for pioglitazone and troglitazone, respectively (Sahi et al., 2003a). These values are much higher than the unbound in vivo concentrations, and therefore, these compounds would not be expected to produce inhibition in vivo. In these cases, the RIS could be used to predict effects due to induction, without the need to incorporate an inhibition term. Work is underway to evaluate the utility of Fa2N-4 cell assay to accurately predict DDI for compounds that are simultaneous inducers and inhibitors of CYP3A4 at clinically relevant concentrations.

It is interesting to note that the most potent clinical inducer published (rifampicin) produced a 97% decrease in midazolam AUC, but only a 67% decrease in ethinylestradiol AUC. Some substrates, such as midazolam, are cleared almost exclusively by CYP3A metabolism, and therefore might be more susceptible to CYP3A induction than other substrates that are cleared via multiple pathways. Ethinylestradiol is cleared by a combination of CYP3A4 metabolism, sulfation, and glucuronidation (Maggs et al., 1983; Guengerich, 1990). It would also be expected that compounds that are cleared by both intestinal and hepatic CYP3A would be more susceptible to the effects of inducers than those cleared by hepatic enzymes only. An example of this is the difference in clinical DDI observed with midazolam depending on whether it is administered orally or intravenously (Gorski et al., 2003). Since most DDI studies with midazolam are conducted using an oral route of administration, we chose to investigate the in vitro-in vivo correlation using oral midazolam data.

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In conclusion, our findings indicate that Fa2N-4 immortalized hepatocytes can be used to identify a wide range of CYP3A4 inducers, including those compounds that are weak in vitro inducers. Moreover, potency and efficacy data from Fa2N-4 cells can be combined with plasma concentration data to generate a RIS that correlates with clinical DDI. The low inter-assay variability of the Fa2N-4 cells relative to primary hepatocytes makes this in vitro-in vivo correlation possible. Future studies will be directed at using these immortalized hepatocytes to develop clinical DDI prediction tools for additional drug-metabolizing enzymes.

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Acknowledgments.

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Footnotes

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Send reprint requests to:

Sonia M. de Morais, Ph.D.

Pfizer Global Research & Development

400 Eastern Pt Rd

Groton, CT 06340

Sonia.m.demorais@pfizer.com

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Fig. Legends

Fig. 1. Maximal fold-induction of CYP3A4 in Fa2N-4 cells treated with various inducers and non-inducers. Gray bars indicate compounds previously identified as negative for CYP3A4 induction in primary hepatocytes, and black bars indicate compounds previously identified as positive for induction of CYP3A4 in primary hepatocytes. Cells were treated for 2 days with compounds in 0.1% DMSO, followed by cell harvest, RNA isolation, and measurement of CYP3A4 mRNA. Data were normalized to vehicle control (0.1% DMSO). Concentrations shown (μM) and are those that produced maximal fold-induction of CYP3A4 for a given compound.

Fig. 2. Concentration-response curves for compounds producing a positive response in Fa2N-4 cells. Carbamazepine, dexamethasone, phenytoin, phenobarbital, sulfinpyrazone, nifedipine, pioglitazone, rosiglitazone, and rifampicin exhibited typical concentration-response relationships that could be fit using a sigmoidal curve-fitting program (symbols denote observed data ($\pm$ S.D.), while line denotes curve fit) (**A**). Reserpine, rifabutin, rifapentine, and ritonavir exhibited bell-shaped concentration response relationships (**B**, left graphs). Attainment of full concentration-response relationships for avasimibe, clotrimazole, efavirenz, mifepristone, and troglitazone was limited by cell toxicity (**B**, right graphs).

Fig 3. Relationship between relative induction score (RIS) and clinical effects on coadministered midazolam (A) and ethinylestradiol (B). Compound abbreviations:

CBM, carbamazepine; CP A, 6-methoxy-1-methyl-1-trifluoromethyl-isochroman-7-

ylmethyl)-(2-phenyl-piperidin-3-yl)-amine; NIF, nifedipine; PB, phenobarbital; PHT,

phenytoin; PIO, pioglitazone; RIF, rifampicin; RSG, rosiglitazone; TRG, troglitazone.

