

USE OF A REPORTER GENE ASSAY TO PREDICT AND RANK THE POTENCY AND EFFICACY OF CYP3A4 INDUCERS

WAFAA EL-SANKARY, G. GORDON GIBSON, ANDY AYRTON, AND NICK PLANT

Molecular Toxicology, School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, United Kingdom (W.E.-S., G.G.G., N.P.); and Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, the Frythe, Welwyn, Herts, United Kingdom (A.A.)

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ABSTRACT:

Regulation of the CYP3A4 gene has been studied using an *in vitro* reporter gene assay. The effect of 17 xenobiotics on ~1 kilobase of the CYP3A4 proximal promoter, upstream of a secretory placental alkaline phosphatase reporter gene was investigated following transfection into the HepG2 cell line. Transfections were carried out either in the basal system or with cotransfection of expression plasmids for the human pregnane X receptor (hPXR) and the human glucocorticoid receptor (hGR), two important receptors in the regulation of CYP3A4 gene expression. Compounds were tested at four concentrations, and the resulting data were used to calculate maximal induction (I_{\max}) and EC_{50} values. An "overall inductive ability" (IA) was derived by dividing I_{\max} by EC_{50} . Of the compounds tested seven were established transcriptional inducers, all of which were positive in the *in vitro* assay. The remaining 10 com-

pounds represented a group with preliminary evidence for CYP3A4 transcriptional activation. Nine of these compounds produced statistically significant inductions *in vitro*, with only pravastatin failing to activate the reporter gene. This is of potential interest in light of the high IA values observed with the other structurally and functionally similar statins tested. We conclude that a four-concentration-point, *in vitro* model is capable of identifying CYP3A4 transcriptional inducers and yields an IA value allowing the ranking of compounds for their overall ability to induce CYP3A4 transcription. In addition, the majority of the compounds tested showed increased IA values in the hPXR/hGR cotransfected system, underpinning the importance of these receptors in CYP3A4 gene transcriptional regulation.

The cytochrome P450 gene superfamily is a group of mixed function oxidases widely expressed in both eukaryotes and prokaryotes (Nelson et al., 1996) responsible for the majority of primary oxidative metabolism of chemicals, both endobiotic and xenobiotic. The cytochrome P450 3A (CYP3A) subfamily represents the most abundant P450s¹ in human liver, comprising approximately 30% of the total P450 content (Watkins, 1994). In addition, approximately 50% of pharmaceutical drugs currently in use that undergo oxidative biotransformation are substrates for CYP3A enzymes (Cholerton et al., 1992), emphasizing the clinical importance of this subfamily in drug biotransformation. The human CYP3A family comprises three enzymes that show variable levels of expression in the population, CYP3A4, CYP3A5 and CYP3A7, of which CYP3A4 is the most prevalent in adults, being present in all but one adult liver sample so far screened.

¹ Abbreviations used are: P450, cytochrome P450; kb, kilobase; bp, base pair; hPXR, human pregnane X receptor; hGR, human glucocorticoid receptor; SPAP, secretory placental alkaline phosphatase; IA, overall inductive ability; Dex, dexamethasone; Rif, rifampicin; PCN, pregnenolone-16 α -carbonitrile; Cipro, ciprofibrate; BNF, β -naphthoflavone; Keto, ketoconazole; PB, phenobarbitone; Clot, clotrimazole; CPA, cyproterone acetate; Carb, carbamazepine; Spir, spironolactone; Phen, phenytoin; Sulf, sulfipyrazone; Met, metyrapone; Lov, lovastatin; Sim, simvastatin; Prav, pravastatin; Trog, troglitazone; Piog, pioglitazone; Fex, SCE, specific chemical effect.

Address correspondence to: Dr. Nick Plant, Molecular Toxicology, School of Biological Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK. E-mail: n.plant@surrey.ac.uk

Hence, CYP3A4 contributes the major CYP3A-mediated metabolism in the population as a whole.

Many compounds that are substrates for CYP3A4 also induce levels of the enzyme. This regulation is mainly transcriptional [e.g., dexamethasone, rifampicin (Ogg et al., 1999)], although increased enzyme activity may also be achieved via mRNA stabilization [e.g., erythromycin (Watkins et al., 1986)]. The wide range of substrates, inducers, and inhibitors of CYP3A4 introduces the possibility of clinically significant drug interactions, both during polypharmacy [e.g., treatment of psychiatric disorders (Ketter et al., 1995)] and environmental/dietary exposures [e.g., terfenadine and grapefruit juice (Bailey et al., 1998)]. It is therefore considered critical to be able to assess the potential of any new compound to induce CYP3A4, both in terms of potency (maximal induction [I_{\max}]) and efficacy (EC_{50}). Taken together, these values allow an approximation of the potential for any given compound to transcriptionally activate the CYP3A4 gene.

