Cytochrome P450 3A4 mRNA Is a More Reliable Marker than CYP3A4 Activity for Detecting Pregnane X Receptor-Activated Induction of Drug-Metabolizing Enzymes

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ABSTRACT:

Induction of cytochrome P450 (P450) activity in the clinic can result in therapeutic failure such as tissue rejection in transplant patients or unwanted pregnancy, among others. CYP3A4 is by far the most abundant isozyme and is responsible for the majority of P450-related metabolism of all marketed drugs. However, it is of importance to understand the significance of induction mediated through other P450 enzymes. The objective of this investigation was to evaluate several known inducers in vitro using cryopreserved human hepatocytes, with the aim of assessing the relevant induction of CYP3A4, CYP2B6, CYP2C9, CYP2C19, and CYP3A5, based on mRNA expression. CYP3A4 induction was also assessed based on enzymatic activity in three different lots to investigate whether mRNA expression data have any advantages over enzymatic activity. In general, the mRNA fold-induction data results were more sensitive compared with activity data, and more informative in cases in which the drug is also a P450 inhibitor. The induction of transcription of other drug-metabolizing enzymes including CYP2B6 and CYP2C enzymes occurred every time that CYP3A4 mRNA levels increased, but to a lesser extent, indicating that measurement of CYP3A4 mRNA is a sensitive marker for the induction of these other enzymes. This was the case even for enzymes and inducers that are known to also act via the constitutive androstane receptor pathway. Finally, the utility of in vitro induction measurements in the identification of clinically meaningful inducers was tested by using two simple binary classification approaches: 1) fold-induction versus vehicle control and 2) induction response relative to rifampin. The best classification was observed when the cutoff criteria based on fold induction relative to the vehicle control, using mRNA data are used.

Introduction

The induction of cytochrome P450 (P450) enzymes can have clinical significance. The expression of the genes for these enzymes is inducible by numerous xenobiotics, resulting in altered drug metabolism and drug-drug interactions (DDIs). DDIs caused by enzyme induction could lead to loss of efficacy and/or increased toxicity via increased formation of reactive metabolites. PXR belongs to the nuclear receptor superfamily of ligand-activated transcription factors that includes vitamin D receptor (NR1I1), the constitutive androstane receptor (CAR; NR1I3), peroxisome proliferator-activated receptor (NR1C), and others (Germain et al., 2006). Members of this NR1 subfamily of nuclear receptors typically function as heterodimers with the retinoid X receptor (RXR; NR2B1). In the unactivated state, the PXR/RXR heterodimers are thought to be retained in the cytoplasm, bound to heat shock proteins and/or various chaperone proteins (Squires et al., 2004). Upon ligand binding, PXR/RXR translocates to the nucleus, binds DNA in the 5’-regulatory region of target genes, and interacts with various coregulatory proteins, thereby effecting gene transcription (Stanley et al., 2006). Like the closely related receptor PXR, CAR exists as a heterodimer with RXR. Once in the nucleus, the CAR/RXR heterodimer is constitutively active; therefore, the translocation event is essentially a receptor activation event. CAR, like PXR, mediates the induction of numerous additional drug-metabolizing enzymes, including phase II enzymes and transporters, such as glutathione transferase A2, multidrug resistance 1, and UDP-glucuronosyltransferase 1A1 (Maglich et al., 2002, 2003). In addition, it was recognized that many of the ligands that activate PXR can also activate CAR and that there is overlap in the target genes of these two receptors (Moore and Kliewer, 2000; Maglich et al., 2002; Wei et al., 2002). Cytochrome P450 enzymes are regulated by both the PXR and CAR pathways. The nuclear receptor CAR is activated by fewer compounds than PXR but is responsible for regulating similar target genes such as CYP3A4 and CYP2B6. Most drugs that induce CYP3A levels are believed to do so primarily via PXR activation; however, there is overlap in activation with CAR in the sets of target genes that are expressed (e.g., CYP2B, CYP3A, and CYP2C). This explains the overlapping P450 induction patterns that exist; for example, rifampin induces CYP2B6, CYP2C9, and CYP3A4 genes in humans. The CYP3A subfamily is involved in the oxidation of the largest range of substrates of all the P450s and is mostly found in the liver. CYP3A4 is by far the most abundant P450 isoform, constituting 30%...
of the P450 liver enzyme total in humans; it is also substantially expressed in the intestine and plays a dominant role in drug clearance, which is responsible for approximately 60% of P450-mediated metabolism of all marketed drugs (Frye, 2004). Thus, induction of CYP3A4 represents a serious issue in pharmacotherapy. Two major forms of CYP3A are expressed in adult human tissues: CYP3A4 and CYP3A5. Whereas the former is found in the liver and small intestine of most individuals, CYP3A5 is polymorphically expressed in the liver and other organs (Lin et al., 2002). Such polymorphisms contribute to interpatient variation and may affect the clinical efficacy and safe doses of a given drug.

