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Upregulation of cytochromes *P*450 2B in rat liver by orphenadrine

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1 The alkylamine drug orphenadrine (ORPH) is an inducer and inhibitor of the microsomal cytochrome *P*450 (CYP) system in mammals. This study evaluated the selectivity of CYP induction by ORPH in rat liver.

2 Immunoblot analysis indicated that ORPH was a selective inducer of the phenobarbitone (PB)-inducible CYP2B in rat liver. CYP2B protein was increased to ~14-fold of levels in untreated rat liver. By comparison PB increased CYP2B expression 40-fold. Corresponding increases in the activity of CYP2B-dependent androstenedione 16β-hydroxylation were measured in microsomes from ORPH and PB-induced rats.

3 Northern analysis indicated that CYP2B1/2 mRNA was increased in ORPH-induced rat liver. Consistent with this finding, ORPH was found to activate a PB-responsive enhancer module in constitutive androstane receptor (CAR)-transfected Hep G2 cells.

4 Other alkylamines like troleandomycin impair CYP turnover. We tested whether ORPH induction of CYP2B may include a post-translational component. In PB-pretreated animals ORPH administration delayed the loss of CYP2B after PB withdrawal, but no evidence for altered turnover was found.

5 These studies establish ORPH as a selective inducer of CYP2B in rat liver. Induction appears to be mediated pretranslationally by CAR activation of CYP2B gene transcription. Post-translational stabilisation by an ORPH metabolite does not elicit induction. Induction of CYP2B may influence pharmacokinetic interactions involving ORPH.
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Keywords: Cytochrome *P*450; orphenadrine; phenobarbitone; enzyme induction; constitutive androstane receptor; protein turnover; drug interactions

Abbreviations: AD, androstenedione; BNF, β-naphthoflavone; CAR, constitutive androstane receptor; C/EBP-α, CCAATT-enhancer binding protein-α; CYP, cytochrome *P*450; DEX, dexamethasone; GH, growth hormone; IgG, immunoglobulin G; MI, metabolite intermediate; NR, nuclear receptor; ORPH, orphenadrine; PB, phenobarbitone; PBREM, phenobarbitone responsive enhancer module; RXR, retinoid X (9-*cis*-retinoic acid) receptor; TCPOBOP, 1,4-*bis*[2-(3,5-dischloropyridyloxy)]benzene; TLC, thin layer chromatography

Introduction

The anticholinergic agent orphenadrine (ORPH; Figure 1) inhibits its own elimination when administered by multiple dose regimen (Labout *et al*., 1982) and prolongs the half-life of coadministered haloperidol (Altamura *et al*., 1986). ORPH is a relatively nonpotent inhibitor of the cytochrome *P*450 (CYP) monooxygenase system that mediates drug biotransformation and undergoes CYP-dependent biotransformation within the alkylamine side chain to generate a potent inhibitory metabolite (Bast & Noordhoek, 1982). The ORPH metabolite binds tightly to the CYP haem, thus generating a metabolite intermediate (MI)-complex similar to those produced by other alkylamines, such as the macrolide antibiotics (Murray & Reidy, 1990; Franklin, 1991). The structurally similar diphenhydramine, a widely used antihistamine, is an inhibitor of human CYP2D6 and precipitates clinically significant pharmacokinetic drug interactions (Hamelin *et al*., 2000). It is recognised that there is a deficiency of information on pharmacokinetic drug interactions elicited by anticholinergic drugs (Brocks, 1999).

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Agents that generate MI-complexes have also been found to induce or upregulate the expression of CYP proteins when administered to mammals *in vivo*. Thus, isosafrole and variously substituted methylenedioxyphenyl compounds, and alkylamine derivatives like ORPH and troleandomycin, are inducers as well as inhibitors of CYPs (Elcombe *et al*., 1975; Ryan *et al*., 1980; Pershing & Franklin, 1982; Murray *et al*., 1983; Reidy *et al*., 1989). Treatment of patients with ORPH decreased plasma concentrations of chlorpromazine and the half-life of antipyrine; these findings are consistent with induction of human CYP enzymes by ORPH (Loga *et al*., 1975). Interestingly, the particular CYP enzymes that are subject to MI-complexation are also often those that are inducible by the same chemicals and drugs (Murray *et al*., 1986; Voorman & Aust, 1988). There is evidence that the MI-complex-forming agents troleandomycin and isosafrole stabilise CYP expression in liver at a post-translational level (Watkins *et al*., 1986; Voorman & Aust, 1988).

The present study addressed the selectivity and mechanism of induction of CYP by ORPH. The principal finding to emerge was that ORPH appeared to be a selective inducer of CYP2B in rat liver and that induction was exerted at a

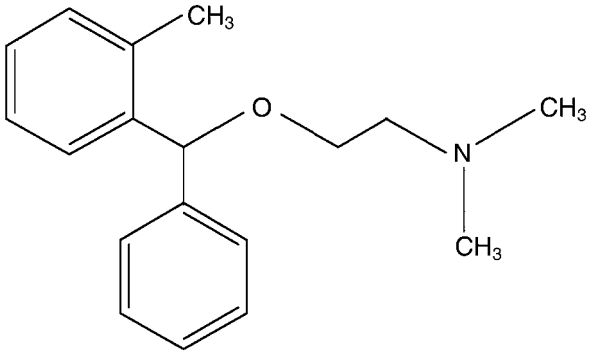

Figure 1 Structure of orphenadrine (ORPH).

pretranslational level. In transient transfection studies, ORPH was found to activate the constitutive androstane receptor (CAR), a nuclear receptor that mediates the induction of phenobarbitone (PB)-responsive genes. In further studies that were undertaken in PB-pretreated rats, the decline in microsomal CYP2B immunoreactive protein content after removal of PB occurred more slowly in rats that were also administered ORPH, but this effect was not due to an impairment in CYP turnover. Thus, the present study establishes that ORPH is a selective PB-like inducer that acts principally at the level of CYP2B gene transcription. Pharmacokinetic interactions with ORPH may be attributed to both increased expression of hepatic CYPs 2B that participate in oxidative drug metabolism and inhibition of the same enzymes.

