EFFECTS OF PROTOTYPICAL MICROSOMAL ENZYME INDUCERS ON CYTOCHROME P450 EXPRESSION IN CULTURED HUMAN HEPATOCYTES

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(Received September 24, 2002; accepted December 20, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
Cultured human hepatocytes are a valuable in vitro system for evaluating new molecular entities as inducers of cytochrome P450 (P450) enzymes. The present study summarizes data obtained from 62 preparations of cultured human hepatocytes that were treated with vehicles (saline or dimethylsulfoxide, 0.1%), β-naphthoflavone (33 μM), phenobarbital (100 or 250 μM), isoniazid (100 μM) and/or rifampin (20 or 50 μM), and examined for the expression of P450 enzymes based on microsomal activity toward marker substrates, or in the case of CYP2C8, the level of immunoreactive protein. The results show that CYP1A2 activity was markedly induced by β-naphthoflavone (on average 13-fold, n = 28 preparations), and weakly induced by phenobarbital (1.9-fold, n = 25) and rifampin (2.3-fold, n = 22); CYP2B6 activity tended to be increased with phenobarbital (n = 7) and rifampin (n = 3) treatments, but the effects were not statistically significant; CYP2B6 was induced by phenobarbital (6.5-fold, n = 13) and rifampin (13-fold, n = 14); CYP2C8 was induced by phenobarbital (4.0-fold, n = 4) and rifampin (5.2-fold, n = 4); CYP2C9 was induced by phenobarbital (1.8-fold, n = 14) and rifampin (3.5-fold, n = 10); CYP2C19 was markedly induced by rifampin (37-fold, n = 10), but relatively modestly by phenobarbital (7-fold, n = 9); CYP2D6 was not significantly induced by phenobarbital (n = 5) or rifampin (n = 5); CYP2E1 was induced by phenobarbital (1.7-fold, n = 5), rifampin (2.2-fold, n = 5), and isoniazid (2.3-fold, n = 5); and, CYP3A4 was induced by phenobarbital (3.3-fold, n = 42) and rifampin (10-fold, n = 61), but not by β-naphthoflavone. Based on these observations, we generalize that β-naphthoflavone induces CYP1A2 and isoniazid induces CYP2E1, whereas rifampin and, to a lesser extent phenobarbital, tend to significantly and consistently induce enzymes of the CYP2A, CYP2B, CYP2C, CYP2E, and CYP3A subfamilies but not the 2D subfamily.

Drugs and NMEs5 are often screened for their ability to induce P450 and other drug-metabolizing enzymes with the aim of predicting or explaining drug-drug interactions and pharmacokinetic tolerance.

A preliminary account of this work was presented at the International Society for the Study of Xenobiotics (ISSX) meeting, 2000 June 11–16, St. Andrews, Scotland.

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5 Abbreviations used are: NME, new molecular entity (also known as NCE, new chemical entity); P450, cytochrome P450; BCA, bicinchoninic acid; ANOVA, analysis of variance; EROD, 7-ethoxyresorufin O-dealkylase; 7-EFCOD, 7-ethoxyflutrimouethylcarbin O-dealkylase; DMSO, dimethylsulfoxide; CAR, constitutive androstane receptor; PXR, pregnane X receptor.

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Primary cultures of human hepatocytes are a reliable in vitro system for evaluating NMEs as inducers of P450 enzymes (Kostrubsky et al., 1999; LeCluyse et al., 2000; Robertson et al., 2000; Parkinson, 2001; Silva and Nicoll-Griffith, 2001). When hepatocytes are cultured under conditions that restore near-normal hepatocellular morphology and the expression of liver-specific genes (e.g., albumin), P450 enzymes can be induced in vitro in a manner that reflects the in vivo situation in terms of the magnitude and specificity of P450 induction (LeCluyse et al., 2000; Runge et al., 2000). In general, the enzyme induction data obtained in vitro appear to correlate well with clinical observations, provided the in vitro experiments are performed at pharmacologically-relevant concentrations of the NMEs (Dilger et al., 1999; LeCluyse et al., 2000). Additionally, species differences in P450 inducibility are retained in the cultured hepatocytes (Silva et al., 1999). For example, cultured hepatocytes correctly predict that 1) rifampin is a potent and effective inducer of human CYP3A4 but not rat CYP3A (Silva et al., 1999; LeCluyse et al., 2000; Zhu et al., 2000), 2) omeprazole is a more effective inducer of human CYP1A2 than of rat CYP1A (Shih et al., 1999; LeCluyse et al., 2000), and 3) even though omeprazole induces
human CYP1A2, it rarely causes significant clinical drug interactions because the concentration of omeprazole required to induce CYP1A2 in vitro generally exceeds the concentrations achieved in vivo (Dujat et al., 1992; Dilger et al., 1999; LeCluyse et al., 2000).