The importance of CYP2B6 in drug metabolism has gained prominence recently. However, only a small proportion of drugs (estimated to be approximately 8%) show some metabolism by CYP2B6 (Nolan et al., 2006), such as bupropion, methadone, efavirenz, ketamine, and tamofoxifen (Kharasch et al., 2008).

The human CYP2C gene subfamily of cytochrome P450 enzymes, composed of at least four distinct genes, namely CYP2C8, CYP2C9, CYP2C18, and CYP2C19, is responsible for the metabolism of approximately 20% of all clinically prescribed therapeutic agents (Ferguson et al., 2005). The CYP2C subfamily constitutes approximately 30 to 40% of hepatic P450 content, with CYP2C9 being the most highly expressed and CYP2C19 having the lowest hepatic expression levels. CYP2C9 is responsible for metabolizing several anti-inflammatory drugs such as celecoxib, ibuprofen, naproxen, and tenoxicam. CYP2B6, CYP2C9, and CYP2C19 are also induced by rifampin and some of the other classic CYP3A4 inducers.

The objective of this investigation was to evaluate several known literature inducers with varying levels of PXR/CAR contributions, with the aim of assessing the relevant induction potency of CYP3A4, CYP2B6, CYP2C9, CYP2C19, and CYP3A5 in vitro, based on mRNA expression. CYP3A4 induction was assessed also based on enzymatic activity to investigate whether mRNA expression data have any advantages over enzymatic activity. Finally, an assessment of previously proposed criteria for the use of in vitro induction data to classify compounds as clinically relevant inducers (Bjornsson et al., 2003) was tested against other binary classification criteria using the data described in this report and clinical induction data gathered from the scientific literature.

Materials and Methods

Cell Culture and Cryopreserved Human Hepatocytes. Leflunomide, roxithromycin, ranitidine, fluoxetine, acetyl acetid acid, flavoxamine, flumazenil, rosiglitazone, omeprazole, dexamethasone, efavirenz, modafinil, naficilin, nimodipine, oxcarbazepine, phenobarbital, phenytoin, rifabutin, rifampin, topiramate, troglitazone, Dulbecco’s phosphate-buffered saline, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, and nifedipine were obtained from Sequoia Research Products, Ltd. (Pangbourne, UK). Aprepitant, ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Ritonavir, fluconazole, gatifloxacin, nitrendipine, pioglitazone, and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO).

Treatment of Cryopreserved Human Hepatocytes. Human cryopreserved hepatocytes were thawed in hepatocyte thawing medium and were seeded in collagen I-precoated 24-well plates, each well having a cell density of 3.5 to 4.2 × 10^5 viable cells in 0.5 ml of hepatocyte plating medium. Viability as determined by trypan blue exclusion was 85% or better for this study. The cells were maintained at 37°C in a humidified incubator with 90% atmospheric air and 5% CO_2. Cells were incubated for 24 h before being treated with compounds. All drugs were dissolved in DMSO and then added to the culture medium (final DMSO concentration, 0.1%). Incubation medium containing 0.1% DMSO served as the vehicle control. After daily treatment for 3 days, the medium was removed, and the cells were washed with saline. Enzyme activity and mRNA levels were then measured as described below.

**Testosterone 6β-Hydroxylation Measurement.** After the incubation phase of the experiment, the medium containing controls or test article was removed, the cells were washed with saline, and fresh medium was added to all wells for a 30-min wash. After removal of the wash medium, 200 μM testosterone in phosphate-buffered saline was added to each well, and the plate was returned to the incubator. After incubation for 30 min, 200 μl of the medium from each well was removed and filtered using a Millipore Multi- screen HA filter plate to remove protein. Samples were directly injected onto the high-performance liquid chromatograph. Testosterone metabolites were analyzed by high-performance liquid chromatography with UV detection at 254 nm (Fahmi et al., 2008a). Concentrations of 6β-hydroxysterosterone were extrapolated via a standard curve (50–0.1 μM) also prepared in phosphate-buffered saline.