Methods

Chemicals

Orphenadrine and biochemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Restriction enzymes were from Boehringer Mannheim (Castle Hill, NSW, Australia). [α-^{32}P] dCTP (3000 Ci mol^{-1}), [4-^{14}C] androst-4-ene-3,17-dione (AD; specific activity 57–59 mCi mmol^{-1}), Hybond N+, Hyperfilm-MP, ACS-II scintillation fluid and reagents for enhanced chemiluminescence were purchased from Amersham Pharmacia Biotech Australia (Castle Hill, NSW, Australia). AD metabolite standards were obtained from Steraloids (Wilton, NH, U.S.A.), Sigma Chemical Co. (St Louis, MO, U.S.A.) or the MRC Steroid Reference Collection (Queen Mary's College, London, U.K.). Protran nitrocellulose membranes (0.2 μm) for Western transfer were purchased from Medos (Lidcombe, NSW, Australia). Reagents used in gel electrophoresis were obtained from Bio-Rad (Richmond, CA, U.S.A.). HPLC-grade solvents were obtained from Rhone-Poulenc Chemicals (Baulkham Hills, Australia) and analytical-grade reagents were from Ajax Chemicals (Sydney, Australia). Merck silica gel 60 thin-layer chromatography (TLC) plates with 254 nm fluorescent indicator were purchased from Alltech (Sydney, NSW, Australia).

Materials for cell culture and molecular biology were purchased from Gibco-BRL (Melbourne, VIC, Australia) and Promega (Annandale, NSW, Australia), respectively. The β-galactosidase plasmid, reagents for luciferase assay and pGL-3 basic vector were from Promega.

Animal treatments

Studies were performed in accordance with the guidelines of the Australian National Health and Medical Research Council and were approved by the Institutional Animal Care and Ethics Committee. Male Wistar rats (ca. 200 g) were held under conditions of constant temperature and lighting (12 h light – dark cycle) and had free access to food and water. Animals received ORPH at a dose of 60 mg kg^{-1} daily in sterile water by i.p. administration on three consecutive days. In the induction profile study, other animals received CYP inducers by i.p. injection over 3 days according to established protocols (Martini & Murray, 1993): phenobarbitone (PB; 100 mg kg^{-1} in saline), dexamethasone (DEX; 100 mg kg^{-1} in corn oil), β-naphthoflavone (BNF; 40 mg kg^{-1} in corn oil) and dimethylsulphoxide (2 ml kg^{-1} twice daily); preliminary experiments confirmed that corn oil and saline did not influence CYP activity or expression in rat liver. Animals were killed 24 h after the final dose of chemical, and livers were removed and perfused with ice-cold saline. In the time course study one, two or three injections of ORPH (60 mg kg^{-1} i.p.) were administered and animals were killed at intervals. In the turnover study, rats received three injections of PB on consecutive days and then a single injection of $NaH^{14}CO_3$ (0.5 mCi) and either ORPH (60 mg kg^{-1}) or no treatment. Animals were killed at 24 h intervals after the final treatment.

Segments of liver tissue were frozen in liquid nitrogen and the remainder was used in the preparation of hepatic microsomes by differential ultracentrifugation. The microsomal pellet was resuspended in homogenisation buffer and treated with potassium ferricyanide (100 μM) to dissociate the ORPH MI-complex. After a 30 min incubation on ice, the suspensions were centrifuged at 105,000 $\times$ g. The microsomal pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, which contained 20% glycerol and 1 mM EDTA and frozen in liquid nitrogen for storage at -70° until required in experiments (Murray *et al*., 1998).

Microsomal steroid hydroxylation

Microsomal AD hydroxylation activities were determined by methods described previously (Martini & Murray, 1993). The [^{14}C]-labelled substrate (50 μM, 0.18 μCi) was incubated with 0.15 mg microsomal protein (Lowry *et al*., 1951) and NADPH (1 mM) at 37°C (0.1 M phosphate buffer, pH 7.4; 0.4 ml). Chloroform (5 ml) was added after 2.5 min, tubes were transferred to ice and then shaken for 10 min. After centrifugation, the organic phase was removed under a stream of N_2. The residue was applied to TLC plates (Merck silica gel 60 F_{254} type; Darmstadt, Germany) in a small volume of chloroform and run twice in chloroform : ethyl acetate (1 : 2) with air drying between (Waxman *et al*., 1983). Autoradiography (Hyperfilm-MP) was used to locate radioactive metabolites. Metabolites were quantified by scintillation spectrometry (ACS II; Amersham). The identity and homogeneity of the products of microsomal AD metabolism was confirmed. Radioactive regions corresponding to individual metabolites were eluted from the silica gel with methanol. Eluates were applied to new TLC plates along with the authentic unlabelled steroids and the plates were resolved in chloroform : ethyl acetate (1 : 2). Unlabelled steroids were visualised under UV light and radioactive products by

autoradiography. Identity was confirmed by HPLC using the methods described by Waxman *et al.* (1983). Intra-assay variation was estimated at 3% after analysis of three samples in triplicate. Interassay variation was 8% when microsomal fractions ($n=3$) were analysed on three consecutive days. The linearity of product formation with protein concentration and time was confirmed for each metabolite; substrate conversion was $15\pm3\%$ in microsomes from control rat liver.

Immunoquantitation of CYPs in rat liver microsomes

The rabbit anti-rat CYP2C11 immunoglobulin G (IgG), anti-rat CYP3A and anti-rat NADPH-CYP-reductase IgGs used in this study have been described previously (Murray *et al.*, 1992; 2001). The rabbit anti-rat CYP2B1 IgG was cross-adsorbed against homogeneous CYP2B1 that was coupled to CNBr-activated Sepharose 4B and the monospecific IgG was eluted from the column with ammonium thiocyanate and was dialysed against saline before use. The rabbit anti-human CYP2E1 IgG, which also detects the rat enzyme, was generously provided by Professor M Ingelman-Sundberg (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden).