Numerous in vitro studies with cultured human hepatocytes have demonstrated the inductive effects of proton-pump inhibitors (e.g., omeprazole) on CYP1A2 and those of macrolide antibiotics (e.g., rifampin) on CYP3A4 (Reviewed by Abdel-Rahman and Leeder, 2000). However, few studies have systematically evaluated the specificity and selectivity of prototypical enzyme inducers on the expression of P450 enzymes in human hepatocytes. The effects of prototypical P450 enzyme inducers on CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 have not been studied in detail. Also, anticonvulsants (e.g., phenobarbital) are thought to induce CYP2C and CYP3A enzymes in vivo, but direct evidence of this is minimal. Additionally, data in the literature can be contradictory. For example, rifampin has been reported to induce CYP2C9 and CYP2C19 in human hepatocytes (Chang et al., 1997; Morel et al., 1990; Gerbal-Chaloin et al., 2001), but other reports suggest that it does not (Li et al., 1997; Runge et al., 2000).

There are several reasons for the lack of availability of comprehensive and reproducible data: 1) the technique of culturing human hepatocytes has been somewhat standardized only in the last 5 years, 2) the availability of livers that yield hepatocytes suitable for preparing primary cultures remains a problem, 3) individual cultures of human hepatocytes exhibit variable responses to known inducers of P450 enzymes, and 4) with the exception of CYP1A2 and CYP3A4, only modest induction of P450 enzymes is generally observed, which makes it difficult to document such effects. It is notable that Donato et al. (1995) have studied the effects of 3-methylcholanthrene, phenobarbital, ethanol, dexamethasone and isosafrole on CYP1A1, CYP2A, CYP2C, CYP2E1 in 14 preparations of human hepatocytes. However, they selected marker substrate reactions for the CYP2A2 and CYP2C2 enzymes that are selective for rat P450 enzymes but not for human P450 enzymes. Additionally, Donato et al. (1995) did not choose rifampin as a prototypical inducer, nor were they able to distinguish between CYP2C9, CYP2C9, and CYP2C19. A recent study with up to 76 preparations of human hepatocytes showed that CYP1A1/2, CYP2A6, and CYP3A4 are inducible by prototypical P450 enzyme inducers (Muenier et al., 2000), but the effects on CYP2B6, CYP2C’s, and CYP2D6 were not evaluated.

During the last few years, we have evaluated the potential of a large number of NMEs (~70) to cause induction of various P450 enzymes in 62 preparations of cultured human hepatocytes. Each experiment included one or more prototypical P450 inducers, namely β-naphthoflavone, phenobarbital, isoniazid and/or rifampin, as positive controls to demonstrate induction of CYP1A2, CYP2B6, CYP2E1, and CYP3A4, respectively. During this process, we have collected data on the effects of these prototypical inducers on CYP1A2, CYP2A6, CYP2B6, CYP2E1, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, which are summarized here.

Materials and Methods

Chemicals and Reagents. The sources of most of the reagents used in the experiments described herein have been published elsewhere (LeCluyse et al., 1999; Madan et al., 1999; Robertson et al., 2000). β-Naphthoflavone, phenobarbital, isoniazid, and rifampin (prototypical inducers studied) were obtained from Sigma-Aldrich (St. Louis, MO).

Source of Human Livers. Human livers were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), the Midwest Transplant Network (Westwood, KS) and the Anatomical Gift Foundation (Woodbine, GA). These livers were originally intended for transplantation but later deemed unsuitable for various reasons (e.g., because of high fat content, improper vasculature, elevated liver function enzyme levels, etc.). The cold-ischemia time for these livers ranged from 1 to 36 h. Additionally, small pieces of liver (50–100 g) were obtained from the Department of Surgical Pathology, University of Kansas Medical Center (Kansas City, KS). These sections of liver were from patients undergoing liver transplantation or resection, and they are normally discarded after pathological examination.

Hepatocyte Isolation, Culture, and Treatment with P450 Inducers. Hepatocytes were isolated and cultured based on a modification of a two-step collagenase digestion method described recently (Madan et al., 1999; LeCluyse et al., 2000; Robertson et al., 2000). Hepatocytes were cultured with serum-free modified Chee’s medium (Waxman et al., 1990) containing 0.1 μM dexamethasone, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium) in a sandwich configuration on a collagen substratum with a Matrigel overlay (Madan et al., 1999; LeCluyse et al., 2000; Robertson et al., 2000). The hepatocytes were allowed to adapt to culture conditions for 2 to 3 days before they were treated with inducers because hepatocytes are often refractory to enzyme inducers during this initial culture period (Schuetz et al., 1984). After the adaptation period, hepatocytes were examined under a light microscope to evaluate cell morphology. Hepatocyte cultures that had either dedifferentiated into fibroblast-like cells or undergone extensive apoptosis were discarded. Both events occur during the first two days of culture and result in hepatocytes that are nonresponsive to P450 enzyme inducers. After this 2 to 3 day adaptation period and morphological examination, acceptable cultures were treated daily for 3 days with saline or DMSO (final concentration 0.1%, v/v), β-naphthoflavone (33 μM), phenobarbital (100 or 250 μM), isoniazid (100 μM), and/or rifampin (20 or 50 μM). β-Naphthoflavone, phenobarbital, and rifampin were dissolved in DMSO, whereas isoniazid was dissolved in saline. Approximately 24 h after the final treatment, the hepatocytes were photographed to document their morphological characteristics and any overt signs of toxicity. Culture dishes within individual treatment groups were scraped and pooled, and microsomes were prepared by differential centrifugation as described previously (LeCluyse et al., 1996). The microsomes were stored frozen at ~80°C until needed. Protein concentration of each microsomal sample was determined by the BCA assay with a BCA protein assay kit from Pierce Chemical Co. (Rockford, IL).