CYP3A4, CYP2B6, CYP3A5, CYP2C9, and CYP2C19 mRNA Preparation and Analysis. The assay was performed according to a previously published method (Fahmi et al., 2008a). The gene-specific primer/probe sets used were as follows: for CYP3A4 target cDNA, Hs00640506_m1; for CYP2B6 target cDNA, Hs03044636_m1; for CYP2C9 target cDNA, Hs00426397_m1; for CYP2C19 target cDNA, Hs00426380_m1; for CYP3A5 target cDNA, Hs01070905_m1; and for glyceraldehyde-3-phosphate dehydrogenase endogenous control, Hs99999905_m1.

### Results

#### Comparison of Induction of CYP3A4 mRNA- and CYP3A4-Catalyzed Testosterone 6β-Hydroxylase Activity. Three lots of cryopreserved human hepatocytes were challenged with 20 known clinical inducers and 15 clinical noninducers, and both CYP3A4 mRNA levels and CYP3A4 catalytic activities were measured (Tables 1 and 2); the maximum fold induction observed is showing for both enzymatic activity and mRNA. The maximum fold increases (E_{max}) in mRNA levels, relative to those in vehicle-treated hepatocytes, were almost always greater than the maximum fold increases in activity, indicating that mRNA is a more sensitive marker for induction. The differences in fold increase in mRNA relative to activity were 6.4 ± 5.4, 4.3 ± 4.1, and 3.1 ± 3.6 for lots Hu4026, RCP, and Hu8020, respectively. The most noteworthy differences were observed for nevirapine, aripiprazol, and naficilin. Some differences in mRNA versus activity were ever observed between the lots. For example, topiramate showed a marked difference in response between mRNA and activity in two lots, but in the third lot the increase in activity approached the same value measured for mRNA.

#### Comparison of the Measurement of Increase in CYP3A4 mRNA with mRNA Increases for Other Inducible Drug-Metabolizing Enzymes. The increases in mRNA observed for five drug-metabolizing enzymes (CYP3A4, 3A5, 2B6, 2C9, and 2C19) upon treatment with 20 different compounds were compared (Table 3). In every instance, the increase in CYP3A4 mRNA was greater than the mRNA increases for all other enzymes. In general, the mRNA for CYP2B6 increased to the next greatest extent. The ratios of increases in CYP3A4 mRNA to CYP2B6 were lowest for phenobarbital, phenytoin, and troglitazone. CYP3A4 mRNA levels increased, on aver-
The in vitro data, two categorizations were used to assign a drug as an inducer or noninducer: 1) a cutoff of maximum induction achieving 40 and 20% of the effect of rifampin (at 25 μM) as assessed by measurement of CYP3A4 catalytic activity (Bjornsson et al., 2003) and CYP3A4 mRNA (Tables 4 and 5); and 2) a cutoff of a mean fold maximum increase in CYP3A4 mRNA (4-fold) and enzymatic activity (2-fold) (Table 6) from three human hepatocyte lots. The fidelity of these cutoff parameters is summarized in Tables 4, 5, and 6, where the rate of correct assignment is defined as shown in the formula below:

Rate of Correct Assignment

\[ \text{Rate of Correct Assignment} = \frac{\text{Agreed True Inducers} + \text{Agreed True Noninducers}}{\text{Total No. of Clinical Inducers} + \text{Total No. of Noninducers}} \]

Based on enzymatic activity and the cutoff of maximum induction at 40% of the effect of rifampin (Table 4), the rates of correct categorization were 55, 46, and 58% for lots Hu4026, RCP, and Hu8020, respectively. The rates of false-positives were 23, 27, and 31% and the rates of false-negatives were 60, 75, and 50% for lots Hu4026, RCP, and Hu8020, respectively. The rates of false-positives were 23, 33, and 38% and the rates of false-negatives were 55, 40, and 39% for lots Hu4026, RCP, and Hu8020, respectively. When the mRNA data were assessed based on the cutoff of maximum induction at 40% of the effect of rifampin, the rates of correct categorization were 61, 69, and 55% for lots Hu4026, RCP, and Hu8020, respectively. Rates of false-positives were 38, 33, and 38% and false-negatives rates were 40, 30, and 50% for lots Hu4026, RCP, and Hu8020, respectively, indicating that this approach is probably not suitable to identify clinical inducers, especially because of the high rates of false-negatives. The same approach was investigated using a more conservative cutoff of 20% relative to the effect of rifampin and led to some improvement in detecting inducers based on mRNA (false-negatives rates were 30, 0, and 22% for lots Hu4026, RCP, and Hu8020, respectively) but no significant improvement based on enzymatic activity data (55, 40, and 39% for lots Hu4026, RCP, and Hu8020, respectively).