Microsomal fractions were heated for 5 min at 100°C with 2% sodium dodecyl sulphate and 5% β-mercaptoethanol and loaded onto vertical polyacrylamide gels (7.5%; 20 lanes per gel). For CYP2B quantitation, 30 μg of microsomal protein was loaded (or 1 μg from ORPH- and PB-induced fractions), for CYP3A 5 μg of microsomal protein (or 2 μg from DEX-induced fractions) was loaded onto each lane and for CYP2C11, CYP2E1 and NADPH-CYP-reductase 5 μg protein from differently induced microsomal fractions was loaded onto gels. In all cases, these quantities elicited responses that were in the linear range. Electrophoresis was conducted overnight at $\sim$15 mA per gel, followed by transfer to nitrocellulose sheets (1.5 h at 70 V; Protran; Schleicher and Schuell, Dassel, Germany). Nitrocellulose sheets were then incubated with the anti-CYP IgG fractions (3.7 μg ml^{-1}, except in the case of the cross-adsorbed anti-CYP2B IgG, which was used at a protein concentration of 0.08 μg ml^{-1}) for 120 min. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL RPN 2106 kit, Amersham Pharmacia Biotech, Australia) and the resultant signals were analysed by densitometry (Bio-Rad GS-700, Richmond, CA, U.S.A.).

Hepatic RNA extraction and analysis of CYP mRNAs

Total RNA was extracted from male rat liver by the guanidinium thiocyanate/CsCl method and quantified spectrophotometrically (Sambrook *et al.*, 1989). For Northern analysis, RNA (10 μg lane^{-1}) was electrophoresed on 1% agarose in the presence of 2.2 M formaldehyde and then transferred to Hybond-N$^+$ nylon filters (0.45 μm, Amersham). Hybridisation and washing conditions were as described previously (Jiang *et al.*, 1994), and signals corresponding to CYP mRNAs were quantified by densitometry after autoradiography on Hyperfim MP (Amersham Pharmacia Biotech, Australia). To demonstrate equivalence of RNA loading between samples, filters were stripped and rehybridised to an 18S ribosomal RNA [α-^{32}P]-labelled oligonucleotide probe.

The CYP2B1 cDNA was a gift from Dr FJ Gonzalez, National Institutes of Health, Bethesda, MD, U.S.A. and the 18S RNA oligonucleotide [5′-CGG-CAT-GTA-TTA-GCT-CTA-GAA-TTA-CCA-CAG-3′] corresponding to the complement of the coding region of the gene from nucleotides 151–180 (Chan *et al.*, 1984) was purchased from Bresatec (Adelaide, South Australia). The cDNA was labelled using [α-^{32}P] dCTP and Klenow enzyme, while the oligonucleotide was labelled using [α-^{32}P] dCTP and deoxynucleotidyl transferase.

Plasmids and cell transfections

Plasmids encoding the mouse CAR and PB-responsive enhancer module (PBREM) from the mouse *cyp2B10* gene were from Dr M Negishi, NIEHS, NC, U.S.A. HepG2 human hepatoma-derived cells (American Tissue Culture Collection, MD, U.S.A.) were incubated at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin–streptomycin antibiotic mix, 26 mM sodium bicarbonate, 20 mM HEPES and additional 1% glutamine until $\sim$80% confluent, which was found in preliminary experiments to be optimal for transfection. Cells were passaged twice weekly and used at passage 4 in all experiments. Cells were seeded at a density of 6×10^5 cells per well in six-well plates 24 h prior to transfection.

Transfections were carried out using the Effectene transfection reagent at a ratio of 10 μl Effectene μg total DNA.$^{-1}$ Briefly, cells were cotransfected with 0.6 μg of plasmids as appropriate and 0.3 μg of pCMV-β-galactosidase expression plasmid as a control for transfection efficiency. Total DNA in each transfection (2 μg) was equalised with salmon sperm DNA. The transfection mixture was prepared essentially according to the manufacturer's instructions (Qiagen) and cells were treated for 24 h. Cells were treated for a further 2 h with 3α-androstanol (8 μM) to repress CAR activity and, either 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP; 250 nM), ORPH (0.1 mM) or no further treatment before harvest. Duplicate transfections were performed in at least three separate experiments.

Assays of luciferase and β*-galactosidase activities*

Luciferase reporter gene activity was measured by established protocols (Steady-Glo assay system, Promega). HepG2 cell suspensions (100 μl) were added to the reconstituted Steady-Glo reagent (100 μl) and the luciferase activity was determined after 60 s in a β-scintillation counter. For the measurement of β-galactosidase activity, cell suspensions (300 μl) were added to reporter lysis buffer and incubated for 15 min, vortexed for 15 s and centrifuged at $2000\times g$ and 4°C for 4 min. Supernatants (150 μl) were mixed with assay buffer (270 μl of 0.1 M sodium phosphate, pH 7.3, containing 1 mM $MgCl_2$, 70 mM β-mercaptoethanol and 3.2 mM 2-nitrophenyl β-D-galactopyranoside) and incubated at 37°C for 2 h. After addition of 1 M sodium carbonate (500 μl), the absorbance at 420 nm was measured. Luciferase activity was normalised to β-galactosidase activity to correct for differences in transfection efficiency.

Statistics

Data are presented throughout as mean$\pm$s.e. All measurements were made in hepatic fractions from individual rats or in

individual transfection assays. Differences between mean values from control and treatment groups were detected using the Student's *t*-test (two group comparisons) or one-way analysis of variance in conjunction with Dunnett's *q'*-test or Fisher's PLSD test (comparison of several treatments with the control group).

Results

Profile of CYP induction elicited in rat liver by ORPH

To evaluate the impact of ORPH on CYP function, male rats were administered the drug according to standard induction regimen (daily dosage over a 3-day period; animals were killed 24 h after the third injection). Preliminary dose–response experiments indicated that CYP2B expression and function was induced by ORPH and that 60 mg kg^{-1} i.p. in saline over 3 days was optimal. At this dosage, ORPH increased AD 16β-hydroxylation activity to about four-fold over that detected in control liver (2.45 ± 0.59 *versus* 0.59 ± 0.04 nmol (mg protein min)$^{-1}$; $P<0.01$, Table 1), but ORPH did not elicit detectable changes in other pathways of microsomal AD hydroxylation. By comparison, other typical CYP inducers exerted different effects on CYP-mediated AD hydroxylation. Thus, PB increased AD 16β-hydroxylation to about 20-fold of control ($P<0.01$), and DEX and PB administration increased AD 6β-hydroxylation, respectively, to about six- and four-fold of control ($P<0.01$). In contrast, dimethylsulphoxide and BNF, which are established inducers of 2E and 1A subfamily CYPs, did not alter AD hydroxylation activities significantly (Table 1).