The molecular mechanisms behind regulation of CYP3A4 gene expression have been studied by several groups. Approximately 1 kb of proximal promoter was initially isolated (Hashimoto et al., 1993), and computer analysis showed the presence of putative transcription factor binding sites for estrogen receptor, COUP-TF, HNF4, HNF5, and Oct-1 (Hashimoto et al., 1993). Importantly, no consensus glucocorticoid receptor binding site was identified within this region despite the fact that CYP3A4 is transcriptionally activated by glucocorticoids (Ogg et al., 1999). This introduced the possibility of activation via a nonconsensus glucocorticoid-responsive unit, as seen

in the rat CYP3A4 ortholog CYP3A23 (Quattrochi et al., 1995; Huss et al., 1996). Later experiments identified a novel nuclear receptor, the human pregnane X receptor (hPXR), which bound to both ER6 and DR3 motifs (Lehmann et al., 1998). Such motifs are present at approximately –150 bp and also as an enhancer module some 8 kbp upstream of the CYP3A4 gene transcription start site (Goodwin et al., 1999). The ER6 motif is present within the CYP3A23 glucocorticoid-responsive unit (Huss et al., 1996), although no direct binding of PXR has been demonstrated yet. The synthetic glucocorticoid dexamethasone, a potent CYP3A4 inducer, has been shown to act as a ligand for hPXR, albeit a poor one (Lehmann et al., 1998), and glucocorticoid induction of CYP3A4 may occur solely through hPXR. However, cotransfection experiments with hGR clearly showed an increase in glucocorticoid responsiveness (El-Sankary et al., 2000) suggesting that the receptor does play a role, although murine knock-outs suggest this role is nonessential (Schuetz et al., 2000). Thus, induction may occur either via direct interaction of hGR with the CYP3A4 proximal promoter, potentially through a nonconsensus GRE, or through an indirect effect on the expression of other transcription factors, such as hPXR (Pascucci et al., 2000). Whichever molecular mechanism predominates *in vivo*, it is clear that both hGR and hPXR contribute to the transcriptional regulation of the CYP3A4 gene by a large number of xenobiotics (Ogg et al., 1999; El-Sankary et al., 2000).

An *in vitro* reporter gene assay has recently been developed to study the molecular mechanisms of CYP3A4 gene transcriptional regulation by both xenobiotics and endogenous steroids (Ogg et al., 1999). This system uses the previously cloned ~1 kb of CYP3A4 proximal promoter linked to a secretory alkaline phosphatase reporter gene (SPAP). When transfected into HepG2 cells, this assay correctly identifies known, clinically significant, CYP3A4 inducers (Ogg et al., 1999). To further expand this model, we studied the dose response relationship of the reporter gene construct to various xenobiotics, allowing the calculation of I_{\max} , EC_{50} , and IA values, the latter representing the overall ability of any given compound to transcriptionally activate the CYP3A4 gene.

Materials and Methods

Chemicals. Dexamethasone (Dex), rifampicin (Rif), pregnenolone-16 α -carbonitrile (PCN), ciprofibrate (Cipro), β -naphthoflavone (BNF), and ketoconazole (Keto) were of cell culture grade and purchased from Sigma Chemical Co. (St. Louis, MO). All other test compounds [phenobarbitone (PB), clotrimazole (Clot), cyproterone acetate (CPA), carbamazepine (Carb), spironolactone (Spir), phenytoin (Phen), sulfapyrazone (Sulf), metyrapone (Met), lovastatin (Lov), simvastatin (Sim), pravastatin (Prav), troglitazone (Trog), pioglitazone (Piog), and fexofenadine (Fex)] were a kind gift from GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). All other chemicals were of cell culture grade and obtained from Sigma Chemical Co.

Plasmids. The secretory placental alkaline phosphatase reporter gene pCMV-SPAP (termed pCMV) was a gift from GlaxoWellcome (Ware, UK). The CYP3A4 reporter construct p3A4-CMV-SPAP (termed p3A4) was previously engineered in our laboratory (Ogg et al., 1999) and contained ~1 kb (–1087 to –57 bp) of the CYP3A4 proximal promoter. The pSG5-hGR α and pSG5-hPXR1 expression plasmids were gifts from Dr. Jonathon Tugwood (Zeneca CTL, Macclesfield, UK) and Dr. Steven Kliewer (GlaxoWellcome, Research Triangle Park, NC), respectively.