Enzyme Assays and Western Immunoblotting. Microsomes were analyzed to determine the rates of 7-ethoxyresorufin O-dealkylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), 7-ethoxy-4-trifluoromethyl coumarin O-dealkylation (CYP1A2/2B6), S-mephenytoin N-demethylation (CYP2B6), diclofenac 4'-hydroxylation (CYP2C9), S-mephenytoin 4'-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A4) according to previously described methods (Robertson et al., 2000). CYP2C8 content was determined by Western immunoblotting with an antibody that selectively recognizes human CYP2C8 as described previously (Robertson et al., 2000). CYP2C8 activity was not determined because the amount of microsomal protein from the hepatocytes was limiting.

Statistical Analysis. To evaluate the statistical significance of differences between group means, equal variance and normality tests were first conducted to determine whether the data were parametrically distributed. For a parametrically distributed data set, a one-way repeated measures analysis of variance (ANOVA) was carried out to determine whether there were significant differences between the group means. For a nonparametrically distributed data set, the Friedman repeated measures ANOVA on ranks was employed. Statistically significant differences from the controls were identified by a Dunnnett’s post hoc test (p < 0.05 or 5% level of significance). A separate test (paired Student’s t test, p < 0.05) was employed for comparing the effects of isoniazid to those of its corresponding vehicle (saline) control.

At times, the presence of a strong inducer (e.g., β-naphthoflavone for CYP1A2) tended to mask the possible statistical significance of the effect of a weak or moderate inducer (e.g., phenobarbital and rifampin for CYP1A2). In such cases, when necessary, the strong inducer treatment group was excluded and repeated measures ANOVA followed by Dunnnett’s test were performed again. Statistical analyses were performed with SigmaStat Statistical Analysis System (SPSS Inc., Chicago, IL).

Limitations of the Experimental Design. Not all human hepatocyte cultures were treated with all three prototypical P450 inducers. Additionally, when they were treated with all three inducers, the microsomes were not...
necessarily analyzed for all P450 enzyme activities. There are two reasons for these limitations: 1) the primary experimental design was specifically used to study the effect of a series of NMEs on CYP3A4 expression, in which case, rifampin was the only necessary positive control; and 2) at times, microsomal protein yield was not sufficient to allow analysis of all P450 enzyme activities. Since most experiments focused on an evaluation of NMEs as inducers of CYP3A4 and CYP1A2, there was a larger sample size for these enzymes relative to others. Omeprazole, 3-methylcholanthrene, and β-naphthoflavone are all inducers of human CYP1A2 in vitro. Omeprazole is a clinically relevant CYP1A2 inducer, and clearly is the prototypical inducer of choice. However, at the time this work was initiated, omeprazole was not commercially available. 3-Methylcholanthrene is a known carcinogen and is light sensitive. For these reasons, β-naphthoflavone was selected as the prototypical CYP1A2 inducer for the present studies. Finally, the concentrations of β-naphthoflavone, phenobarbital, and rifampin used in these experiments have been shown previously to cause maximal induction of CYP1A2, CYP2B6, and CYP3A4, respectively (LeCluyse et al., 2000; Meunier et al., 2000). It is not known if these concentrations cause maximal induction of other enzymes. Therefore, when comparisons among inducers were made, efficacy of the inducer was compared instead of potency, because the latter requires an understanding of the dose-response curves for all enzymes.

Results

7-Ethoxyresorufin O-Dealkylase (EROD) (CYP1A2) Activity. The effects of treating human hepatocyte cultures with β-naphthoflavone, phenobarbital, isoniazid, or rifampin on microsomal EROD activity are shown in Fig. 1A to D. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 1E) and were within the range of high and low EROD activities determined in a bank of human liver microsomes (Fig. 1F). Treatment with β-naphthoflavone caused a statistically significant increase in EROD activity, ranging from 2.3- to 56-fold (average = 13-fold, n = 28), whereas isoniazid had little or no effect. Treatment with phenobarbital and rifampin caused a 1.9-fold (n = 25) and 2.3-fold (n = 22) increase in CYP1A2 activity, respectively, but these effects were statistically significant only when the β-naphthoflavone treatment group was excluded from the statistical analysis.

Coumarin 7-Hydroxylase (CYP2A6) Activity. The effects of treating human hepatocyte cultures with β-naphthoflavone, phenobarbital, or rifampin on coumarin 7-hydroxylase activity are shown in Fig. 2A to C. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 2D), with the exception of two cases where the samples were below the limit of quantification (see Fig. 2, A and B), and they were within the range of high and low coumarin 7-hydroxylase activities determined in a bank of human liver microsomes (Fig. 2E). Treatment with β-naphthoflavone tended to decrease CYP2A6 activity, whereas treatment with phenobarbital and rifampin tended to increase coumarin 7-hydroxylase activity. However, these effects were not statistically significant, perhaps due to the small sample sizes.