None of these treatments exerted any discernible effects on the activity of the 3-ketosteroid-Δ^4-oxidoreductase enzyme that catalyses the reduction of the 4,5-double bond of AD and generates 5α-dihydroAD (Table 1). In contrast, DEX and ORPH decreased the activity of the 17β-oxidoreductase that generates testosterone from AD in the microsomal reaction to 58 and 67% of control, respectively ($P<0.01$; Table 1).

Since ORPH appeared to increase selectively CYP2B-mediated AD 16β-hydroxylation, further studies explored these findings in immunoquantitation experiments. CYP2B immunoreactive protein was increased by both ORPH and PB administration over the 3-day treatment regimen to 14- and 40-fold of control, respectively (Figures 2 and 3). Consistent with the findings from AD hydroxylation measurements, neither CYP2C11 nor total CYP3A immunoreactive protein expression was altered in rat liver by ORPH treatment (Figures 2 and 3). In contrast, DEX and BNF decreased CYP2C11 slightly and DEX and PB increased CYP3A-immunoreactive protein expression to about 4.6- and two-fold of control (Figure 3). Unlike dimethylsulphoxide, ORPH administration had no effect on the expression of CYP2E1 in liver, which is consistent with the lack of effect of ORPH on aniline 4-hydroxylation (not shown). NADPH-CYP-reductase was increased by only two treatments, DEX and PB, to about 1.5- and 1.6-fold of control ($P<0.01$; Figure 3); ORPH was ineffective in this regard.

Pretranslational mechanisms of CYP2B induction by ORPH

The mechanisms by which ORPH selectively modulated CYP2B protein expression were evaluated further. As shown in Figure 4, administration of ORPH to male rats for 3 days increased the hepatic content of mRNAs that encode CYPs 2B1/2, thus establishing the fact that the induction of CYP2B by ORPH is exerted at least in part at a pretranslational level. Transcriptional activation of CYP2B genes is mediated by the CAR, which acts at a 51-bp PBREM in the 5′-flanking regions. In transiently transfected HepG2 cells, it was found that ORPH (0.1 mM) stimulated reporter gene activity controlled by the mouse *cyp2B10* PBREM to about 2.5-fold of control ($P<0.05$; Figure 5); somewhat higher concentrations of ORPH (0.5–1 mM) appeared to be cytotoxic in HepG2 cells. TCPOBOP (250 nM) stimulated CAR-controlled PBREM reporter activity to six-fold of control (Figure 5). These findings indicate that, like TCPOBOP and a number of other PB-like inducers in rat liver, ORPH activates the PBREM in CAR-transfected HepG2 cells.

Evaluation of post-translational mechanisms of CYP2B induction during ORPH treatment

ORPH has been shown to generate an MI-complex with CYPs 2B *in vivo* and *in vitro*, which contributes significantly to the inhibitory effect of the drug on enzymic oxidation (Bast *et al*., 1983b; 1984; Reidy *et al*., 1989). There is evidence that MI-complexation may stabilise CYP proteins against proteolytic degradation and inhibit their turnover in liver (Voorman & Aust, 1988; Watkins *et al*., 1986). In the present study, we tested whether the complex formed between an ORPH metabolite and CYP2B may impair protein turnover. A single dose of ORPH to rats did not elicit induction in hepatic

Table 1 Formation of AD metabolites in hepatic microsomes from control and differently pretreated rats

	AD metabolite				
Rat treatment	*5α-dihydroAD*	*Testosterone*	*6β-HydroxyAD*	*16α-HydroxyAD*	*16β-HydroxyAD*
	(nmol mg protein^{-1} min^{-1})				
None (control)	1.17±0.06	2.36±0.38	2.36±0.18	2.69±0.15	0.59±0.04
ORPH	1.25±0.15	1.59±0.09**	2.81±0.92	3.00±0.24	2.45±0.59**
PB	1.77±0.12	2.61±0.18	9.17±0.56**	2.68±0.25	13.38±0.72**
BNF	1.34±0.11	2.67±0.05	3.05±0.27	1.84±0.22**	0.55±0.02
DEX	1.34±0.23	1.36±0.05**	13.08±1.47**	3.41±0.25*	1.48±0.13
Dimethylsulphoxide	1.40±0.23	2.98±0.14*	1.94±0.15	2.68±0.24	0.60±0.07

Data are mean±s.e.m. of measurements made in microsomal fractions from individual rat livers ($n=3$–6 per group). Different from control: *$P<0.05$, **$P<0.01$.

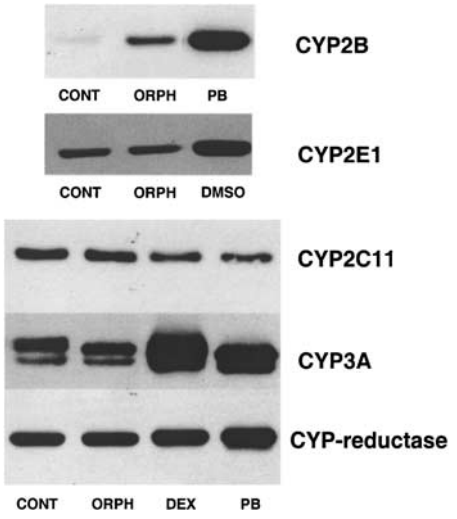

Figure 2 Representative immunoblots of CYP2B, CYP2E1, CYP2C11, CYP3A and NADPH-CYP-reductase expression in hepatic microsomes from untreated rats (CONT) and rats that have received ORPH (60 mg kg^{-1} daily for 3 days). The effects of prototypic CYP-inducing chemicals are included for comparison: PB, CYP2B; dimethylsulfoxide (DMSO), CYP2E1; DEX, CYPs 2C11, 3A and CYP-reductase.

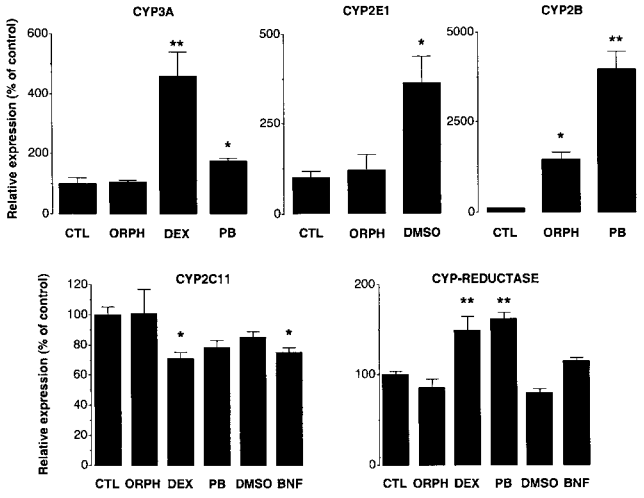

Figure 3 Relative microsomal CYP and CYP-reductase expression in control (CTL) rat liver and after administration of ORPH and other inducing chemicals. $*P<0.05$, $**P<0.01$ ($n=5$ animals in each treatment group).