All plasmids were grown in the *Escherichia coli* strain TOP10F' (Invitrogen, Leek, The Netherlands) and purified using Endo-free maxi preps QIAGEN (Dorking, Surrey, UK) according to manufacturers instructions. Removal of endotoxin contamination of DNA before transfection has previously been shown to be critical for optimal transfection of cell lines.

Cell Culture and Transient Transfection. All cell culture media and supplements were purchased from Invitrogen. HepG2 cells, a human hepatocyte carcinoma cell line, were obtained from the European Collection of Animal Cell Cultures (ECACC 85011430, Porton Down, UK). HepG2 cells

were routinely cultured in 75 cm² vented tissue culture flasks (Nunc, Loughborough, UK) using minimal essential media with Earle's salts supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 μ g/ml gentamycin, and 10% Australasian fetal bovine serum. To maintain HepG2 phenotypic consistency, HepG2 cells were only used up to passage 13 after receipt from ECACC (received from ECACC at passage 90).

Transient transfection was based upon the calcium phosphate precipitation methodology of Jordan (Jordan et al., 1996) and described in detail elsewhere (Ogg et al., 1999). Briefly, the methodology was as follows.

Day 1: Seeding of HepG2 cells. Cells in medium were seeded into 96-well plates (Nunc) at a concentration of 0.6×10^6 cells/ml (final volume, 120 μ l/well). Plates were placed in a humidified container in a cell culture incubator at 37°C in 5% CO₂.

Day 2: Transfection. One hour before transfection, medium was replaced with fresh, and the cells returned to the incubator. The transfection mix was prepared on ice, with a standard precipitation formation time of 1 min. Plasmid DNA was used at a fixed concentration of 12.5 μ g/ml during precipitate. Where indicated, the hGR and hPXR plasmids were added to the transfection mix at a concentration of 12.5 μ g/ml each. The precipitation reaction was terminated by further dilution of plasmid DNA to 830 ng/ml using fresh medium. After precipitate formation, medium in the 96-well plate was replaced with transfection mix (120 μ l; equivalent to 100 ng/plasmid/well).

Day 3: Xenobiotic dosing. Xenobiotics were dissolved in the relevant solvent and used in final concentrations of 1, 10, 30, and 50 μ M for main experiments. Validation experiments were carried out at eight points over a concentration range that covered the full response of the reporter gene. Xenobiotics were dissolved such that final solvent concentration was 0.1%, and solutions were prepared fresh on the day of dosing. Solvents used were dimethyl sulfoxide (Dex, Rif, PCN, PB, Phen, Sulf, Met, and BNF), ethanol (Clot, CPA, Carb, Spir, Lov, Sim, Prav, Cipro, and Keto), or methanol (Trog, Piog, and Fex). Medium was removed from wells and stored at –20°C for later assay of SPAP activity. Fresh medium was then added to the wells, and xenobiotic solution and solvent controls were added as required. Each experimental condition was carried out in replicate in eight separate wells.

Day 4: Cells incubated.

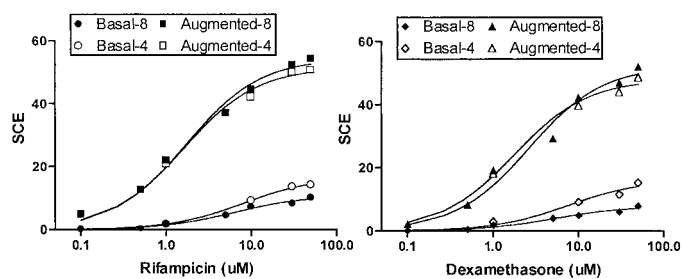
Day 5: SPAP sample. Medium was removed from the wells and stored at –20°C for later measurement of SPAP activity.

SPAP Activity Determination. Aliquots of cell culture medium (25 μ l/well) were transferred into 96-well Optiplates (Packard BioScience Ltd., Pangbourne, Berkshire, UK). Endogenous alkaline phosphatase activity was abolished by heat-treatment of the medium at 65°C for 30 min. During this procedure, plates were sealed to prevent loss of sample by evaporation. SPAP activity was then assayed using the AURORA system (ICN, Thame, UK), according to the manufacturers protocol. Chemiluminescent output was measured using a LumiCount automated plate reader (Packard BioScience Ltd.).