S-Mephentoin N-Demethylase (CYP2B6) Activity. The effects of treating human hepatocyte cultures with β-naphthoflavone, phenobarbital, isoniazid, or rifampin on S-mephentoin N-demethylase activity are shown in Fig. 3A to D. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 3E) and were within the range of high and low S-mephentoin N-demethylase activities determined in a bank of human liver microsomes (Fig. 3F). Treatment with phenobarbital and rifampin caused a statistically significant increase in S-mephentoin N-demethylase activity, whereas treatment with β-naphthoflavone and isoniazid had little or no consistent effect. (Note: β-naphthoflavone tended to increase CYP2B6 activity, but this effect was not statistically significant even when analyzed by a Student’s t test.) The inductive effects of phenobarbital and rifampin on CYP2B6 activity were variable, ranging from 1.3- to 17-fold (average 6.5-fold, n = 13) and 3.0- to 71-fold (average 13-fold, n = 14), respectively.

7-Ethoxy-4-trifluoromethylcoumarin O-Dealkylase (7-EFCD) (CYP2B6 and CYP1A2) Activity. The effects of treating human hepatocyte cultures with β-naphthoflavone, phenobarbital, or rifampin on 7-EFCD activity are shown in Fig. 4A to C. In human liver microsomes, 7-EFCD is catalyzed by both CYP1A2 and CYP2B6 (possibly with a minor contribution from CYP2C enzymes; Madan et al., 2002), which complicates the use of 7-EFCD as a marker of CYP2B6 activity in cultures expressing CYP1A2. However, in cultures treated with phenobarbital or rifampin, where the level of CYP1A2 is low, 7-EFCD is markedly inhibited to control levels by inhibitory antibodies against CYP2B1 (unpublished results). This suggests that, in phenobarbital- and rifampin-treated cultures, the increase in 7-EFCD activity largely reflects the activity of CYP2B6. Reaction rates determined from individual hepatocyte preparations
were averaged (Fig. 4D) and were within the range of high and low 7-EFCOD activities determined in a bank of human liver microsomes (Fig. 4E). The inductive effects of phenobarbital and rifampin on 7-EFCOD activity were variable, ranging from no increase to 7.8-fold (average 3.8-fold, \( n = 16 \)) and from no increase to 14-fold (average 4.1-fold, \( n = 22 \)), respectively. Additionally, 7-EFCOD activity was induced up to 9.9-fold (average 4.6-fold, \( n = 10 \)) by \(-naphthoflavone, an effect that is attributable, however, to its previously discussed induction of CYP1A2.

**CYP2C8 Immunoreactive Protein Levels.** The effects of treating human hepatocyte cultures with phenobarbital or rifampin on CYP2C8 content are shown in Fig. 5A and B. Immunoreactive CYP2C8 protein content determined from individual hepatocyte preparations was averaged (Fig. 5C). The levels of CYP2C8 in hepatocytes treated with vehicle only, phenobarbital or rifampin tended to be low relative to those in microsomes prepared directly from human liver (Fig. 5D). However, treatment with phenobarbital and rifampin caused statistically significant increases in immunoreactive CYP2C8 content relative to that in the vehicle controls. The inductive effects of phenobarbital and rifampin on CYP2C8 content ranged from 3.0- to 5.7-fold (average 4.0-fold, \( n = 4 \)) and 2.7- to 9.9-fold (average 5.2-fold, \( n = 4 \)), respectively.

**Diclofenac 4'-Hydroxylase (CYP2C9) Activity.** The effects of treating human hepatocyte cultures with \(-naphthoflavone, phenobarbital, isoniazid, or rifampin on diclofenac 4'-hydroxylase activity are shown in Fig. 6A to D. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 6E). Hepatocytes treated with phenobarbital and rifampin exceeded the highest activity determined from the bank of human liver microsomes (Fig. 6F). Treatment with phenobarbital and rifampin caused a statistically significant increase in diclofenac 4'-hydroxylase activity, whereas \(-naphthoflavone and isoniazid had little or no effect. (It is notable that, in one of five preparations of human hepatocytes, CYP2C9 was induced 8.9-fold by isoniazid.) The inductive effects of phenobarbital and rifampin on CYP2C9 activity were apparent in all hepatocyte preparations examined, ranging from no change to 2.5-fold (average 1.8-fold, \( n = 14 \)) and 1.3-fold to 10-fold (average 3.5-fold, \( n = 10 \)), respectively.

**S-Mephenytoin 4'-Hydroxylase (CYP2C19) Activity.** The effects of treating human hepatocyte cultures with \(-naphthoflavone, phenobarbital, isoniazid or rifampin on S-mephenytoin 4'-hydroxylation activity are shown in Fig. 7A to D. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 7E) and were within the range of high and low S-mephenytoin 4'-hydroxylation activities determined from a bank of human liver microsomes (Fig. 7F). Treatment with rifampin caused a statistically significant increase in S-mephenytoin 4'-hydroxylation activity, ranging from 4.9- to 190-
Enzymatic rates determined from a bank of human liver microsomes (F). Rates expressed as the mean ± S.D. for each treatment group (E). The average for treated samples were compared with DMSO control. BLQ, below limit of detection. Treatment of human hepatocyte cultures with phenobarbital, isoniazid, and rifampin showed markedly higher CYP2E1 activity than hepatocytes treated with saline (the vehicle used for isoniazid).

