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Effect of bupropion on CYP2B6 and CYP3A4 catalytic activity, immunoreactive protein and mRNA levels in primary human hepatocytes: comparison with rifampicin

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Abstract

Animals treated with multiple doses of bupropion have had increased bupropion clearance or increased liver weight, suggesting induction of drug-metabolizing activity. The possibility of cytochrome p450 (CYP) induction by bupropion (10 μM) was evaluated in-vitro by comparing catalytic activity, immunoreactive protein and CYP mRNA levels from human hepatocytes in primary culture versus cells treated with vehicle (0.5% methanol) and with rifampicin (rifampin) as a positive control. mRNA levels were analysed using a branched DNA luminescent assay. CYP2B6 activity, protein and mRNA levels were increased by 2.5, 1.5 and 2.1 fold, respectively, by 20 μM rifampicin. However, 10 μM bupropion minimally altered CYP2B6 (1.4, 1.1, 0.8 fold). Although CYP3A4 activity, protein, and mRNA levels were increased by 4.0, 2.3, and 14.0 fold, respectively, by 20 μM rifampicin, 10 μM bupropion minimally altered CYP3A4 (1.4, 1.0, 0.8 fold). Rifampicin (20 μM) increased CYP2E1 protein by 2.1 fold, while 10 μM bupropion minimally altered CYP2E1 protein (1.2 fold). Overall, results of this study suggest that multiple doses of bupropion are not likely to induce CYP2B6, 3A4 or 2E1 in-vivo in man.

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Introduction

Bupropion is an antidepressant and non-nicotine aid to smoking cessation that acts by inhibiting the reuptake of dopamine and noradrenaline (norepinephrine) (Cooper et al 1994). Bupropion also may be used to treat attention-deficit hyperactivity disorder when other agents are not effective (Cantwell 1998). Adverse drug effects associated with bupropion include agitation, anxiety, insomnia, hypertension and seizures. The incidence of seizures is 0.1% at doses up to 300 mg per day of sustained-release bupropion, and 0.4% at doses up to 450 mg per day of the immediate-release formulation (Dunner et al 1998). The aetiology of seizure induction is unknown but appears to be associated with high plasma concentrations of bupropion or a metabolite (Welch et al 1987; Preskorn 1991).

In-vivo in man, bupropion is extensively metabolized to three principal metabolites: hydroxybupropion (morphinol), threohydrobupropion and erythrohydrobupropion (Schroeder 1983; Golden et al 1988; Preskorn 1991). Hydroxybupropion appears to be the most important metabolite, being a more potent antagonist of dose-dependent sedation produced by tetrabenazine and having a lower LD50 value than the erythro and threo metabolites in mice (Welch et al 1987). Furthermore, plasma levels of hydroxybupropion greatly exceed those of bupropion in humans. In-vitro experiments using human liver microsomes have shown that bupropion is mainly metabolized to hydroxybupropion via cytochrome p450 (CYP) 2B6, although CYP2E1 has a minor role in hydroxybupropion formation, as shown in-vitro using individual cDNA-expressed CYPs (Faucette et al 2000; Hesse et al 2000). It is not known if CYP2B6

has the major role in hydroxybupropion formation in-vivo. There is concern that altering CYP2B6 catalytic activity while taking bupropion may alter plasma levels of bupropion or hydroxybupropion, leading to increased risk of toxicity.

Animal studies have suggested that bupropion may induce CYPs (Schroeder 1983; Tucker 1983). Tucker (1983) reported increased liver weight in rats and dogs after chronic dosing that was attributed to enzyme induction. AUC and plasma half-life of bupropion was significantly decreased in rats, mice and dogs after 14 days of administration (Welch et al 1987), and plasma levels of bupropion were greatly decreased after one year of bupropion treatment in dogs and rats (Schroeder 1983), suggesting induction of drug-metabolizing enzymes. However, a human study in which healthy subjects received doses up to 450 mg daily of bupropion for more than two weeks showed no evidence of self-induction (Data on file, Glaxo Wellcome Inc. THRS/92/0016). Another human study in which healthy subjects received 300 mg daily of bupropion for 14 days showed no evidence of self-induction (Posner et al 1985). Sweet et al (1995) reported a clinical study in which the half-life of bupropion after 7–11 days of therapy decreased in only 1 of 6 subjects. Clinical studies have not determined whether bupropion induces other CYPs in-vivo, such as CYP3A4, the most abundant CYP isoform (Shimada et al 1994).

Although CYP3A4 has been studied extensively both in-vivo in humans and in-vitro in human liver hepatocytes, CYP2B6 induction has only recently been studied in primary human hepatocytes because few CYP2B6 substrates have been identified. Clinical studies have shown that rifampicin (rifampin) induces CYP3A4 in-vivo in humans, leading to increased clearance of CYP3A4 substrates when co-administered (Backman et al 1996; Villikka et al 1997). Rifampicin may induce other CYPs besides CYP3A4, including 2B6 (Bachmann & Jauregui 1993; Kerbusch et al 2001).

In this study, the effect of 10 μM bupropion on CYP2B6 and 3A4 expression and function was determined in-vitro in primary human hepatocytes by measuring changes in CYP2B6 and CYP3A4 catalytic activity, immunoreactive protein and mRNA levels. The effect of 10 μM bupropion on CYP2E1 protein expression was also determined. Rifampicin (20 μM) was used as a positive control, since rifampicin has been shown to induce CYP2B6, 3A4 and 2E1 in studies involving primary human hepatocytes (Kostrubsky et al 1995, 1999; Chang et al 1997; Li 1997). According to prescribing information, plasma concentrations of rifampicin after a single 600-mg oral dose range from 5 to 39 μM in healthy fasting adults, and a 30-minute infusion of a 600-mg dose of rifampicin resulted in a peak plasma concentration of 21 μM.

Materials and Methods

Materials

Bupropion hydrochloride and bupropion internal standard (GW340416A, also named A234U) were provided by Glaxo Wellcome (Research Triangle Park, NC). Rifampicin, triazolam and phenacetin (acetophenetidin) were obtained from Sigma (St Louis, MO). Western-blotting anti-peptide antibody to CYP2B6 (catalogue no. 458226) was purchased from Gentest Corp. (Woburn, MA) and polyclonal antibody against rat CYP3A1/3A2 (catalogue no. A3005) was purchased from Xenotech (Kansas City, KS). Plating media, incubation media, and Krebs-Henseleit bicarbonate (KHB) media were kindly provided by In Vitro Technologies (Baltimore, MD).