Data Analysis. The relative change in SPAP activity between day 3 (before dose) and day 5 (after dose) was calculated for both p3A4 and pCMV in the presence and absence of xenobiotic. These measurements allow for the control of variation in cell seeding, transfection efficiency, and cytotoxic or proliferative effects of xenobiotics that might otherwise produce anomalous results.

A specific chemical effect (SCE) is calculated using the values mentioned above, and the statistical significance of this value over solvent control is tested as described previously (Plant et al., 2000). The SCE is therefore a refinement of the fold induction value, additionally taking into account any confounding factors, as described above. I_{\max} (representing the overall maximal induction produced, encompassing ligand-receptor, receptor-receptor, receptor translocation, and receptor-DNA interactions caused by the inducer) and EC_{50} values were calculated using nonlinear regression analysis (GraphPad Prism v2.0, GraphPad Software, San Diego, CA), and an indication of the overall effect of the xenobiotic was calculated by dividing I_{\max} by $EC_{50} \cdot I_{\max}$ and EC_{50} values were only calculated if the data fitted several criteria, namely:

- A significant induction was observed for at least two of the four tested concentrations ($p < 0.05$).
- The coefficient of fit (r^2) for a nonlinear regression, as defined by the equation below, was greater than that for a linear regression.
- The coefficient of fit (r^2) of the nonlinear regression, as defined by the equation below, was equal to, or greater than, 0.9.



Compound	hGR/hPXR co-transfected	I_{max} (SCE)		EC_{50} (μ M)		IA (SCE/ μ M)	
		4-Point	8-Point	4-Point	8-Point	4-Point	8-Point
Rifampicin	-	16.8 \pm 0.4	11.0 \pm 0.6	8.0 \pm 0.8	6.1 \pm 1.3	2.1	1.8
Rifampicin	+	51.6 \pm 1.5	54.4 \pm 1.7	1.6 \pm 0.3	1.7 \pm 0.8	32.3	32.0
Dexamethasone	-	16.3 \pm 2.3	7.9 \pm 0.6	7.5 \pm 4.2	5.2 \pm 1.5	2.2	1.5
Dexamethasone	+	48.1 \pm 1.7	52.3 \pm 2.8	1.7 \pm 0.4	2.6 \pm 0.6	28.3	20.1

Fig. 1. Concentration response curves for CYP3A4 induction.

HepG2 cells were transfected with p3A4 or pCMV reporter gene constructs either in the presence (augmented) or absence (basal) of hGR/hPXR expression plasmids, as described under *Materials and Methods*. Cells were exposed to varying concentrations of rifampicin or dexamethasone, and the SCE, I_{max} , EC_{50} , and IA were calculated as described under *Materials and Methods*. The results are derived from a representative experiment in which each data point is the mean of eight separate determinations. Statistical significance, compound dosed versus vehicle dosed, is not shown on the graph for clarity, but all points are statistically significant ($p < 0.05$) for rifampicin concentrations greater than 10 μ M (1 μ M in receptor-augmented system) and dexamethasone concentrations greater than 30 μ M (1 μ M in receptor-augmented system).

$$\text{specific chemical effect} = \frac{I_{max} * \text{drug concentration}}{EC_{50} + \text{drug concentration}}$$

This equation describes the activation of a reporter gene by a compound that follows the law of mass action for binding of the ligand to the receptor [$Y = I_{max} * X/(EC_{50} + X)$]. I_{max} is the maximal activation, and EC_{50} is the concentration of compound required to reach half-maximal activation.

Results

System Controls. To control for the validity of the reporter gene assay, the system was tested with three compounds that are established noninducers of CYP3A4. HepG2 cells were transfected with the CYP3A4 reporter gene assay in the presence or absence of hGR and hPXR and exposed to BNF (CYP1A inducer), Cipro (CYP4A inducer), or Keto (CYP3A inhibitor). These compounds failed to cause a significant induction of the CYP3A4 reporter gene assay at any of the doses tested (data not shown), demonstrating the specificity of the assay.

Validation of Four-Point Assay. To validate the use of four points in calculating I_{max} , EC_{50} , and IA values, two compounds were examined in more detail, using 0.1, 0.5, 1, 5, 10, 30, 50, and 100 μ M drug concentrations to cover the full dose response curve. Rif and Dex were chosen as compounds that fell within the prediction envelope of the four-point model. Figure 1 shows the dose response curves for the two compounds and confirms the use of a four-point assay in deriving I_{max} , EC_{50} , and IA values, as per the above criteria.