**Testosterone 6β-Hydroxylase (CYP3A4) Activity.** The effects of treating human hepatocyte cultures with β-naphthoflavone, phenobarbital, isoniazid, or rifampin on testosterone 6β-hydroxylase activity are shown in Fig. 10A to D. Due to the large sample size (up to 62 samples), data from only 25 samples were plotted in Fig. 10, B and C, however, all data were used for statistical analysis. Reaction rates determined from individual hepatocyte preparations were averaged (Fig. 10E) and were within the range of high and low testosterone 6β-hydroxylase activities determined in a bank of human liver microsomes (Fig. 10F), with the exception of liver number 57 (β-naphthoflavone and phenobarbital groups), which was below the limit of detection. Treatment of human hepatocyte cultures with phenobarbital or rifampin caused statistically significant increases in testosterone 6β-hydroxylase activity. Treatment with β-naphthoflavone and isoniazid tended to cause a slight decrease, but these effects were not statistically significant even when analyzed by Student’s t test. The inductive effects of phenobarbital and rifampin on CYP3A4 activity were variable, ranging from 1.2- to 12-fold (average 3.3-fold, n = 42) and no increase to 145-fold (average 10-fold, n = 61), respectively.

**Discussion.**

Enzyme induction enables some xenobiotics to accelerate their own biotransformation (auto-induction) or the biotransformation and elimination of other drugs. A number of P450 enzymes in human liver microsomes are inducible, including various members of the CYP1A, CYP2A, CYP2B, CYP2C, and CYP3A subfamilies (Kostrubsky et al., 1999; LeCluyse et al., 2000; Robertson et al., 2000; Parkinson, 2001; Silva and Nicoll-Griffith, 2001). Induction of the majority of P450 enzymes analyzed in this study occurs by increase in the rate of gene transcription and involves ligand-activated transcription factors aryl hydrocarbon receptor, constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (Quattrrochi et al., 1994; Savas et al., 1999; Waxman, 1999; Xie and Evans, 2001). In general, binding of the appropriate ligand to the receptor initiates the induction process that cascades through a dimerization of the receptors, their translocation to the nucleus and binding to specific regions in the promoters of P450s.

The potential for NMEs to cause P450 enzyme induction is generally evaluated in primary cultures of human hepatocytes. To control interindividual differences, each preparation of human hepatocytes must be treated with known prototypical P450 enzyme inducers so that the response to the NMEs can be benchmarked against a known positive response. β-Naphthoflavone (and omeprazole) and rifampin (and phenobarbital) are often used as positive control inducers for CYP1A2 and CYP3A4, respectively. Although, there are plenty of data documenting the expected response to these prototypical inducers in human hepatocytes, there is only limited information on their effects on other P450 enzymes. This study summarizes data obtained...
from 62 preparations of human hepatocytes that were treated with vehicle (0.1% DMSO or saline), β-naphthoflavone, phenobarbital, isoniazid and/or rifampin, and their effects on the activity or content of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4.

All mammalian species apparently possess two inducible CYP1A enzymes, namely CYP1A1 and CYP1A2, although human liver contains only CYP1A2. In addition to cigarette smoke, inducers of the CYP1A enzymes include charcoal-broiled meat (a source of polycyclic aromatic hydrocarbons), cruciferous vegetables (a source of various indoles), and omeprazole (Parkinson, 2001). Polycyclic and polyhalogenated aromatic hydrocarbons appear to induce CYP1A enzymes in all mammalian species. In contrast, omeprazole is an effective inducer of CYP1A enzymes in humans but not in mice, rats or rabbits (Diaz et al., 1990; Daujat et al., 1992; Shih et al., 1999). As expected, treatment of cultured hepatocytes with β-naphthoflavone caused, on average, a 13-fold induction of CYP1A2 activity (Fig. 1). These results confirm previous observations that β-naphthoflavone induces CYP1A2 in cultured human hepatocytes (Maurice et al., 1992; Silva et al., 1999; LeCluyse et al., 2000; Sahi et al., 2000).

Phenobarbital and rifampin previously have been reported to have no effect on CYP1A2 (Mates and Li, 1997; Meunier et al., 2000; Runge et al., 2000). Our data suggest that both compounds may have the ability to weakly induce CYP1A2 (as measured by EROD activity); although this induction was significant only when β-naphthoflavone was removed from the statistical analysis. Similar modest induction of CYP1A2 by rifampin was also observed in a recent study in human hepatocytes (Rae et al., 2001). It is possible that other enzymes that are induced by phenobarbital or rifampin have the ability to catalyze EROD. This might explain the increase in EROD activity in hepatocyte cultures treated with phenobarbital or rifampin. However, preliminary Western immunoblotting data suggests that phenobarbital and rifampin does increase the immunoreactive CYP1A2 protein levels in some human hepatocytes cultures (data not shown). Additionally, these results are consistent with clinical reports that theophylline and caffeine clearances are increased, and their plasma half-lives decreased, in patients treated with phenobarbital or rifampin (Landay et al., 1978; Wietholtz et al., 1995). The degree to which variability in the inducibility of CYP1A2 in vitro reflects variability in vivo is not known, but there is clinical evidence to suggest omeprazole increases the CYP1A2-dependent N-demethylation of caffeine to paraxanthine in most, but not all, subjects (Rost et al., 1994). The range of the absolute CYP1A2 activities (in absence or presence of an inducer) was comparable with the range of P450 activities from microsomes prepared directly from human livers. This suggests, but does not prove, that the variation in CYP1A2 activity in

Figure 4. Effect of treating primary cultures of human hepatocytes with prototypical cytochrome P450 enzyme inducers on the O-dealkylation of 7-ethoxy-4-trifluoromethylcoumarin (CYP1A2 and CYP2B6 activity).
vivo is largely determined by environmental factors. In other words, individuals with high CYP1A2 activity might be those exposed to the enzyme-inducing effects of chemicals in the environment and food. 