Primary human hepatocytes (preparation and treatment)

Fresh human liver tissue from two different donors was obtained. There was no indication of exposure to CYP2B6 or 3A4 inducers before tissue harvesting (Table 1). The hepatocytes were isolated and cultured in either 24-well plates or 96-well plates first with plating media for a 24-h attachment period at 37 °C and 5% CO_2. Hepatocytes were used for induction studies if they were sufficiently confluent after the attachment period, and were then cultured with incubation media. Hepatocytes were exposed to substrate by the addition of media containing dissolved substrate. Cells were exposed in incubation media containing bupropion (0.1–100 μM and 0.5% methanol) or rifampicin (10–100 μM and 0.5% DMSO) or vehicle control (0.5% methanol or 0.5% DMSO) for 48 h at 37 °C and 5% CO_2. Incubation media was changed approximately every 12 h during the 48-h induction period due to bupropion degradation in solution (Laizure & DeVane 1985).

Measurement of CYP catalytic activity

Probe substrates for CYP3A4 and CYP2B6 activity were 250 μM triazolam (Kronbach et al 1989; von Moltke et al 1996, 1998; Perloff et al 2000) and 500 μM bupropion (Faucette et al 2000; Hesse et al 2000), respectively, prepared in KHB media with 0.5% methanol. Cells in 24-well

Table 1 Demographics of the two liver donors.

Donor	Age	Sex	Race	Drug history	Cause of death
1	36	Female	Caucasian	5 cigarettes/day; marijuana; alcohol rehabilitation for 11 months	Basilar artery infarction
2	61	Female	Caucasian	No alcohol or drugs; no smoking	Cerebrovascular accident

plates were incubated for 1.5 h at 37 °C and 5% CO_2. Reactions were stopped by the addition of 100 μL of 1 M HCl to wells exposed to bupropion (as a CYP2B6 activity probe) or 200 μL of acetonitrile to wells exposed to triazolam. Plates containing media were frozen at −80 °C until HPLC analysis.

HPLC analysis of hydroxybupropion

Hydroxybupropion was extracted from the media of cells exposed to bupropion as a CYP2B6 activity probe using a modified protocol from Cooper et al (1984). First, the media was thawed at room temperature. The internal standard for bupropion (GW340416A) was added to glass extraction tubes (15 μL of 5 mg GW340416A per 50 mL methanol). The methanol was evaporated to dryness in a heated vacuum oven. Media (550 μL) from each well was added to a tube containing the dried internal standard. Carbonate buffer (pH 9.5; 0.6 M; 600 μL) was added to the media. Heptane (4 mL, with 1.5% isoamyl alcohol) was added to the media and buffer mixture. The mixture was vortexed for 10 min and centrifuged for 10 min. The organic layer was transferred to a new tube and 250 μL of a phosphoric acid buffer (pH 2.5) was added to the organic layer. The mix was vortexed for 45 s and centrifuged for 10 min. The aqueous phase was removed and transferred to autosampling vials for HPLC analysis.

The HPLC mobile phase consisted of 500 mL of a 50 mM potassium phosphate buffer and 110 mL of acetonitrile containing 0.5 mL 85% H_3PO_4, 0.6 mL triethanolamine and 3.5 mL of 20% heptane sulfonic acid. A reverse-phase Nova-Pak C18 column (Waters Associates, Milford, MA) was used with a flow rate of 2 mL min^{-1} and a detection wavelength of 214 nm. Peak height ratios of hydroxybupropion and the internal standard were determined (Figure 1A).

HPLC analysis of alpha-hydroxytriazolam

Media was thawed and the internal standard phenacetin (50 μL of 4.5 mg per 100 mL methanol) was added directly to the media in the plate wells and mixed by pipetting. Media with internal standard was transferred directly to autosampling vials for HPLC analysis. The HPLC mobile phase consisted of 710 mL of 10 mM potassium phosphate buffer, 200 mL acetonitrile and 100 mL methanol. A reverse-phase Nova-Pak C18 column (Waters Associates, Milford, MA) was used with a flow rate of 1.5 mL min^{-1} and a detection wavelength of 220 nm. Peak height ratios of alpha-hydroxytriazolam and internal standard phenacetin were determined (Figure 1B).

HPLC analysis of bupropion

Media (450 μL) containing dissolved bupropion was removed (from the culture wells containing the hepatocytes) immediately before changing the media and added to 75 μL of 1 M HCl. GW340416A (75 μL of 5 mg/50 mL methanol) was added and the mixture was transferred to autosampling vials for HPLC analysis. The HPLC mobile phase consisted of 850 mL of 50 mM potassium phosphate buffer (pH 3) and 150 mL acetonitrile. A reverse-phase μBondapak C18 column (Waters Associates, Milford, MA) was used with a flow rate of 2 mL min^{-1} and a detection wavelength of 214 nm. A standard curve of bupropion was generated by adding various amounts of bupropion (0.2–222 μM final concentration) and GW340416A (75 μL of 5 mg/50 mL methanol), evaporating off the methanol, reconstituting the drugs in cell media and analysing the drugs by HPLC. Concentrations of bupropion in the media were determined using peak height ratios of bupropion versus the internal standard.

Lysing of cells

Media was aspirated from the cells, and the cells were stored on the plates at −80 °C until protein analysis. For the lysis procedure, cells were thawed in the plates on ice and 100 μL of lysis buffer was added to the cells for at least 20 min. Lysis buffer consisted of 50 mM Tris (hydroxymethyl) aminomethane (Sigma, St Louis, MO) at pH 8, containing 150 mM sodium chloride, 3 mM sodium azide (Fisher Scientific Company, Fair Lawn, NJ) and 0.6 mM phenylmethylsulfonyl fluoride (ICN Biomedicals, Aurora, OH) added in a solution of 100 mg mL^{-1} dimethyl formamide and 1% Triton X-100 (Sigma). The contents of each well were scraped and similar treatments were pooled in the same tube. Lysates were sonicated and centrifuged for 30 s at 2000 rev min^{-1}. Protein concentration in the supernatant was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Bovine serum albumin was used as a standard. The lysates were stored at −80 °C.

Immunoquantification of CYP2B6, 3A4 and 2E1

Microsomal protein (0.016–0.250 pmol lymphoblast-expressed CYP2B6 or CYP3A4 or 0.015–1 pmol CYP2E1, Gentest Corporation, Woburn, MA) and cell lysate (1–2 μg μL^{-1} protein) were denatured for 5 min at 100 °C in 100 mM Tris buffer containing 10% glycerol, 2% β-mercaptoethanol, 2% SDS and 5 μg mL^{-1} pyronin Y (pH 6.8) or in ImmunoPure Lane Marker Reducing Sample Buffer (Pierce, Rockford, IL). Approximately 20, 50 or 24 μg of protein from cell lysates were loaded per well for CYP2B6, CYP3A4 and CYP2E1 blots, respectively. Protein was separated by SDS-PAGE in 7.5% polyacrylamide gels (Biorad, Hercules, CA). Samples were run at 100 V for 60–90 min in 25 mM Tris buffer–0.192 M glycine–0.1% SDS buffer. Then samples were transferred to Immobilon-P (PVDF membrane) (Millipore, Bedford, MA) for 1 h at 100 V in 25 mM Tris buffer with 20% methanol. Blots were fixed in an acetic acid solution for 60 s.