Examination of Classical CYP3A4 Inducers. To further validate the in vitro model and to examine the role of hGR/hPXR in mediating CYP3A4 induction, five additional established CYP3A4 inducers were examined. PB, Spir, Phen, Carb, and Met are established CYP3A4 inducers with good literature evidence for induction (Thummel et al., 1992; Kocarek et al., 1995; Lake et al., 1996; Tomlinson et al., 1996; Harvey et al., 2000, respectively). The compounds were examined for their ability to induce the CYP3A4 reporter gene at concentrations of 1, 10, 30, and 50 μ M. Specific chemical effect

TABLE 1

Kinetic values for induction of the CYP3A4 reporter gene by classical CYP3A4 inducers

I_{max} , EC_{50} , and IA values were derived from concentration response curves for each compound. Four xenobiotic concentrations (1, 10, 30, and 50 μ M) were used in the construction of each curve, and each data point was derived from eight replicates in a representative experiment. I_{max} , EC_{50} , and IA values were only derived if the curve fitting passed strict criteria, as described under *Materials and Methods*.

Compound	hGR/hPXR Co-Transfected	I_{max} (SCE)	EC_{50} (μ M)	IA (SCE/ μ M)
Carbamazepine	-	8.1 \pm 0.5	1.6 \pm 0.6	5.0
Carbamazepine	+	14.1 \pm 0.5	0.9 \pm 0.2	16.4
Metyrapone	-	12.8 \pm 1.3	6.4 \pm 2.8	2
Metyrapone	+	20.9 \pm 3.0	6.6 \pm 4.0	3.2
Phenytoin	-	15.8 \pm 1.1	17.6 \pm 3.3	0.9
Phenytoin	+	27.5 \pm 8.7	25.1 \pm 18.4	1.1
Phenobarbital	-	16.9 \pm 2.6	17.2 \pm 7.3	1.0
Phenobarbital	+	41.3 \pm 15.3	9.7 \pm 12.7	4.3
Spironolactone	-	9.0 \pm 0.9	9.1 \pm 3.4	1.0
Spironolactone	+	13.2 \pm 0.7	1.1 \pm 0.4	11.7

levels were calculated as previously described (Plant et al., 2000), and the I_{max} , EC_{50} , and IA values given in Table 1. All compounds examined produced significant transcriptional activation in the reporter gene assay, an effect that was amplified in the presence of cotransfected hGR/hPXR.

Examination of Putative CYP3A4 Inducers. Following examination of the classical CYP3A4 inducers, ten compounds were studied in which putative evidence of CYP3A4 induction in humans exists. PCN has been shown to transcriptionally activate CYP3A23 in rat hepatocytes (Barwick et al., 1996) and induce CYP3A-mediated testosterone metabolism in humans (Lake et al., 1998). Cyproterone acetate (Kocarek et al., 1995), lovastatin (Kocarek et al., 1995), and clotrimazole (Schuetz et al., 1993) all cause increased CYP3A activity in primary human hepatocytes. Sulfapyrazone (Upton, 1991), simvastatin (Horsmans et al., 1993), pravastatin (Horsmans et al., 1993), and troglitazone (Yamazaki et al., 2000) all cause in vivo effects on the metabolism of known CYP3A substrates, and pioglitazone is of interest as a member of the same structural family as troglitazone. Finally, fexofenadine is one of the active metabolites of terfenadine, which is known to effect in vivo metabolism of CYP3A substrates (Wang et al., 2000).

PCN, CPA, Clot, Fex, Piog, Lov, Prav, Sim, Sulf, and Trog were all examined for their ability to induce the CYP3A4 reporter gene assay at concentrations of 1, 10, 30, and 50 μ M. Specific chemical effect levels were calculated as previously described (Plant et al., 2000), and I_{max} , EC_{50} , and IA values derived as described under *Materials and Methods* (Table 2). Compounds that did not satisfy the requirements of fit for the four-point model, as described under *Materials and Methods*, were excluded from analysis and noted as such in the table.

As can be seen from Table 2, two compounds, lovastatin and troglitazone, did not fit the four-point model and, hence, could not be further analyzed. Because both of these compounds are of substantial pharmaceutical interest, they were re-examined over a full dose response curve, and I_{max} , EC_{50} , and IA values were calculated from the full dose response curves. Figure 2 shows the dose response curves and derived data for these two compounds.