β-Naphthoflavone tended to suppress CYP2A6 expression, whereas phenobarbital and rifampin tended to increase CYP2A6 activity (Fig. 2), however, these effects were not statistically significant (perhaps due to the small sample size). These findings are consistent with a study in Cynomolgus monkey, in which CYP2A6 (coumarin 7-hydroxylase) was induced by phenobarbital, but suppressed by β-naphthoflavone (Bullock et al., 1995). This observation of induction by rifampin supported by studies in which rifampin was found to induce CYP2A6 (Mates and Li, 1997; Picard-Garcia et al., 2000; Rae et al., 2001).

Recent studies have suggested that, like the corresponding rat enzyme, CYP2B6 is an inducible enzyme in humans, and is present in most, possibly all, samples of human liver microsomes (Gervot et al., 1999; Hanna et al., 2000; LeCluyse et al., 2000; Sahi et al., 2000; Madan et al., 2001). Treatment of human hepatocytes with phenobarbital and rifampin, but not β-naphthoflavone, caused a marked increase in CYP2B6 activity based on S-mephenytoin N-demethylase and 7-EFCOD activities (Figs. 4 and 5). The induction of CYP2B6 by rifampin and phenobarbital was also accompanied by an increase in 7-EFCOD (Fig. 4). This activity was also increased by β-naphthoflavone because CYP1A2, like CYP2B6, catalyzes 7-EFCOD (Madan et al., 2001). The fold-induction of CYP2B6 by phenobarbital and rifampin was highly variable and ranged from no increase to a 14-fold increase. Although phenobarbital and rifampin have been in clinical use for decades, little information exists on their effects on CYP2B6 activity in vivo because of the unavailability of selective in vivo probes for CYP2B6 activity. However, induction of CYP2B6 by phenobarbital in vivo has been inferred from the inductive effect of phenobarbital on the metabolism and activation of cyclophosphamide and ifosfamide (Chang et al., 1997; Granvil et al., 1999). The absolute CYP2B6 activities (in absence or presence of an inducer) was at least a factor of 10 lower than those observed in microsomes prepared

![Fig. 5. Effect of treating primary cultures of human hepatocytes with prototypical cytochrome P450 enzyme inducers on CYP2C8 content.](image-url)

Human hepatocytes from several donors (liver number) were placed in culture for 3 days before the initiation of treatment with phenobarbital (250 μM) (A) or rifampin (50 μM) (B), with renewal of the medium and inducer every 24 h. The final concentration of the solvent (DMSO) in the medium was 0.1%. Microsomes were prepared for each hepatocyte culture and used to determine CYP2C8 content by Western immunoblotting. Average CYP2C8 expressed as the mean ± S.D. for each treatment group (C). The average for DMSO group is from all the hepatocyte preparations studied. Low and high CYP2C8 immunoreactive protein levels determined from a bank of human liver microsomes (D). PB, phenobarbital; RIF, rifampin.

![Fig. 6. Effect of treating primary cultures of human hepatocytes with prototypical cytochrome P450 enzyme inducers on the 4'-hydroxylation of diclofenac (CYP2C9 activity).](image-url)

Human hepatocytes from several donors (liver number) were placed in culture for 3 days before the initiation of treatment with β-naphthoflavone (33 μM) (A), phenobarbital (250 μM) (B), rifampin (50 μM) (C), isoniazid (100 μM) (D), with renewal of the medium and inducer every 24 h. All inducers were dissolved in DMSO (final concentration 0.1%) except isoniazid, which was dissolved in saline. Microsomes were prepared for each hepatocyte culture and used to determine the rate of diclofenac 4'-hydroxylation. Average diclofenac 4'-hydroxylation rates expressed as the mean ± S.D. for each treatment group (E). The average for DMSO group is from all the hepatocyte preparations studied. Low and high enzymatic rates determined from a bank of human liver microsomes (F). *p < 0.05 in the comparison of treated samples with DMSO control. BNF, β-naphthoflavone; PB, phenobarbital; INH, isoniazid; RIF, rifampin.
directly from human livers, which suggests that either an optimal induction of CYP2B6 was not achieved in culture or that environmental factors alone may not explain the wide variation in CYP2B6 activity in vivo.

Induction of CYP2C enzymes by phenobarbital and rifampin has been implicated in a variety of drug interactions (Abdel-Rahman and Leeder, 2000; Jang and Maurel, 2000). Such induction has been corroborated by in vitro studies in human hepatocytes in which phenobarbital and rifampin have been reported to induce CYP2C8, CYP2C9, and CYP2C19 (Morel et al., 1990; Chang et al., 1997; Gerbal-Chaloin et al., 2001). However, two other studies have reported that rifampin does not induce CYP2C9 or CYP2C19 (Li et al., 1997; Runge et al., 2000).