Blots were blocked with 0.5% (CYP2B6), 3% (CYP3A4) or 1% (CYP2E1) powdered non-fat milk in TBS–Tween (0.15 M NaCl, 0.04 M Tris-HCl pH 7.7 and 0.06% Tween 20) for 1 or 2 h at room temperature (CYP3A4) or at 4 °C overnight (CYP2B6, CYP2E1). CYP2B6 blots were incubated with a 1:500 dilution of a

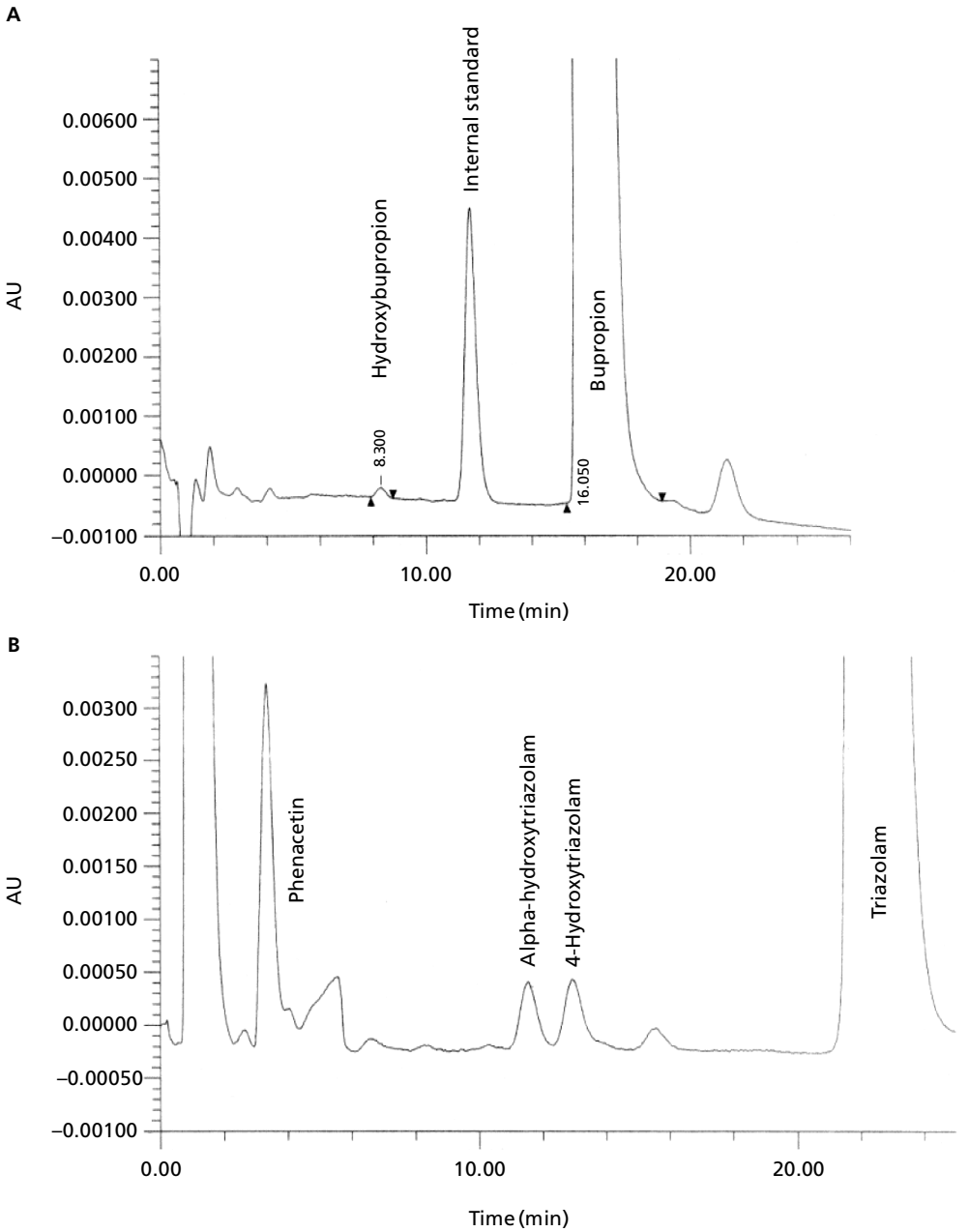

Figure 1 Representative HPLC tracings showing hydroxybupropion formation (CYP2B6 activity) (A), and alpha-hydroxytriazolam formation (CYP3A4 activity) (B). The retention times of hydroxybupropion and the internal standard were 8.3 and 12 min, respectively. The retention times of phenacetin and alpha-hydroxytriazolam were 3.5 and 11.5 min, respectively.

polyclonal antipeptide CYP2B6 antibody (Stresser & Kupfer 1999) (Gentest, Woburn, MA) in TBS–Tween containing 0.1% BSA for 1 h at room temperature. CYP3A4 blots were incubated with a 1:1500 dilution of a polyclonal antibody against CYP3A1/3A2 (XenoTech, Kansas City, KS) containing 1% milk in TBS–Tween for 5 h at room temperature and also at 4 °C overnight. CYP2E1 blots were incubated with a 1:1000 dilution of anti-rat CYP2E1 (Gentest, Woburn, MA) containing 1% milk in TBS–Tween for 2 h at room temperature. Blots were washed with TBS–Tween. CYP2B6 blots were then incubated with a 1:500 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Gentest, Woburn, MA) in 0.5% powdered non-fat milk in TBS–Tween for 1 h at room temperature. CYP3A4 blots were re-blocked for 30 min in 3% milk in TBS–Tween and then incubated with a 1:10 000 dilution of HRP-conjugated donkey anti-rabbit IgG (Pierce, Rockford, IL) in 1% powdered non-fat milk in TBS–Tween for 1 h at room temperature. CYP2E1 blots were then incubated with a 1:4000 dilution of HRP-conjugated anti-goat IgG (Product no. A 5420,

Sigma-Aldrich) in 1% powdered non-fat milk in TBS–Tween for 1 h at room temperature.