Based on enzymatic activity of 2-fold, the rates of correct categorization (Table 6) were 69, 74, and 81% for lots Hu4026, RCP, and

### TABLE 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hu4026 mRNA Activity</th>
<th>RCP mRNA Activity</th>
<th>Hu8020 mRNA Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (50-100 μM)</td>
<td>12 14.2 11 5.3</td>
<td>9.1 7.0</td>
<td></td>
</tr>
<tr>
<td>N.A., not available.</td>
<td></td>
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</tbody>
</table>

N.A., not available.
The rates of false-positives were 27, 27, and 36% and the rates of false-negatives were 35, 25, and 6% for lots Hu4026, RCP, and Hu8020, respectively. When the mRNA data were assessed using a mean cutoff of 4-fold increase, the rates of correct categorization were 79, 83, and 81% for lots Hu4026, RCP, and Hu8020, respectively. Rates of false-positives were 46, 40, 46, and false-negatives rates were 5, 0, and 0% for lots Hu4026, RCP, and Hu8020, respectively. The rates of false-positives were 27, 27, and 36% and the rates of false-negatives were 35, 25, and 6% for lots Hu4026, RCP, and Hu8020, respectively. When the mRNA data were assessed using a mean cutoff of 4-fold increase, the rates of correct categorization were 79, 83, and 81% for lots Hu4026, RCP, and Hu8020, respectively. Rates of false-positives were 46, 40, 46, and false-negatives rates were 5, 0, and 0% for lots Hu4026, RCP, and
Hu8020, respectively. When in vitro induction data were used to inform a clinical DDI study strategy, the latter criteria offer the most conservative approach (i.e., may conduct clinical studies that come as negative but will not miss any inducers). This conclusion is best informed by Table 7, in which the specificity and sensitivity of the approach of using 4-fold induction as the cutoff value, based on mRNA, show 98% sensitivity but 69% selectivity.

**Discussion**

Induction of drug-metabolizing enzymes is an important mechanism underlying some drug-drug interactions. Increases in expression levels of important enzymes, such as CYP3A4, can cause increases in the intrinsic clearance of many drugs, resulting in decreases in their plasma concentrations and in some cases a loss of efficacy. Thus, it is important to be able to find agents capable of causing induction early in the drug research process to identify those compounds most prone to this activity and select alternate agents that will lack this activity. Several types of assays have been described for this purpose, such as mRNA, show 98% sensitivity but 69% selectivity.

**TABLE 6**

Truth tables for categorizing clinically meaningful inducers (20 inducers and 15 noninducers), using in vitro induction data (lots Hu4026, RCP, and Hu8020, respectively) and cutoff values of 4-fold increase in CYP3A4 based on CYP3A4 mRNA (a, c, and e for lots Hu4026, RCP, and Hu8020) or 2-fold increase in enzymatic activity (b, d, and f for lots Hu4026, RCP, and Hu8020).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Predicted</th>
<th>Non-Inducer</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>c</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>e</td>
<td>18</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Predicted</th>
<th>Non-Inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>d</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>f</td>
<td>17</td>
<td>6</td>
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</table>

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
</tr>
<tr>
<td>e</td>
<td>9</td>
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</table>

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Actual</th>
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<td>b</td>
<td>8</td>
</tr>
<tr>
<td>d</td>
<td>9</td>
</tr>
<tr>
<td>f</td>
<td>7</td>
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**TABLE 7**

Summary of the mean specificity and sensitivity for the two binary classification approaches, fold induction versus vehicle control and induction relative to rifampin, based on mRNA and activity data.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>4-Fold Cutoff</th>
<th>40% Relative to Rifampin</th>
<th>20% Relative to Rifampin</th>
<th>Enzymatic Activity</th>
<th>2-Fold Cutoff</th>
<th>40% Relative to Rifampin</th>
<th>20% Relative to Rifampin</th>
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<tr>
<td></td>
<td>specificity</td>
<td>specificity</td>
<td>specificity</td>
<td>sensitivity</td>
<td>sensitivity</td>
<td>sensitivity</td>
<td>sensitivity</td>
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<tr>
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<td>55</td>
<td></td>
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</table>

The data clearly show that measurement of CYP3A4 mRNA levels represents the most sensitive marker for detecting induction in human hepatocytes. Several compounds were shown to cause marked increases in CYP3A4 mRNA, whereas the increases in catalytic activity were much lower. For example, aprepitant caused 7.6- to 16-fold increases in CYP3A4 mRNA, whereas the increases in catalytic activity were much lower. For example, aprepitant caused 7.6- to 16-fold increases in CYP3A4 mRNA, but activity levels actually decreased.
This finding suggests that arepitant does induce expression of drug-metabolizing enzymes and that one might expect a clinical drug interaction between arepitant and other drugs. In the clinic, arepitant has been shown to cause a decrease in midazolam clearance but induction of ethinyl estradiol clearance (Majumdar et al., 2003) and (New Drug Application 021549 to the Food and Drug Administration).