In the present study, all three CYP2C activities or content (namely, CYP2C8, CYP2C9, and CYP2C19) were induced by phenobarbital and rifampin, although not by β-naphthoflavone or isoniazid. Rifampin and phenobarbital were similarly effective in inducing CYP2C8, but rifampin was more effective in inducing CYP2C9 and especially CYP2C19. (The levels of immunoreactive CYP2C8 in cultures treated with rifampin and phenobarbital did not exceed those seen in human liver microsomes with low CYP2C8 (paclitaxel 6-hydroxylase) activity. Therefore, it is possible that these inducers are simply returning the already suppressed CYP2C8 to basal levels. However, a recent study by Raucy et al. (2002) showed increases in CYP2C8 mRNA in human hepatocyte cultures after treatment with phenobarbital and rifampin, suggesting that the induction of CYP2C8 is not an experimental artifact.) The extent of induction of CYP2C19 activity by rifampin was an average of 37-fold over that in the solvent control. It should be noted, however, that both CYP2C9 and CYP2C19 are polymorphically expressed enzymes (Ingelman-Sund-
Human hepatocytes from several donors (liver number) were placed in culture for 3 days before the initiation of treatment with β-naphthoflavone (33 μM) (A), phenobarbital (250 μM) (B), rifampin (20 or 50 μM) (C), isoniazid (100 μM) (D), with renewal of the medium and inducer every 24 h. All inducers were dissolved in DMSO (final concentration 0.1%) except isoniazid, which was dissolved in saline. Microsomes were prepared for each hepatocyte culture and used to determine the rate of chlorozoxazone 6-hydroxylation. Average chlorozoxazone 6-hydroxylation rates expressed as the mean ± S.D. for each treatment group (E). The average for DMSO group are from all the hepatocyte preparations studied. Low and high enzymatic rates determined from a bank of human liver microsomes (F).

**Fig. 9.** Effect of treating primary cultures of human hepatocytes with prototypical cytochrome P450 enzyme inducers on the 6-hydroxylation of chlorzoxazone (CYP2E1 activity).

In humans, CYP3A4 is inducible by numerous drugs, such as ethanol and isoniazid (Hayashi et al., 1991; Inoue et al., 2000). CYP2E1 is regulated by transcriptional activation, mRNA stabilization, increased mRNA translation, and decreased protein degradation (Koop and Tierney, 1990). CYP2E1 is induced by many xenobiotics (e.g., ethanol and isoniazid), as well as starvation and diabetes (Koop and Tierney, 1990). As expected, in human hepatocyte cultures, CYP2E1 was modestly induced by isoniazid, and by phenobarbital and rifampin. It is notable that DMSO (the vehicle used for treatment of hepatocytes with NMEs) also markedly increased the levels of CYP2E1, which is consistent with the effects of organic solvents (including DMSO) on rat CYP2E1 (Koop and Tierney, 1990).

In humans, CYP3A4 is inducible by numerous drugs, such as

with the large number of CYP2D6 allelic variants that have been identified to date (Ingelman-Sundberg et al., 1999).

Although CYP2E1 is polymorphically expressed, the majority of the CYP2E1 polymorphisms have been shown to occur in introns and seem not to be associated with functional consequences with respect to protein expression or enzyme activity (Hayashi et al., 1991; Inoue et al., 2000). CYP2E1 is regulated by transcriptional activation, mRNA stabilization, increased mRNA translation, and decreased protein degradation (Koop and Tierney, 1990). CYP2E1 is induced by many xenobiotics (e.g., ethanol and isoniazid), as well as starvation and diabetes (Koop and Tierney, 1990). As expected, in human hepatocyte cultures, CYP2E1 was modestly induced by isoniazid, and by phenobarbital and rifampin. It is notable that DMSO (the vehicle used for treatment of hepatocytes with NMEs) also markedly increased the levels of CYP2E1, which is consistent with the effects of organic solvents (including DMSO) on rat CYP2E1 (Koop and Tierney, 1990).

In humans, CYP3A4 is inducible by numerous drugs, such as
rifampin, phenobarbital, troglitazone, and phenytoin (Pichard et al., 1990; Meunier et al., 2000; Runge et al., 2000; Sahi et al., 2000), but CYP3A5 does not appear to be induced by similar drugs (Schuetz et al., 1993). The results of the present study are consistent with a vast body of literature from both in vitro and in vivo studies that shows that phenobarbital and rifampin are effective inducers of CYP3A4, whereas β-naphthoflavone is not. The data presented herein show that phenobarbital and rifampin are, on average, equally effective as inducers at the concentrations studied, however, there were hepatocyte preparations where phenobarbital was a better inducer of CYP3A4 than rifampin and vice versa. Curiously, high levels of DMSO, the vehicle used to dissolve rifampin and other drugs that are evaluated as inducers of CYP3A4 in vitro, increases CYP3A4 to levels that can reach as high as those achieved with rifampin (LeCluyse et al., 2000), a phenomenon that has also been observed in rat hepatocytes (Zangar and Novak, 1998). It would appear that 0.1% DMSO (the concentration used in the present evaluations) has minimal effect on CYP3A4 expression. It is therefore advisable to keep the concentration of DMSO in the culture medium as low as possible.