Blots were rinsed with TBS–Tween and the Super Signal Cl-HRP Substrate System (Pierce, Rockford, IL) was used for enhanced chemiluminescence detection. Blots were analysed by a Kodak Image Station 440 and quantitation of CYP content was completed using KODAK 1D Image Analysis Software (Kodak, Rochester, NY). A standard curve of arbitrary units of net or mean intensity versus picomoles of CYP was created and fit to one of the following equations: $y = m*x + B$ or $y = m*\ln(x) + B$ using non-linear least-squares regression.

mRNA quantification (branched DNA assay)

mRNA was quantified using a branched DNA signal amplification assay (QuantiGene bDNA Signal Amplification Kit; Bayer Diagnostics, East Walpole, MA). CYP3A4, CYP2B6, and GAPDH probe sets (catalogue nos B0160, B0125 and B0960, respectively) were purchased from XenoTech (Kansas City, KS). The probe set consisted of capture probes, blocker probes and label probes that were used at 50, 100 and 200 fmol μL^{-1}, respectively. Reagents consisted of lysis buffer, capture hybridization buffer, amplifier/label probe buffer, washes A and D and substrate solution.

Primary hepatocytes were plated at a density of 35 000 per well in a 96-well plate. Cells were incubated with 10 μM bupropion or 20 μM rifampicin, or vehicle control (0.5% methanol or 0.5% DMSO, respectively) as described above for 48 h. Incubation media with inducer or vehicle was changed approximately every 12 h.

After 48 h, lysis buffer containing the appropriate probes was added to cells for 30 min at 37–53 °C (100 μL/well). Lysis buffer without capture extenders was added to cells of all different induction conditions to determine background luminescence. Cell lysates (90 μL) were transferred to capture wells containing 100 μL capture buffer provided with the kit, and wells were incubated overnight at 53 °C. For the remainder of the experiment, the instructions provided with the kit were closely followed, as described in Zhou et al (2000). Luminescence from the capture wells was measured on a luminometer (Wallac VICTOR^2; Perkin Elmer Life Sciences). Luminescence was expressed in relative light units and CYP luminescence was adjusted for background and then normalized to GAPDH luminescence for each treatment group. Results were expressed as an average of the ratios of normalized luminescence from the induced cells versus the average normalized luminescence from vehicle control cells.

Statistical methods

Activity

Activity was assayed in individual wells. Similar treatments were performed in 3 or 4 wells for donor 1 and in 7 or 8 wells for donor 2. Activity measurements in wells of control cells were averaged. Activity measurements in wells incubated with bupropion or rifampicin ($n = 3$ or 4 for donor 1 and $n = 7$ or 8 for donor 2) were first individually expressed as a ratio relative to the average control value. These ratios were averaged for each treatment. Differences between bupropion and rifampicin were compared using a Student's *t*-test (Table 2, Figure 2). A significance level of $P < 0.05$ denoted significance in all cases.

Protein

Cells in consecutive wells received the same treatment ($n = 4$ for donor 1, $n = 6$ for donor 2). After incubation with rifampicin or bupropion, cell lysates from similar treatments were pooled and mixed to give a single value, as shown in Figure 4.

mRNA

Similar treatments were performed in four different wells. The effect of concentration (0 and 10 μM) and drug (rifampicin and bupropion) on the ratio of CYP relative light units to GAPDH relative light units was statistically analysed using a 2-way analysis of variance. In all cases, post-hoc comparisons of the means of individual groups were performed using Tukey's test. A significance level of $P < 0.05$ denoted significance in all cases.

Table 2 Effect of 10 μM bupropion and 20 μM rifampicin on CYP2B6 and 3A4 catalytic activity in primary human hepatocytes from two different donors.

Donor	Ratio of CYP2B6 catalytic activity (treated cells vs vehicle control)		Ratio of CYP3A4 catalytic activity (treated cells vs vehicle control)	
	Rifampicin	Bupropion	Rifampicin	Bupropion
1	2.9 ± 0.4 (n = 4)	1.2 ± 0.6 (n = 3)	4.8 ± 0.4 (n = 4)	2.0 ± 0.3 (n = 4)
2	2.0 ± 0.1 (n = 8)	1.5 ± 0.1 (n = 8)	3.1 ± 0.3 (n = 7)	0.87 ± 0.04 (n = 7)
Average	2.5	1.4	4.0	1.4

The ratio of CYP2B6 or 3A4 catalytic activity from treated cells versus vehicle control (0.5% methanol or 0.5% DMSO, respectively, without inducer) is given ± the standard deviation (n = 3–8 for the same liver donor). The effect of rifampicin differed from the effect of bupropion in the same donor ($P < 0.01$).

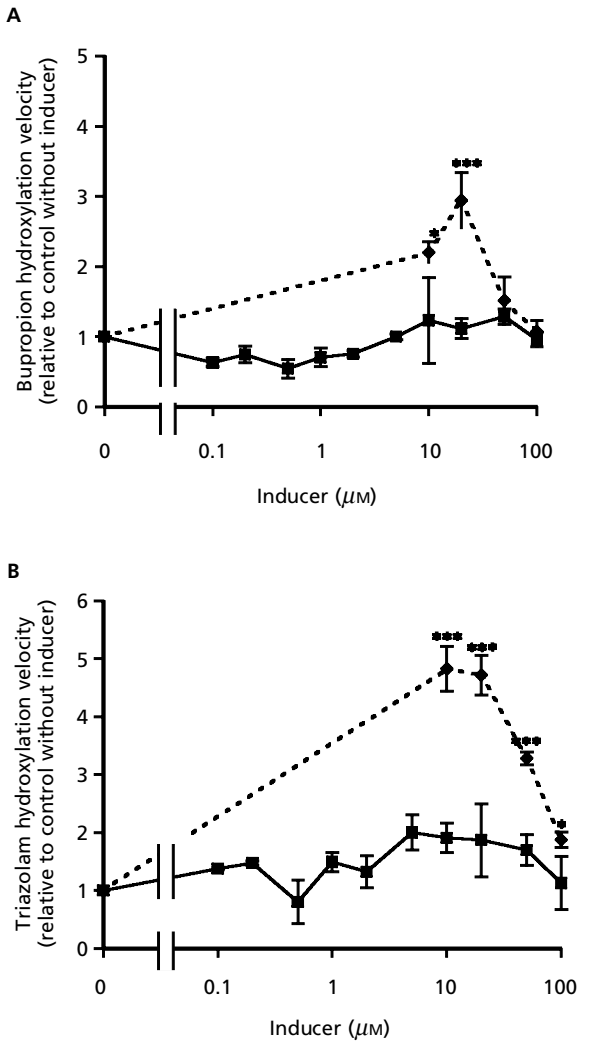

Figure 2 Effect of bupropion (■) or rifampicin (◆) on CYP2B6 (A) and CYP3A4 (B) activity. Various concentrations of inducer (bupropion at 0.1–100 μM or rifampicin at 10–100 μM), as shown on the x-axis, were dissolved in cell media and incubated with hepatocytes from donor 1. The y-axis shows bupropion hydroxylation velocity (CYP2B6 activity, A) or triazolam hydroxylation velocity (CYP3A4 activity, B) as compared with vehicle control (0.5% methanol or 0.5% DMSO, respectively). Error bars denote standard deviations (n = 3 or 4). $*P < 0.05$, $***P < 0.001$, CYP activity of cells treated with rifampicin vs CYP activity of cells treated with bupropion.