Discussion

The ability to rapidly and accurately assess drug induction potential is considered essential in both new drug development and clinical management of existing drug treatment regimes. There are several factors key in this assessment including pharmacokinetics, pharma-

TABLE 2

Kinetic values for induction of the CYP3A4 reporter gene by putative CYP3A4 inducers

I_{\max} , EC_{50} , and IA values were derived from concentration response curves for each compound. Four xenobiotic concentrations (1, 10, 30, and 50 μM) were used in the construction of each curve, and each data point was derived from eight replicates in a representative experiment. I_{\max} , EC_{50} , and IA values were only derived if the curve fitting passed strict criteria, as described under *Materials and Methods*.

Compound	hGR/hPXR Co-Transfected	I_{\max} (SCE)	EC_{50}	IA
CPA	–	9.0 \pm 0.2	17.7 \pm 1.0	0.5
CPA	+	11.2 \pm 0.2	1.6 \pm 0.2	6.9
Clotrimazole	–	8.6 \pm 0.4	6.2 \pm 1.1	1.4
Clotrimazole	+	15.2 \pm 0.3	1.1 \pm 0.1	14.0
Fexofenadine	–	17.9 \pm 2.8	7.0 \pm 4.4	2.6
Fexofenadine	+	47.0 \pm 0.9	2.4 \pm 0.3	19.8
Lovastatin	–	DNF		
Lovastatin	+	DNF		
PCN	–	13.2 \pm 1.1	4.5 \pm 1.9	2.9
PCN	+	44.3 \pm 2.5	2.5 \pm 0.8	17.7
Pioglitazone	–	12.5 \pm 2.5	6.0 \pm 5.3	2.1
Pioglitazone	+	31.2 \pm 0.8	1.1 \pm 0.2	28.4
Pravastatin	–	NI		
Pravastatin	+	NI		
Simvastatin	–	31.3 \pm 4.8	17.4 \pm 7.3	1.8
Simvastatin	+	81.5 \pm 8.3	0.8 \pm 0.6	100.5
Sulfinpyrazole	–	19.6 \pm 1.7	15.7 \pm 3.9	1.3
Sulfinpyrazole	+	13.9 \pm 2.8	7.9 \pm 6.1	1.8
Troglitazone	–	DNF		
Troglitazone	+	DNF		

NI, no statistically significant induction observed at any dose; DNF, data did not fit criteria for calculation of I_{\max} , EC_{50} , and IA values, as described under *Materials and Methods*.

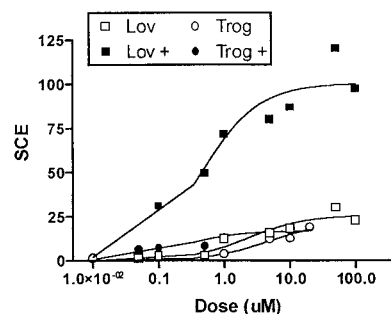
codynamics, efficacy, and potency. Although in vitro systems cannot assess pharmacokinetics/pharmacodynamics parameters, they are ideally suited for initial analysis of the ability of a compound to induce drug-metabolizing enzymes. The CYP3A4 reporter gene assay system developed in our laboratory allowed medium throughput assessment of the intrinsic inductive ability of xenobiotics and endogenous compounds (Ogg et al., 1999), and the addition of a formal mathematical model allowed full statistical analysis of the results, taking into account possible confounding factors, such as toxic or proliferative effects of compounds, plasmid effects, and transfection efficiency (Plant et al., 2000). The receptor-augmented system, using both double cotransfection hGR and hPXR expression plasmids, allows the identification of compounds in which effects are mediated through these interacting transcription factors. Transfection using single expression plasmids may also be carried out to increase information on the role of the individual receptors in the observed induction (El-Sankary et al., 2000). However, given the high level of interaction of PXR and GR, both in terms of regulation of their expression (Pascucci et al., 2000) and overlap of ligands (Lehmann et al., 1998; Ogg et al., 1999), such an approach may not fully represent the in vivo situation.

Although simple induction data can be useful in assessing a compounds' potential effects on CYP3A4, it is more informative to know not only if a compound can induce but also the rank order potency and efficacy of this induction. The use of four concentration points is the minimum required to create a concentration response curve and, hence, the calculation of I_{\max} and EC_{50} values. By applying strict criteria (as described under *Materials and Methods*), it is possible to associate a high degree of confidence with the figures derived from four-point concentration curves. The data from Fig. 1 clearly shows that a four-point curve is sufficient to accurately calculate values when compared with a comprehensive eight-point concentration curve. Although such data will always have the caveat that it is derived from a small data-set, the experimental evidence presented

here suggests that, as long as strict acceptance criterion are imposed on the predictions, meaningful conclusions can be drawn. The fact that both lovastatin and troglitazone did not fit the four-point model demonstrates that, although the chosen concentrations are suitable for the majority of test compounds, they may be inappropriate for very potent (or potentially very weak) inducers. In these cases, the points would not produce a sufficient concentration response curve to allow calculation I_{\max} , EC_{50} , or IA values. However, these points will demonstrate whether the compound is an inducer, and then a repeat experiment over a more appropriate concentration range can be carried out if the more fully descriptive values are desired.