Data points were fitted to a 3-parameter Hill function, $y=(E_{max}*x^{\gamma})/(RIS_{50}^{\gamma}+x^{\gamma})$. For graph

A, $E_{max}=97.0$, $\gamma=1.24$, and $RIS_{50}=0.031$. For graph B, $E_{max}=65.6$, $\gamma=0.997$, and

$RIS_{50}=0.0924$.

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TABLE 1

Intra-day (shaded) and inter-day (italicized) assay variability from 5 rifampicin

concentration-response experiments conducted in triplicate

Exp't #	1 uM RIF		10 uM RIF		30 uM RIF		50 uM RIF		Curve fit parameters[a]			
	AVG	CV[b]	AVG	CV	AVG	CV	AVG	CV	EC_{50}	E_{max}	r^2	γ
1	4.1	6%	7.4	3%	8.2	2%	9.0	11%	2.1	10	0.98	0.56
2	5.5	27%	12	23%	13	23%	14	22%	1.7	14	0.99	0.93
3	5.4	17%	10	14%	10	14%	12	13%	1.3	12	0.98	0.76
4	6.3	23%	12	15%	16	15%	16	16%	3.1	19	0.99	0.62
5	3.8	15%	8.1	25%	11	16%	8.1	29%	1.5	9.7	0.95	1.1
AVG	5.0		9.9		12		12		1.9	13		
CV	21%		22%		25%		28%		37%	28%		

[a]Data were fit using a Hill function (3-parameter) according to the following equation: y = $(E_{max} * x^\gamma)/(EC_{50}^\gamma + x^\gamma)$. [b]CV = coefficient of variation, (standard deviation/average).

TABLE 2. List of compounds with corresponding in vitro induction data and published clinical data used to generate

relative induction scores (RIS).

Drug	Dose[a] (mg)	Total C_{max}[a] (μM)	Free C_{max}[a] (μM)	EC_{50}[b] (μM)	E_{max}[b] (fold-induction)	RIS	Clinical DDI (Decrease in AUC of Coadministered Drug)		
							Midaz-olam	Ethinyl-estradiol	References for Clinical DDI Data
Carbamaze-pine	400-800	34	8.5 (f_u=0.25)[c]	62	2.5	0.30	94%	41%	Backman et al., 1996b Crawford et al., 1990
Nifedipine	30-60	0.29	0.014 (f_u=0.05)	17	8.3	0.0068	Lack of clinical induction with other CYP substrates		Horsmans et al., 1991 Sachse et al., 1998
Phenobarbital	75	52	26 (f_u=0.50)	222	5.5	0.58	NA[d]	70%	Back et al., 1980
Phenytoin	200-300	70	5.2 (f_u=0.075)	44	5.2	0.55	NA	50%	Crawford et al., 1990
Pioglitazone	15-45	2.8	0.028 (f_u<0.01)	4.6	4.1	0.025	26%	11%	pioglitazone product label
Rifampicin	300-600	10	2.5 (f_u=0.25)	1.9	13	7.4	95%	64%	Backman et al., 1996a LeBel et al., 1998
Rosiglitazone	8	1.4	0.0028 (f_u=0.002)	14.5	2.9	0.00056	No effect on ethinylestradiol or nifedipine PK		Harris et al., 1999 Inglis et al., 2001
Troglitazone[e]	600	6.3	0.063 (fu=0.01)	3.0	2.4	0.049	NA	30%	Loi et al., 1999
Compound A	40	0.028	0.0028 (f_u=0.1)	1.3	4.8	0.010	39%	NA	Mcrobie et al., 2004

| Compound A | 120 | 0.106 | 0.011 (f$_u$=0.1) | 1.3 | 4.8 | 0.039 | 62% | NA | |

[a]Dosage and plasma concentration information obtained from product labels and/or cited DDI studies. [b]EC$_{50}$ and E$_{max}$ parameters generated by curve-fitting of Fa2N-4 cell data, with the exception of troglitazone. [c]f$_u$ = unbound fraction in plasma. [d]NA = no data available. [e]Concentration-response curves for troglitazone were limited by toxicity at higher concentrations, therefore, E$_{max}$ was estimated as the highest fold-induction at a non-toxic concentration, and EC$_{50}$ the concentration producing half maximal fold induction.

Fig. 1

Fig. 2A

Fig. 2B

Fig. 3

A

B