Results

When hepatocytes from donor 1 were incubated with various concentrations of bupropion (0.1–100 μM), CYP2B6 activity relative to vehicle-treated cells was minimally altered compared with cells treated with rifampicin. Rifampicin increased CYP2B6 catalytic activity in the hepatocytes from donor 1 by as much as 3 fold (at 20 μM) compared with vehicle control cells (0.5% DMSO) (Figure 2A). Previous studies have validated bupropion hydroxylation as an index reaction for CYP2B6 activity (Faucette et al 2000; Hesse et al 2000); therefore, it was also used as a probe for CYP2B6 activity in hepatocytes in this study.

Similarly, when hepatocytes from donor 1 were incubated with various concentrations of bupropion (0.1–100 μM), CYP3A4 catalytic activity (based on triazolam alpha-hydroxylation) was minimally altered compared with hepatocytes treated with rifampicin, which increased CYP3A4 catalytic activity by as much as 5 fold (at 20 μM) compared with vehicle control (0.5% DMSO) (Figure 2B). Effects on the parallel pathway of triazolam 4-hydroxylation, also mediated by CYP3A, were essentially identical (results not shown). To verify activity results, hepatocytes from donor 2 were incubated with 10 μM bupropion and 20 μM rifampicin (Table 2).

Laizure & DeVane (1985) reported that bupropion stability was time and temperature dependent, being stable at 0°C or at pH 3. Their study showed that in plasma at 37 °C and pH 7.4, bupropion degraded with a half-life of approximately 11 h to a product that was not one of the three major metabolites, and the degradation product may be a spontaneous hydrolysis reaction because the reaction occurred in saline (Laizure & DeVane 1985). In our study, bupropion degraded in the media, so media containing dissolved substrate was changed twice a day. During the 48-h induction period, the concentration of bupropion was measured immediately before changing media. The fresh media contained 10 μM bupropion, but due to substrate degradation, the concentrations of bupropion decreased to 4.5, 3.4 and 5.2 μM at the time of media change. The concentration of bupropion that is typically encountered clinically is 0.6 μM (Hsyu et al 1997). Nonetheless in-vitro concentrations of bupropion, even after degradation, still exceed concentrations encountered clinically. The effect of the degradation product on hepatocytes is unknown.

Representative western blots for CYP2B6 and 3A4 immunoquantification in donor 1 are shown in Figure 3. Changes in CYP protein expression were calculated as the ratio of immunoquantified CYP protein in treated cells versus cells treated with vehicle control. After incubation with bupropion (5–50 μM), immunoreactive CYP2B6 was minimally altered compared with treatment with rifampicin (10–50 μM), which increased protein by at least 1.5 fold at 20 μM (Figure 4A). Treatment with bupropion (5–50 μM) did not increase immunoreactive CYP3A4, while treatment with rifampicin (10–50 μM) increased CYP3A4 by almost 3 fold at 20 μM (Figure 4B). To verify results from donor 1, hepatocytes from donor 2 were incubated with 10 μM bupropion and 20 μM rifampicin, and immunoblot analysis showed that bupropion changed CYPs 2B6 and 3A4 by 1.1 and 1.0 fold, respectively, while rifampicin increased CYPs 2B6 and 3A4 by 1.4 fold and 1.7 fold, respectively (data not shown).

Western blot analysis of CYP2E1 from hepatocytes from both donors 1 and 2 is shown in Figure 5. Rifampicin (20 μM) increased CYP2E1 in hepatocytes from donors 1 and 2 by 1.8 and 2.3 fold compared with control, respectively. Bupropion, 0.5, 1, 5, 10, 20, 50 and 100 μM, changed CYP2E1 protein from donor 1 by 1.2, 1.4, 1.5, 1.1, 1.0, 1.2 and 1.1 fold compared with control,