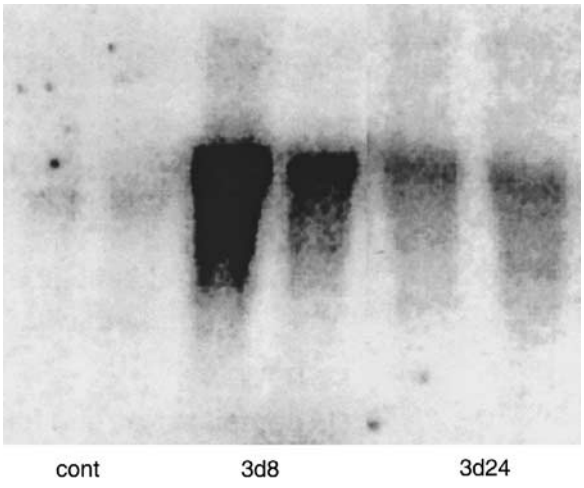

Figure 4 Northern analysis of CYP2B1/2 mRNA expression control (cont) in rat liver and either 8 or 24 h after three injections of ORPH.

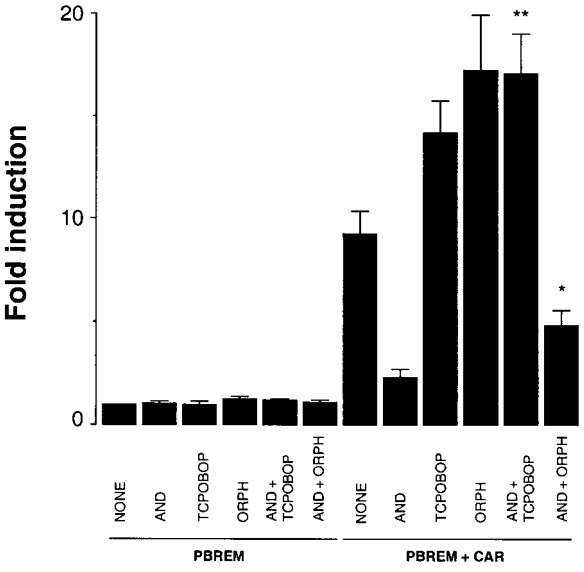

Figure 5 HepG2 cells were transfected with the cyp2b10 gene PBREM-luciferase reporter construct or cotransfected with the PBREM-luciferase reporter construct and a plasmid encoding the mCAR. In addition, all cells were cotransfected with a plasmid encoding β-galactosidase. Transfected cells were treated with androsten-3α-o1 (AND; 8 μM) to suppress endogenous CAR activation, ORPH (0.1 mM) or TCPOBOP (250 nM), or combinations of these reagents. Results are fold induction of reporter-gene activity in AND-treated cells after correction for pGL3 alone; data derived from three to four separate transfections. Different from AND-treated cells: $**P<0.01$, $*P<0.05$.

microsomes but the administration of subsequent doses increased CYP2B protein expression (Figure 6a and b) and CYP2B-dependent AD 16β-hydroxylation (Figure 6c). Thus, 24 h after the administration of two doses of ORPH the microsomal expression of CYP2B was about 50% of that observed after three doses. AD 16β-hydroxylation was increased correspondingly except that the activity after two doses was about 75% of that in microsomes from rats that received three doses of ORPH. The short-term induction of CYP2B by ORPH was evaluated 8 h after the third dose of ORPH. As shown in Figure 6, CYP2B protein expression at that time point was lower than that observed 24 h after the third injection of ORPH. Northern analysis of CYP2B1/2 mRNA after three doses indicated that expression was greater at 8 h than at 24 h after the third dose (Figure 4). It emerged from these studies that the pretranslational induction of CYP2B/1 by ORPH is of relatively short duration. Thus, the lower levels of CYP2B protein and activity observed 8 h after the third dose of ORPH are probably because of the continued decline in expression after the second dose and before the third dose has taken effect.

The time course studies indicated that ORPH was an effective inducer of CYP2B, but that the duration of induction

was dependent upon the number of doses of the drug. Thus, measurement of CYP2B turnover in ORPH-treated rats was likely to be complicated by the time dependence of optimal induction and the low level of expression of CYP2B protein in control liver. To test the possibility that ORPH may stabilise CYP2B protein against proteolysis, further *in vivo* studies were conducted in which ORPH was administered to PB-pretreated male rats (in which microsomal CYP2B expression was already maximal). In these experiments, administration of ORPH to PB-pretreated rats prevented the decline in CYP2B expression and function in rat liver that occurs on removal of inducer. Thus, compared with microsomal CYP2B in the PB-pretreated animals (the reference treatment group in this experiment), the additional ORPH treatment significantly enhanced CYP2B protein (Figures 7 and 8a) and activity (Figure 9) at each time point (24–96 h post-PB). However, the slope of the terminal phase of loss of CYP2B and AD 16β-hydroxylation activity was not different between PB-treated and ORPH/PB-treated animals. Similar findings were made in the case of CYP3A protein (Figures 7 and 8b) and its associated AD 6β-hydroxylation activity (Figure 9), which are also PB-inducible, although to a lesser extent than CYP2B. The minor PB-inducible CYP2A-dependent AD 7α-hydroxylation pathway also exhibited a time-dependent return to control activity over the 4 days of the study; ORPH did not affect this process (Figure 9). In the case of CYP2C11-mediated AD 16α-hydroxylation, effects of PB and combined PB/ORPH treatment on activity were minimal (Figure 9), which is consistent with the fact that this CYP is constitutively expressed in male rat liver and is not inducible by PB (Figure 2). Finally, overall protein turnover was also followed in these experiments by administration of ^{14}C-labelled sodium bicarbonate (Figure 8c). Incorporation of label in microsomal fractions was unchanged by ORPH administration, which is consistent with the notion that protein turnover is refractory to the drug.