It is of potential interest that EC_{50} values vary markedly for some inducers between basal and receptor-augmented systems, suggesting the involvement of several activation pathways. This may be due to the relatively low abundance of PXR and/or GR in HepG2 cells (K. Swales, unpublished data) allowing inducers to act via receptors that would normally play a secondary role, as evidenced by their generally higher EC_{50} values. In the receptor-augmented system, levels of PXR and/or GR are not rate limiting and induction may occur via these receptors, resulting in lower EC_{50} values. Such a system may more closely reflect the in vivo situation in which PXR and GR levels are relatively higher (K. Swales, unpublished data).

Using data derived from this model, a rank order for the overall effect of the compound in the reporter gene assay can be produced, as summarized in Fig. 3. In the construction of rank-order tables, it is important to remember that IA values include the errors associated with the individual I_{\max} and EC_{50} values. Therefore, an I_{\max} value of 10 (± 1) and EC_{50} value of 5 (± 0.5) could lead to IA values in the range 2.4 \rightarrow 1.7 (11/4.5 \rightarrow 9/5.5). Rank order in Fig. 3 is initially derived from IA, but compounds are further subdivided into poor, medium, and good inducers based on broader categories. It is of interest to note that generally recognized potent CYP3A4 inducers, such as rifampicin (Damkier et al., 1999), produce high IA values (2.1 in basal system) when compared with classically poor inducers, such



Compound	hGR/hPXR co-transfected	I_{\max} (SCE)	EC_{50} (μM)	IA (SCE/ μM)
Lovastatin	–	25.7 \pm 2.6	2.3 \pm 1.0	11.2
Lovastatin	+	100.4 \pm 6.8	0.5 \pm 0.2	200.8
Troglitazone	–	21.7 \pm 3.8	4.5 \pm 2.4	4.8
Troglitazone	+	17.1 \pm 1.4	0.2 \pm 0.1	85.5

Fig. 2. Concentration response curves for lovastatin and troglitazone.

HepG2 cells were transfected with p3A4 or pCMV reporter gene constructs either in the presence (augmented) or absence (basal) of hGR/hPXR expression plasmids, as described under *Materials and Methods*. Cells were exposed to varying concentrations of lovastatin or troglitazone, and the SCE, I_{\max} , EC_{50} , and IA were calculated as described under *Materials and Methods*. The results are derived from a representative experiment in which each data point is the mean of eight separate determinations. Statistical significance, compound dosed versus vehicle dosed, is not shown on the graph for clarity, but all points are statistically significant ($p < 0.05$) for lovastatin concentrations greater than 5 μM (1 μM in receptor-augmented system) and troglitazone concentrations greater than 10 μM (0.5 μM in receptor-augmented system).

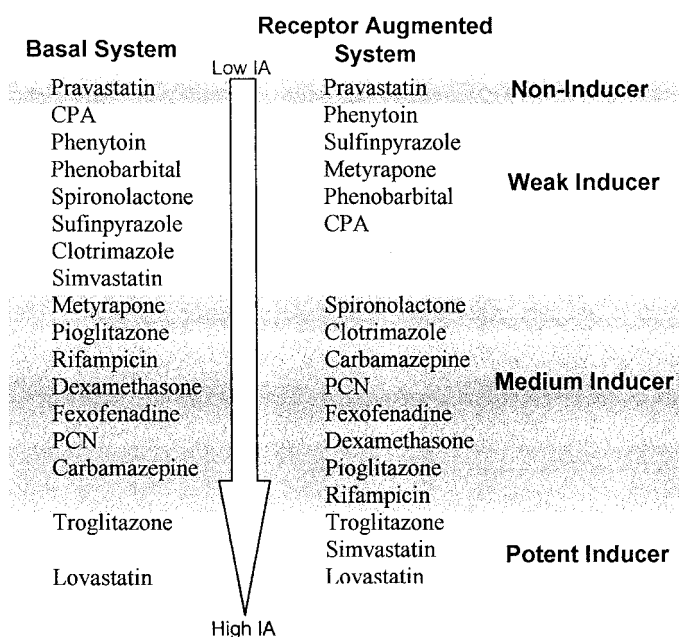


FIG. 3. Relative rank order of CYP3A4 inducers.