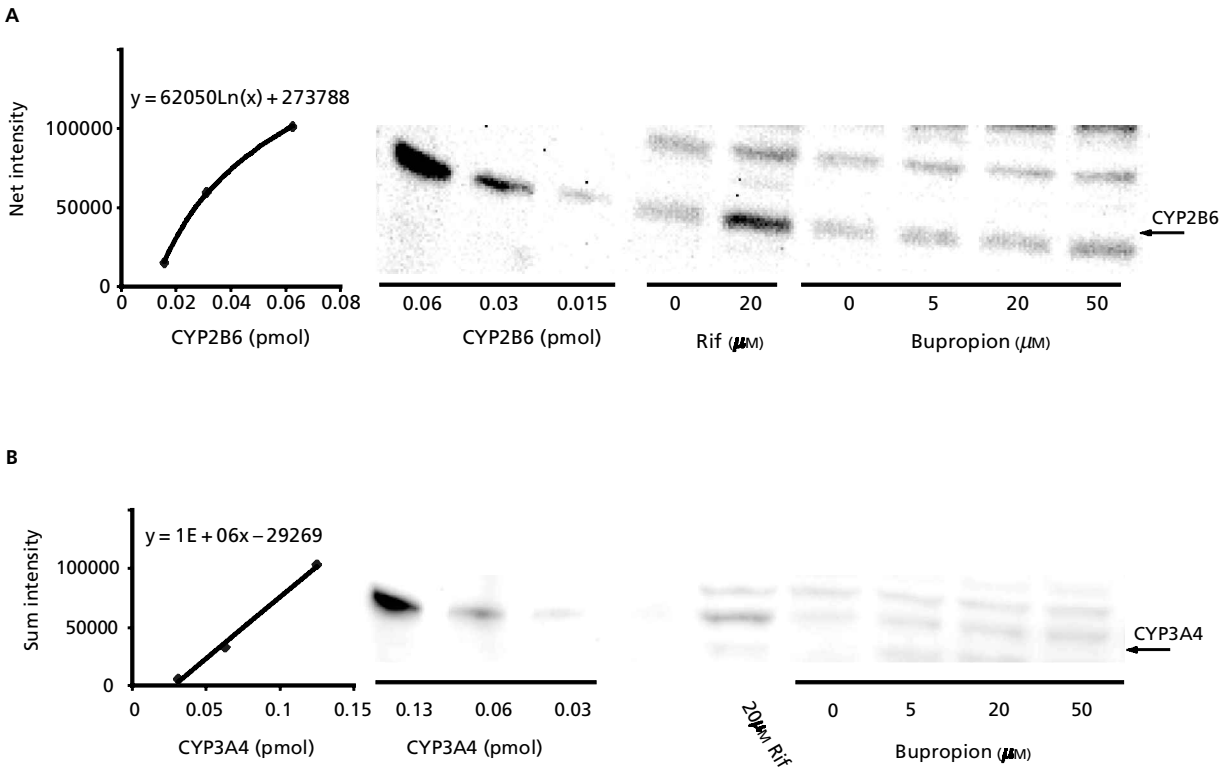

Figure 3 Representative western blots to immunoquantify CYP2B6 (A) and CYP3A4 (B). Hepatocytes from donor 1 were treated with 5–50 μM bupropion or 20 μM rifampicin (Rif), or vehicle control (0.5% methanol or 0.5% DMSO, respectively). Cell lysate protein (20 or 50 μg) was loaded into gels A and B, respectively. cDNA-expressed CYP2B6 or 3A4 was loaded onto the appropriate gel (left three lanes) to generate a standard curve and therefore determine that the measured intensities from the cell lysates were within either a linear or a logarithmic range of the standard curve. Blots were probed with anti-CYP2B6 antibodies (A) or anti-CYP3A1/3A2 antibodies (B). The ratios of immunoquantified CYP2B6 protein from cells treated with inducer (5, 20 and 50 μM of bupropion) vs vehicle control (without inducer) were 1.1, 1.0 and 1.2, respectively, as determined using a logarithmic fit to $y = m*\ln(x) + B$, where y is the net intensity of the band and x is the amount of cDNA-expressed CYP2B6 (pmol) loaded on the gel. The ratios of immunoquantified CYP3A4 protein from hepatocytes treated with inducer (5, 20 and 50 μM bupropion) vs control were 1.1, 1.1 and 1.2, respectively, as determined using a linear fit to $y = m*x + B$, where y is the sum intensity of the band and x is the amount of cDNA-expressed CYP3A4 (pmol) loaded on the gel.

respectively. Bupropion (10 μM) changed CYP2E1 protein from donor 2 by 1.2 fold compared with control.

CYP mRNA levels from hepatocytes (from donor 2) treated either with inducer or vehicle control were measured in arbitrary light units using the bDNA assay, and normalized to GAPDH mRNA levels in each treatment group. Bupropion (10 μM) had minimal effect on CYP2B6 and CYP3A4 mRNA levels, since the ratios of GAPDH-normalized CYP2B6 and 3A4 mRNA levels in treated cells versus vehicle control cells (0.5% methanol) were both 0.8. Rifampicin (20 μM) increased GAPDH-normalized CYP2B6 and 3A4 mRNA levels in treated cells versus vehicle control cells (0.5% DMSO) by ratios of 2.1 and 14.0, respectively (Figure 6).

Discussion

In this study using primary human hepatocytes, 10 μM bupropion changed CYP2B6 or 3A4 catalytic activity, immunoreactive protein or mRNA levels by no more than 2 fold compared with vehicle control after a 48-h incubation period. Likewise, 10 μM bupropion increased CYP2E1 immunoreactive protein by no more than 1.2 fold. In contrast, 20 μM rifampicin produced large increases based on all parameters. Our results are consistent with results from clinical studies that did not find evidence for increased bupropion clearance in patients taking multiple doses of bupropion (Schroeder 1983; Posner et al 1985). Sweet et al (1995) observed a decrease in the half-life of bupropion in one of six patients, suggesting that CYP2B6 protein in a small fraction of the population may be minimally increased. Alternatively, the decreased half-life of bupropion in this subject could be direct effect of a co-administered drug since all of the subjects in the study were currently taking other medications. We note that there is not a clearly established criterion for assigning clinical importance to increases in catalytic activity, protein expression and mRNA levels observed in this cell culture model.

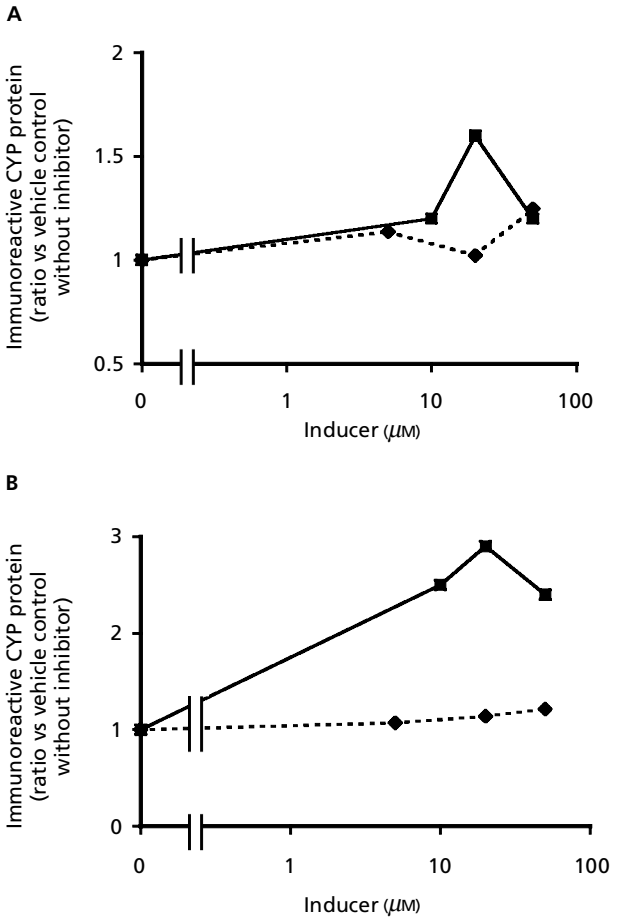

Figure 4 Effect of 5–50 μM bupropion (◆) or 10–50 μM rifampicin (■) on CYP2B6 (A) and CYP3A4 (B) protein levels in primary human hepatocytes from donor 1. The ratio of CYP protein levels of the treated cells vs vehicle control cells (0.5% methanol or 0.5% DMSO, respectively) is given on the y-axis and concentration of inducer is given on the x-axis.

We confirmed induction by rifampicin, with a maximal increase of CYP protein and catalytic activity at 10–20 μM rifampicin, and decreasing CYP activity and protein levels toward baseline levels at higher rifampicin concentrations. Kostrubsky et al (1999) observed the same pattern on induction of CYP3A protein and activity by taxol, suggesting that higher concentrations of taxol are toxic to hepatocytes based on a decrease in total protein synthesis at higher taxol concentrations, shown by pulse-labelling hepatocytes with labelled leucine. Although the physiological concentration of rifampicin is rarely hepatotoxic in humans (Durand et al 1996), higher concentrations of rifampicin (>20 μM) in cell culture may be toxic to hepatocytes, suggesting the need for toxicity checks in studies involving primary human hepatocytes.