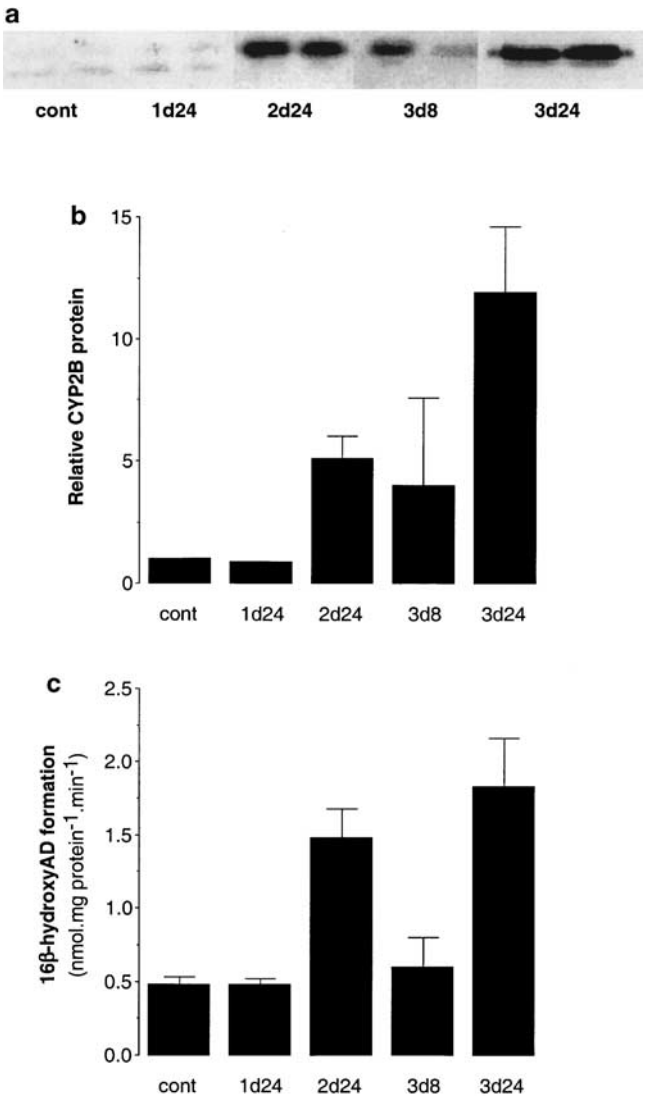

Figure 6 (a) Immunoblot analysis of CYP2B protein expression in rat liver after ORPH administration. 1d24, 2d24 and 3d24 indicates that animals received one, two or three doses of ORPH and were killed 24 h later, whereas 3d8 indicates that three doses were administered and animals were killed 8 h after the third dose. (b) Relative CYP2B protein expression and (c) AD 16β-hydroxylation activity in these fractions ($n=5$ animals in each treatment group).

Impact of ORPH treatment in vivo *on CYP inhibition by ORPH* in vitro

The preceding studies indicated that CYP2B was selectively and markedly induced by ORPH administration *in vivo*. Induction occurred rapidly at the mRNA level and was most pronounced after multiple exposures. Since CYP2B has also been implicated in the formation of inhibitory MI-complexes with ORPH (Bast *et al*., 1983b; 1984; Reidy *et al*., 1989), we tested whether the *in vitro* potency of ORPH against CYP2B reactions in microsomes from ORPH-induced rats might be greater than in microsomes from untreated animals. The preincubation of ORPH with microsomes prior to the measurement of AD hydroxylation activity was used to generate reactive ORPH metabolites *in situ*. In control microsomes, ORPH was a nonpotent inhibitor ($IC_{50}s > 50\ \mu M$) of all pathways of AD hydroxylation catalysed by CYPs 2C11,

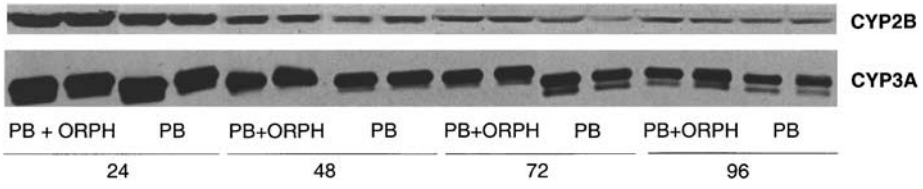

Figure 7 Immunoblot analysis of CYP2B and CYP3A protein expression in hepatic microsomal fractions from PB-pretreated animals (100 mg kg^{-1} daily on three consecutive days) that then received either a single injection of ORPH (60 mg kg^{-1}) or no further treatment ($n=4$ animals in each treatment group). Animals were killed at 24 h intervals thereafter and CYP expression was determined in microsomal fractions; representative blots from two individual livers in each group.