The increase in EROD activity, which is an activity marker for liver CYP1A2, a member of CYP1A, occurred in response to treatment of the hepatocyte cultures with β-naphthoflavone and is consistent with localization of xenobiotic responsive element in the 5’ region of human CYP1A2 gene (Quattrochi et al., 1994). Both rifampin and phenobarbital induced members of CYP2 and CYP3 families, with expected exception of CYP2D6 and CYP2E1, in human hepatocytes. This effect is consistent with identification of CAR and PXR-responsive elements in the sequences of these genes and also with the cross talk between the two pathways (Xie et al., 2000; Smiris et al., 2001). The weak response of CYP2A6 to rifampin or phenobarbital, unexpected in light of report by Meunier et al. (2000), can be attributed to different composition of media used for culturing the hepatocytes, particularly effects of dexamethasone on PXR-mediated enzyme induction (Meunier et al., 2000; Pascussi et al., 2001). CYP2E1, which appeared to be induced by isoniazid, rifampin, and phenobarbital, may be regulated by post-transcriptional changes in translational efficiency, messenger RNA stability, and ligand stabilization, although these processes have been studied predominantly in the rat (Song et al., 1986; Chien et al., 1997; Kocarek et al., 2000). Neither CAR nor PXR responsive elements have been identified in the promoter of CYP2E1.

Understanding of the mechanism of induction of P450 enzymes has led to development of novel in vitro assays predictive of binding of drugs to nuclear receptors and induction of human P450s via the aryl hydrocarbon receptor (Allen et al., 2001) and the PXR (El-Sankary et al., 2001; Raucy et al., 2002; Schuetz et al., 2002). Recently reporter-gene assay technology designed to detect binding ligands to PXR has been coupled with optical imaging for noninvasive in vivo evaluation of CYP3A4 induction (Schuetz et al., 2002).

Inasmuch as the effect of β-naphthoflavone, phenobarbital, and rifampin were studied in a large number of hepatocyte preparations (n = 62), it is possible to make some generalizations regarding the usefulness and limitations of cultured hepatocytes in evaluating drugs as inducers of P450 enzymes. It is apparent that there is enormous variability in the induction of P450 enzymes. However, there is enormous variability in the control activities also, that is, the activity present after 5–6 days of culture. It is well established that placing rat hepatocytes in culture results in loss of P450 enzyme activity, although the factors affecting this loss are poorly understood (Silva et al., 1998). Unlike rat hepatocyte cultures, where almost a complete loss of certain P450 activities is seen (Kocarek et al., 1993; Madan et al., 1999), human hepatocytes tend to retain considerable (albeit low) enzyme activity in culture. The variability in the remaining activity of control cultures can complicate interpretation of the data. For example, if the control enzyme levels decline by 80% when hepatocytes are placed in culture, such that the enzyme activity remaining at the end of the in vitro experiment are one-fifth of the level originally present in vivo, then a 5-fold induction simply restores the enzyme level to that originally present in vivo. This brings into question whether the in vitro system is responding to the enzyme inducer in vitro as it does in vivo. If, however, the enzyme levels increase 5-fold above normal levels, then the apparent induction observed in vitro is 25-fold because the constitutive activity in culture was reduced by 80%. Additionally, as the control activity becomes lower, the accuracy of the analytical techniques used is often compromised, which can result in an erroneous fold-induction calculation. It is for this reason that one must compare the level of induction observed with an NME to that observed with a prototypical inducer (positive controls) in the same preparation of hepatocytes. In many respects, lowering of the control activity is advantageous because it increases the sensitivity (or the dynamic range) for evaluation of new compounds that may not be as effective as the positive controls, and hence would cause only a marginal induction. Such induction may be detected in vitro, but may go unnoticed in vivo, especially when one considers the large interindividual variability in P450 enzyme expression.

In addition, the magnitude of P450 induction obtained after treatment with the prototypical inducers was highly variable from one hepatocyte preparation to the next. Hence, if an NME were evaluated as an inducer of CYP3A4, for example, in a preparation of hepatocytes that showed a weak inductive response to rifampin, it would be difficult to ascertain whether the NME was an inducer or not, especially if the NME is not effective as rifampin. It is therefore recommended that NMEs be evaluated in multiple preparations of human hepatocytes, preferably n = 5, to minimize the effects of intersample variability of the conclusions from the study.

When cultured under appropriate conditions, human hepatocytes appear to respond to P450 enzyme inducers in a manner that is consistent with the clinical effects of these inducers. The response of hepatocytes is specific (i.e., not all enzymes are induced by a given inducer) and the magnitude of response is generally consistent with the cross-variability observed in vivo. Therefore, cultured human hepatocytes are a reliable system to evaluate NMEs as inducers of P450 enzymes, provided the data are interpreted with the limitations of the system in mind.

In conclusion, we generalize that β-naphthoflavone induces CYP1A2 and isoniazid induces CYP2E1, whereas rifampin and, to a lesser extent phenobarbital, tend to significantly and consistently induce enzymes of the CYP2A, CYP2B, CYP2C, CYP2E, and CYP3A subfamilies but not the 2D subfamily.

Acknowledgments. The authors are grateful to the National Disease Research Interchange (Philadelphia, PA), the Midwest Transplant Network (Westwood, KS), and the Anatomical Gift Foundation (Woodbine, GA) for providing the human livers for this study. We also thank Dr. Joel S. Owen for providing scientific input toward the experimental design, and analysis of the data. Additionally, the assistance of Ms. Cory Johnson with the statistical analysis of the data is gratefully acknowledged.

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