In our study, catalytic activity may have been lowered due to the presence of solvent (0.5% methanol or DMSO) in media when incubating cells with inducer or determining CYP activity. It has been shown that solvent can affect the activity of CYPs in-vitro using human liver microsomes or primary human hepatocytes (Chauret et al 1998; Easterbrook et al 2001). Chauret et al (1998) reported that 0.5% DMSO decreased CYP3A4 microsomal activity (testosterone beta-hydroxylation) by more than 30%, while 0.5% methanol decreased activity to a much lesser extent. We added solvent to microsomal assays and compared CYP3A4 activity (triazolam alpha-hydroxylation using 250 μM triazolam) and 2B6 activity (bupropion hydroxylation using 500 μM bupropion) to control assays without solvent. There was a decrease of approximately 50% in CYP3A4 activity when the incubation buffer contained 0.5% DMSO and a decrease of approximately 10% in CYP2B6 or 3A4 activity when the buffer contained 0.5% methanol (data not shown). In agreement with in-vitro microsomal studies, Easterbrook et al (2001) reported that 1% DMSO inhibited CYP3A4 activity to almost 50% in primary human hepatocytes, while methanol had much less of an inhibitory effect. Therefore, the presence of a solvent, especially 0.5% DMSO, may have lowered CYP activity in primary human hepatocytes.

We compared mRNA levels of treated cells to levels in vehicle control cells using a novel method of analysing mRNA levels in primary human hepatocytes based on the QuantiGene kit from Bayer, which may be as sensitive as RT-PCR for mRNA quantification (Hartley & Klaassen 2000; Warrior et al 2000; Zhou et al 2000; Sen et al 2001). The kit has an ELISA-like format and provides a solid support phase which binds RNA via probes specific for the gene of interest (capture extenders). Additional probes (label extenders) bind both the specific gene of interest and the branched DNA (bDNA) molecules available with the kit. Blocker probes bind RNA at gaps between capture and label extender probes. Alkaline phosphatase is conjugated to an oligonucleotide (label probe), which hybridizes to branches of the bDNA molecules. When dioxetane is added, a chemiluminescent signal is emitted. This method has advantages over the traditional way of measuring mRNA, such as quantitative RT-PCR, because the product measured represents the actual amount of target mRNA, meaning that if a standard curve is generated, it is linear as opposed to being logarithmic (Hartley & Klaassen 2000).

Although results indicated that rifampicin is a strong CYP3A inducer, it is not clear from our activity and RNA assay which specific CYP3A isoforms were induced. Patki et al (2003) reported that the CYP3A index reaction of triazolam hydroxylation is not specific to CYP3A4 because triazolam is hydroxylated by both CYP3A4 and 3A5. Additionally, the RNA probes purchased from XenoTech were not specific for CYP3A4, but also bound CYPs 3A5, 3A7 and 3A43. Czerwinski et al (2002) reported in abstract form that rifampicin induced only CYP3A4 but not 3A5 mRNA in primary liver hepatocytes as determined using the QuantiGene kit with newer specific probes to CYP3A4 and 3A5 mRNA. CYP3A7 and 3A43 mRNA are induced by rifampicin but CYP3A43 comprises approximately 0.2% of CYP3A4 mRNA, and CYP3A7 from adult liver comprises approximately 1.7% of the level of CYP3A7