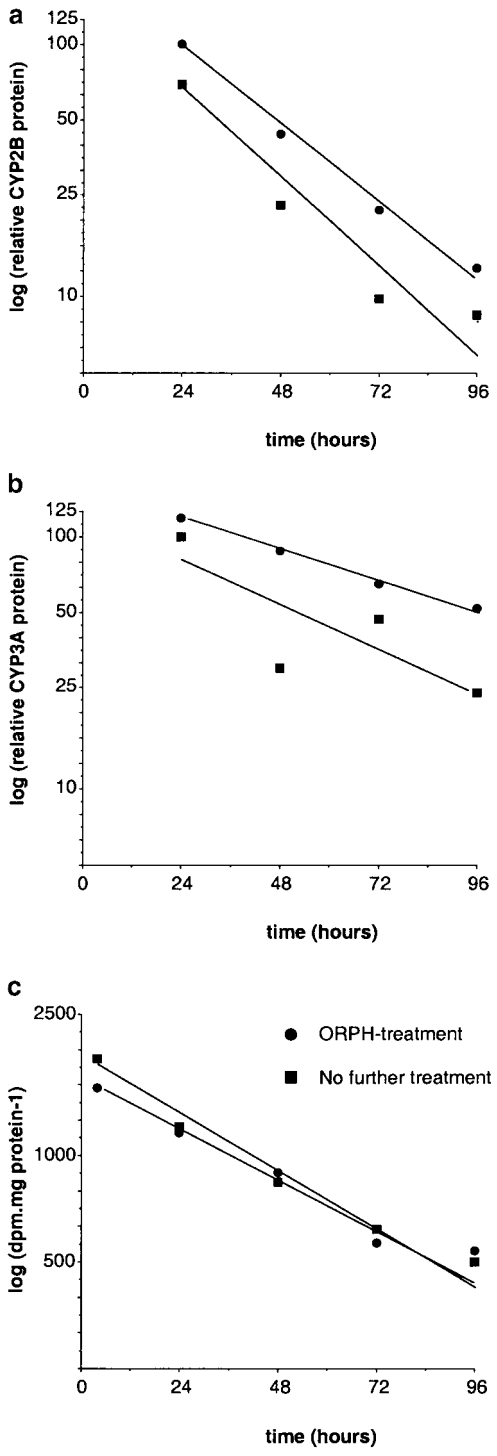

Figure 8 Effect of a single dose of ORPH (60 mg kg^{-1}) to PB-pretreated rats on the relative microsomal expression of (a) CYP2B and (b) CYP3A immunoreactive proteins and (c) ^{14}C-incorporation (dpm) into total microsomal protein (logarithmic scales on the y-axes). Data were derived with four animals in each treatment group.

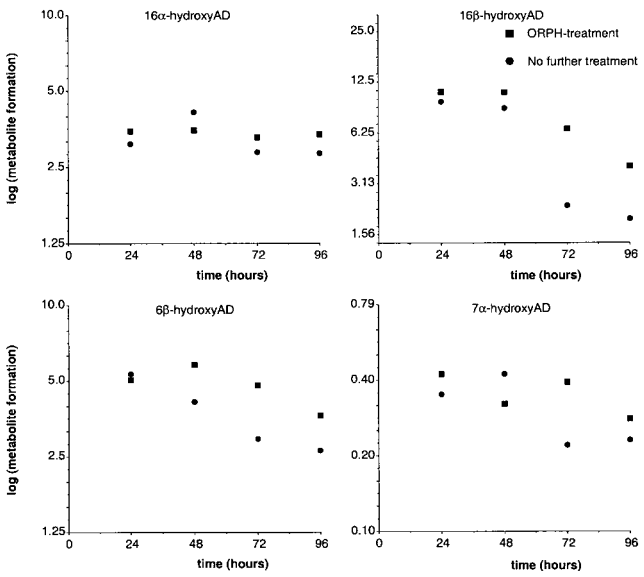

Figure 9 Semilogarithmic plots of time-dependent changes in AD hydroxylation pathways in rat liver after ORPH treatment. Animals (four animals in each treatment group) had been pretreated for 3 days with PB to induce CYP2B optimally and received a single injection of ORPH (60 mg kg^{-1}) or no treatment. Animals were killed at 24 h intervals, hepatic microsomes were prepared and AD hydroxylation activity was measured as described in Materials and methods.

2B and 3A; preincubation slightly increased the potency of ORPH as an inhibitor of AD 16β-hydroxylation (IC_{50} 26 μM compared with $>50\,\mu$M in the absence of preincubation; Table 2). In microsomal fractions from PB-induced rat liver, ORPH was relatively ineffective in the absence of preincubation against all pathways of AD hydroxylation. However, the preincubation of ORPH with NADPH-supplemented PB-microsomes in order to generate the inhibitory metabolite markedly enhanced the extent of inhibition of CYP2B-dependent AD 16β-hydroxylation (IC_{50} 0.8 μM; Table 2). CYP2B also participates in 16α-hydroxylation of AD in these fractions, which could account for the more potent effect of ORPH on that activity following preincubation with NADPH-fortified microsomes (IC_{50} 2.4 μM; Table 2). In hepatic microsomes from ORPH-pretreated rats potentiation of inhibition of AD 16β-hydroxylation was observed when ORPH was preincubated with these fractions *in vitro* in the presence of NADPH (IC_{50} 11 μM; Table 2). Again, some inhibition of AD 16α-hydroxylation was noted, which is consistent with the contribution of CYP2B to this pathway in induced microsomes (Waxman *et al.*, 1983). From these studies, it emerged that the inhibition capacity of ORPH was related to the relative expression of CYP2B in the microsomes: greater CYP2B expression enhanced the inhibitory effectiveness of ORPH (smaller IC_{50} values).

Discussion

The principal finding to emerge from the present study is that the administration of ORPH upregulates hepatic microsomal CYP2B expression in male rats. Thus, CYP2B protein was increased to 14-fold of control by 3 days of intraperitoneal ORPH administration. By comparison, microsomal CYP2B was increased to 40-fold of control by PB. Consistent with these findings, CYP2B function, as reflected by microsomal AD 16β-hydroxylation activity, was increased to a greater extent after PB induction than after ORPH induction (to four-fold of control compared with 20-fold of control after PB). The finding that PB, but not ORPH, increased NADPH-CYP

Table 2 Effect of preincubation of ORPH with microsomal fractions from differently pretreated rats on the potency of AD hydroxylation inhibition

Rat treatment	*Preincubation*	*AD hydroxylation pathway IC50* (μM) *6β-*	*16α*	*16β*
None (control)	No	>50	>50	>50
	Yes	>50	>50	26
PB	No	>50	>50	>50
	Yes	5.0	2.4	0.8
ORPH	No	>50	>50	>50
	Yes	>50	26	11

reductase activity is also consistent with the more pronounced increase in AD 16β-hydroxylation activity in rat liver after PB. No evidence was found for altered regulation or function of several other CYPs, including CYPs 2C11, 2E1 and 3A, following PB administration. CYP2B induction by ORPH is in accord with earlier reports that multiple administrations of ORPH increased the microsomal *N*-demethylations of aminopyrine and ethylmorphine in rat liver (Bast *et al*., 1983a); both of these xenobiotics are substrates for CYP2B (Guengerich *et al*., 1982).

Further studies evaluated potential mechanisms by which ORPH stimulated CYP2B expression in liver. CYP2B1/2 mRNA was increased in ORPH-treated rat liver, which indicates that the drug activates CYP2B gene expression at a pretranslational level. Since most CYPs are regulated transcriptionally, this suggests that CYP2B gene transcription is stimulated by ORPH treatment. Recent work has shown that rodent CYP2B genes possess highly conserved PBREM elements in their distal 5′-promoters that are critical for induction by PB (Trottier *et al*., 1995; Park *et al*., 1996; Honkakoski *et al*., 1998a). The PBREM includes two nuclear receptor (NR) sites and a nuclear factor−1 binding site that accommodates basic leucine zipper transcription factors (Honkakoski & Negishi, 1997; Liu *et al*., 1998; Muangmoonchai *et al*., 2001).