CYP3A4 inducers were ranked according to their IA value, both in the basal and receptor-augmented system. These orders were further subdivided into poor, medium, and strong inducers based on IA values of 0.1 to 2, 2 to 4, and 4+ for the basal system and 0.1 to 10, 10 to 50, and 50+ for the receptor-augmented system.

as phenobarbital (1.0 in basal system) (Pichard et al., 1995). In addition, double cotransfection with expression plasmids for hGR and hPXR cause a marked increase in IA for rifampicin (16-fold increase in SCE from 2→32), which is an established hPXR ligand (Lehmann et al., 1998). In contrast, phenobarbital, a putative CAR ligand (Honkakoski et al., 1998) only exhibits a small increase in IA following cotransfection (4-fold increase in SCE from 1→4). Although phenobarbital is a putative CAR ligand, it also acts as a poor ligand for hPXR (Lehmann et al., 1998), and this may explain the observed slight increase in I_{max} observed between the basal and receptor-augmented system. It is of interest that lovastatin, simvastatin, and troglitazone all produced higher IA values (in both basal and cotransfected systems) than the classically "potent" inducer rifampicin. Both lovastatin and troglitazone have been shown to induce CYP3A in human hepatocytes (Kocarek et al., 1995; Sahi et al., 2000, respectively), but this is the first demonstration of their high overall potency of CYP3A4 induction.

Although lovastatin and simvastatin were demonstrated to have the highest IA values, the structurally similar compound pravastatin was the only putative CYP3A inducer examined that did not cause an induction in this assay. Reasons for this difference are unclear, although two potential explanations exist. First, it is a well established paradigm that substrates for enzymes are often inducers of these enzymes as well. Both lovastatin and simvastatin are substrates for CYP3A4 (Cheng et al., 1992), and although pravastatin is metabolized by CYP3A (Jacobsen et al., 1999), the binding constant of pravastatin to P450s and the Michaelis-Menten constants are 3 orders of magnitude higher than for other statins (Jacobsen et al., 1999). An alternative explanation is that pravastatin is poorly taken up by HepG2 cells and, hence, is not available for induction. Sugiyama and coworkers (Suzuki et al., 1998) have demonstrated that pravastatin is transported by liver organic anion transport protein, and this system may be absent in HepG2 cells (Ziegler et al., 1994).

Clotrimazole has previously been shown by us to be a noninducer

(Ogg et al., 1999) in this reporter gene assay, with its inductive effect thought to occur via mRNA stabilization. However, we now clearly show its ability to induce the CYP3A4 reporter gene construct both in the basal and receptor-augmented system. Advances in the sensitivity of the reporter system used, along with a formal statistical model, allow the identification of this weak inductive effect in the basal system, previously missed. In contrast, the receptor-augmented system shows a substantial induction, probably caused by the inclusion of hPXR in the receptor cotransfection, where previously only hGR had been used. Clotrimazole has been demonstrated to be an hPXR ligand (Bertilsson et al., 1998; Lehmann et al., 1998), and induction presumably occurs via this receptor. Such a finding underpins the importance of using formal mathematical models to analyze induction, as previous systems may under- or overestimate conclusions, resulting in false positives and negatives.

A potential addition to this system would be the replacement of the CMV enhancer with the xenobiotic-responsive module enhancer identified approximately 7500 bp upstream of the CYP3A4 transcription start site by Goodwin and coworkers (1999). This module contains four sites of protein-DNA interactions, two of which are PXR binding sites. Hence, this element may provide a differential-enhancing effect, increasing PXR-mediated inductions more than non-PXR-mediated inductions and could, therefore, potentially alter the rank order observed. However, it is unclear at this time whether such an effect would be large, subtle, or nonexistent, and ongoing studies are assessing this issue.

In conclusion, we have extended our current CYP3A4 reporter gene assay to allow the rapid determination of I_{max} , EC_{50} , and IA values. Such data give a more accurate assessment of the effect any chemical may have on CYP3A4 gene expression and represent an important tool for both new compound screening and research into the molecular mechanisms underlying xenobiotic induction of CYP3A4. Using this system, we have examined a number of compounds with preliminary evidence for CYP3A induction or that are structurally similar to compounds known to induce CYP3A. The majority of these compounds were shown to be inducers of CYP3A4 of varying potency, with the exception of pravastatin, which failed to induce at any of the doses tested. This is of potential interest because the other statins examined (lovastatin and simvastatin) were among the compounds with the highest overall inductive ability.

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