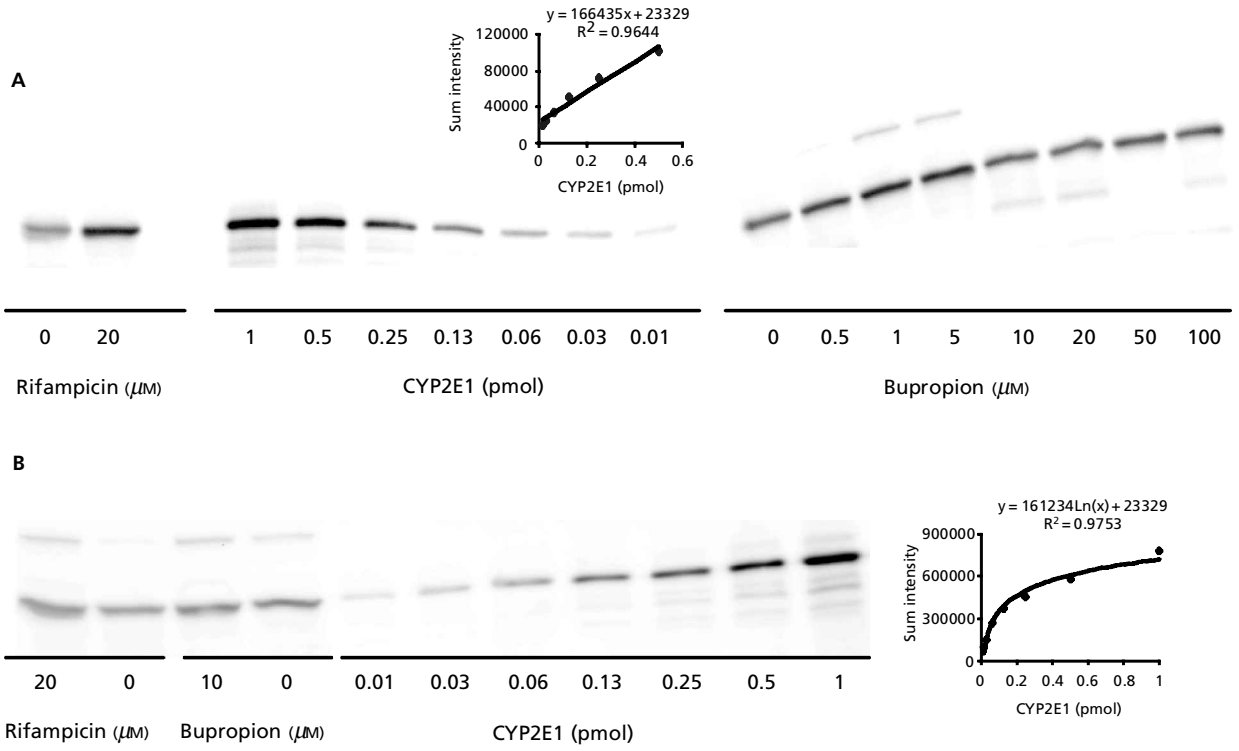

Figure 5 Western blots to immunoquantify CYP2E1. A. Hepatocytes from donor 1 were treated with 0.5–100 μM bupropion or 20 μM rifampicin, or vehicle control (0.5% methanol or 0.5% DMSO, respectively). B. Hepatocytes from donor 2 were treated with 10 μM bupropion or 20 μM rifampicin or vehicle control (0.5% methanol or 0.5% DMSO, respectively). Cell lysate protein (24 μg) was loaded into gels. cDNA-expressed CYP2E1 was loaded onto each gel to generate a standard curve and therefore determine that the measured intensities from the cell lysates were within either a linear or a logarithmic range of the standard curve. Blots were probed with anti-CYP2E1 antibodies. The ratios of immunoquantified CYP2E1 protein from cells treated with inducer (0.5, 1, 5, 10, 20, 50 and 100 μM of bupropion; and 20 μM rifampicin) vs vehicle control (without inducer) in gel A were 1.2, 1.4, 1.5, 1.1, 1.0, 1.2 and 1.0; and 1.8, respectively, as determined using a linear fit to $y = m*x + B$, where y is the sum intensity of the band and x is the amount of cDNA-expressed CYP2E1 (pmol) loaded on the gel. The ratios of immunoquantified CYP2E1 protein from hepatocytes treated with inducer (10 μM bupropion and 20 μM rifampicin) vs control in gel B were 1.2 and 2.3, respectively, as determined using a logarithmic fit to $y = m*\ln(x) + B$, where y is the sum intensity of the band and x is the amount of cDNA-expressed CYP2E1 (pmol) loaded on the gel.

mRNA found in fetal liver (Greuet et al 1996; Gellner et al 2001). Therefore, although other CYP3A isoforms may have been detected, a change in CYP3A activity, protein and mRNA levels in human hepatocytes from the two adult donors in our study may mostly reflect a change in CYP3A4 levels.

The reason for increased liver weight and decreased AUC and plasma half-life of bupropion in animals treated with multiple doses of bupropion is not known, but the difference in induction between animals and humans may be species-specific or may be due to differences in metabolism. Additionally, increased liver weight may be attributed to induction of other enzymes involved in drug metabolism. Lu & Li (2001) compared the inductive effects of rifampicin, omeprazole and dexamethasone on CYPs in hepatocytes from different species including humans, rats, minipigs and beagle dogs. While rifampicin and omeprazole induced CYP3A in all species except rats, dexamethasone was a potent 3A inducer in rats, a moderate to weak inducer in humans and dogs, but did not induce in minipigs. These results indicate that induction is species-specific and that only human hepatocytes should be used to predict human CYP induction in-vivo.

Another reason that bupropion may induce enzymes in animals and not in humans may be due to the difference in metabolism between species. Schroeder (1983) reported that the metabolism of bupropion in man is different to that in rats and dogs because the animals appear to biotransform bupropion predominantly by side-chain oxidative cleavage, while reduction of bupropion to an aminoalcohol (hydroxybupropion) is a major pathway in man. Therefore, induction in animals may be due to a metabolite found at higher concentrations in animals than in humans.

Conclusion

In conclusion, our results indicate that 10 μM bupropion produces only small changes in CYP2B6 or 3A4 expression and function in primary human hepatocytes. Our results support the lack of evidence of CYP2B6 induction in clinical studies of chronic bupropion administration

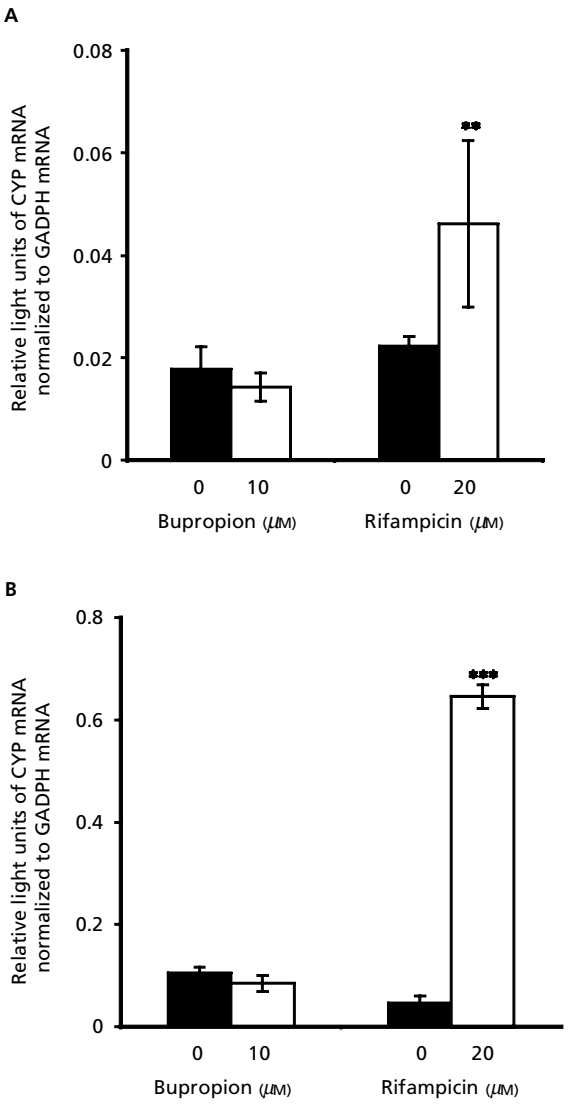

Figure 6 Effect of 10 μM bupropion or 20 μM rifampicin on CYP2B6 (A) and CYP3A4 (B) mRNA levels in primary human liver hepatocytes from donor 2. Levels were determined using the QuantiGene kit (Bayer). Relative light units reflecting the level of CYP mRNA were normalized to the level of GAPDH mRNA for each induction condition, as shown on the y-axis. The inducer is given below the x-axis. Bupropion 0 μM or rifampicin 0 μM reflects vehicle controls of 0.5% methanol or 0.5% DMSO, respectively, n = 3 or 4 for each sample, and error bars denote standard deviation. Ratios of normalized CYP2B6 mRNA levels from cells treated with 20 μM rifampicin and 10 μM bupropion vs normalized vehicle control cells are 2.1 and 0.8, respectively. Ratios of normalized CYP3A4 mRNA levels from cells treated with 20 μM rifampicin and 10 μM bupropion vs normalized vehicle control cells are 14.0 and 0.8, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$, CYP mRNA levels of drug-treated (10 μM rifampicin or 20 μM bupropion) cells vs CYP mRNA levels of untreated (0 μM rifampicin or 0 μM bupropion) cells (Tukey's test).

(Glaxo Wellcome NN1997/00010/00; Posner et al 1985; Sweet et al 1995). Human CYP induction may be better predicted in-vitro using human liver hepatocytes rather than in-vivo using an animal model.

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