The role of the CAR in PB-mediated gene induction is now established (Honkakoski *et al*., 1998b). The CAR is a member of the NR superfamily and forms heterodimers with the retinoidXreceptor (RXR) that bind and activate the NR−1 site within the PBREM of target genes, including rat CYPs 2B. The transcription factor Sp1 is also important and an unidentified protein binds at NR-2, but the entire assembly is required for recruitment of the transcriptional coactivator SRC−1 (Muangmoonchai *et al*., 2001). The potent PB-activator TCPOBOP and a wide range of other PB-like inducers stimulate the nuclear translocation of the CAR for binding and activation of the PBREM (Honkakoski & Negishi, 1997; Honkakoski *et al*., 1998a; Tzameli *et al*., 2000). The present findings suggest that ORPH also stimulates CYP2B gene transcription because it activates the PBREM in CAR-transfected cells.

PB enhances histone acetylation (Procaccini & Bresnick, 1975) and the phosphorylation of acidic proteins in nuclei (Blankenship & Bresnick, 1974). PB induction in hepatocytes was impaired when the phosphorylation status of proteins was modified (Sidhu & Omiecinski 1995; 1997). Thus, increasing intracellular cAMP concentrations by treatment of primary rat hepatocytes with cAMP analogues and/or activators of adenylate cyclase diminished the response of CYP2B genes to PB (Sidhu & Omiecinski, 1995). Okadaic acid and other inhibitors of phosphatases−1 and 2A similarly decreased PB induction, which suggests that regulated phosphorylation/dephosphorylation is important for optimal induction by PB (Sidhu & Omiecinski, 1997).

CYP turnover by phosphorylation, ubiquitylation and proteasomal degradation is accelerated when CYPs are damaged by reactive xenobiotic metabolites (Korsmeyer *et al*., 1999). Other drugs that cause MI-complexation, such as troleandomycin and isosafrole, stabilise CYP proteins against catabolic processes and so decrease CYP turnover (Watkins *et al*., 1986; Voorman & Aust, 1988). Thus, several fold longer half-lives for CYP3A protein and haem were measured in turnover experiments in troleandomycin-treated cells. Similarly, in rats treated with isosafrole *in vivo*, the degradation of CYP1A2 protein, but not CYP1A1, was decreased to 60% of control. These reports prompted us to test whether ORPH, which also forms an MI-complex, may impede CYP catabolism and stabilise CYP. However, it emerged from the study in PB-treated rats that, although ORPH administration clearly induced additional CYP2B protein expression and function in microsomes, there was no evidence for altered protein turnover. Instead, this appears to be more closely related to the pretranslational effects of ORPH.

The results of the present study provide mechanistic information to account for the multiple pharmacokinetic effects of ORPH. Thus, ORPH has been shown to increase plasma chlorpromazine and antipyrine elimination in subjects when administered according to standard therapeutic dosage regimen; the magnitude of the apparent CYP-induction effect was similar to that observed with PB (Loga *et al*., 1975). In addition, ORPH impairs its own elimination in humans (Labout *et al*., 1982), and that of coadministered haloperidol (Altamura *et al*., 1986), which is consistent with CYP inhibition. These findings of bimodal pharmacokinetic effects of ORPH on human CYPs are in accord with the present observations that the drug is both an inducer and inhibitor of CYPs in rat liver. Interestingly, diphenhydramine, which differs from ORPH only in the absence of the aromatic methyl group (Figure 1), has also been shown recently to be an inhibitor of the important human CYP2D6 (Hamelin *et al*., 2000).

In the rat, ORPH would be expected to alter its own oxidative biotransformation to reactive metabolites that

impair CYP2B activity. There has been some controversy in this area, with earlier studies implicating CYPs 2B in the formation of inhibitory metabolites of ORPH (Bast *et al*., 1984; Reidy *et al*., 1989). These studies monitored MI-complexation by the measurement of residual holo-CYP, and not 452-nm spectral intermediate formation. Direct CYP measurement overcomes the problems of CO-release from catabolised haem in the presence of ORPH and NADPH. More recently, it has been suggested that CYP3A may also be involved in *in vitro* interactions with ORPH (Roos & Mahnke, 1996). In this regard, it is noteworthy that reconstituted enzyme systems containing CYP2B1 and NADPH-CYP reductase did not support the formation of an ORPH MI-complex (unpublished observations). It is therefore feasible that CYPs other than CYP2B may catalyse an earlier enzymic oxidation step, such as the *N*-demethylation of ORPH to tofenacine, which rapidly forms MI-complexes (Bast *et al*., 1983b; 1984). As a target for ORPH MI-complexation, CYP2B catalyses the final oxidative step in the multistep conversion of the drug to a reactive metabolite. However, rat strain differences in the expression of CYPs that catalyse other steps leading to MI-complex formation may contribute to apparent variation in the CYPs involved in the process.

PB-like induction occurs with some alkylamine derivatives that are structurally similar to ORPH. Doxylamine possesses a pyridyl system in place of one of the phenyl rings in ORPH (Figure 1), and is a prominent inducer of CYP2B in mice (Bookstaff *et al*., 1996). Tamoxifen also shares some structural features with ORPH, and induces CYP2B and 3A mRNAs in rodent liver (Hellriegel *et al*., 1996; Cotreau *et al*., 2001). Despite the formation of CYP-MI-complexes by some of these alkylamine drugs, it appears that pretranslational induction mechanisms may predominate. At least in part, this process may well involve activation of the CAR, which has a pivotal role in PB-like induction in mammals.

The present findings are relevant for understanding the toxicological impact of ORPH. The drug may have a significant drug abuse potential, as suggested by the report of Slordal & Gjerden (1999), which indicated that ORPH was detected in 57% of autopsies conducted in Norway in 1997. Further, recent studies have suggested that ORPH may also be of value in the treatment of neurodegenerative disorders associated with activation of *N*-methyl-d-aspartate (NDMA) receptors (Pubill *et al*., 2001). Application of ORPH, or structurally similar molecules, to the treatment of such conditions would need to consider the potential toxic actions of the drug resulting from CYP induction and inhibition.

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