In addition, compared with mRNAs for other drug-metabolizing enzymes (CYP3A5, 2B6, 2C9, and 2C19), the mRNA for CYP3A4 increases more in all cases. This result suggests that if one measurement should be chosen for testing for induction, that measurement should be CYP3A4 mRNA. Genes for enzymes proposed to also be regulated by the CAR, such as CYP2B6 or CYP2C19, demonstrate increased transcription upon treatment with known CAR mechanism inducers such as phenobarbital and phenytoin. However, these inducers still caused an even greater increase in mRNA levels for CYP3A4, supporting the fact that just the measurement of CYP3A4 mRNA is adequate to determine the possibility that a compound will be an inducer. Thus, measurement of mRNA for these other drug-metabolizing enzymes should not be necessary to identify inducers, even if a compound were to only activate CAR.

Finally, the utility of in vitro induction measurements in the identification of clinically meaningful inducers was tested. As opposed to more sophisticated approaches for quantitatively predicting clinical drug interactions caused by induction (Persson et al., 2006; Ripp et al., 2006; Sinz et al., 2006; Fahmi et al., 2008a,b, 2009; Hariparsad et al., 2008; Kanebratt and Andersson, 2008; Shou et al., 2008), in this report a simple binary classification of inducers was attempted. Similar approaches such as this are more amenable to early drug research efforts when hundreds of compounds must be assessed. In addition, an approach whereby a simple comparison of induction responses for new compounds with that of rifampin as a positive control had been previously proposed (Bjornsson et al., 2003; U.S. Food and Drug Administration, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf). As with any pharmacological effect, an induction response will be dictated not only by the maximum possible effect but also by the relationship between the concentration in vivo and the intrinsic binding potency (Smith et al., 2007). Thus, the use of simple, high concentrations of test compounds in in vitro induction assays may yield a high rate of false-positive results, because test concentrations far exceed those that are achieved in vivo. Nevertheless, testing of this binary classification was attempted. The results demonstrate that there are high false-negative rates if the cutoff based on fold induction relative to the positive control is applied. The best classification was observed when mRNA data for which the cutoff (4-fold) is based on absolute fold induction (Table 6) relative to the vehicle control are used, as no false-negatives were observed in two of the three lots tested. However, terbinafine, which is a weak clinical inducer suggested by the observed decrease of midazolam exposure of 24.5%, was not identified as an in vitro inducer in one of the lots tested. This observation confirms the need to evaluate new chemical entities with three different human hepatocytes lots as recommended by the U.S. Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf). It is also worth mentioning that notable differences were observed with the three human lots of hepatocytes used in this study. For example, lot RCP seemed to give rise to less false-positives based on mRNA with fold induction and the 20% cutoff criteria approaches.

Even though using the cutoff of 4-fold based on mRNA shows no false-negatives, there is a relatively high percentage of false-positive results (approximately 43%) (Table 7), if absolute fold induction is considered regardless of the in vivo plasma concentration. Some of the clinical noninducers such as rosiglitazone, nifedipine, omeprazole, nitrendipine, pioglitazone, and fluvoxamine show induction in vitro (Table 2). However, a drug such as rosiglitazone shows induction in vitro only at concentrations of 30 μM and above, which far exceed the actual Cmax of 1.4 μM (Fahmi et al., 2008a). Another example is omeprazole, for which induction in vitro is observed at concentrations of 25 μM and above, which far exceed the plasma concentration of 0.68 μM. Therefore, it is crucial to design the in vitro induction study around expected in vivo plasma concentration.

In conclusion, the measurement of CYP3A4 mRNA as a marker for PXR-based induction serves as the most sensitive indicator for the potential for induction. This endpoint has the advantage over measurement of CYP3A4 activity in that compounds that activate PXR but also inhibit/inactivate CYP3A4 will not be missed. The induction of transcription of other drug-metabolizing enzymes such as CYP2B6 and CYP2C2 showed every time that CYP3A4 mRNA levels increased, albeit to a lesser extent, indicating that measurement of CYP3A4 mRNA is a sensitive marker for the induction of these other enzymes. This was the case even for enzymes and inducers that are known to also act via the CAR pathway. Although a binary approach to assignment of inducers versus noninducers from CYP3A4 mRNA tends to overpredict the frequency of clinically relevant inducers, this is probably due to the use of single, high concentrations that far exceed clinically relevant exposure levels.

References


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