

Inhibition of Hepatobiliary Transport as a Predictive Method for Clinical Hepatotoxicity of Nefazodone

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Treatment with the antidepressant nefazodone has been associated with clinical idiosyncratic hepatotoxicity. Using membranes expressing human bile salt export pump (BSEP), human sandwich hepatocytes, and intact rats, we compared nefazodone and its marketed analogs, buspirone and trazodone. We found that nefazodone caused a strong inhibition of BSEP ($IC_{50} = 9 \mu\text{M}$), inhibition of taurocholate efflux in human hepatocytes ($IC_{50} = 14 \mu\text{M}$), and a transient increase in rat serum bile acids 1 h after oral drug administration. Buspirone or trazodone had no effect on biliary transport system. Nefazodone produced time- and concentration-dependent toxicity in human hepatocytes with $IC_{50} = 18 \mu\text{M}$ and $30 \mu\text{M}$ measured by inhibition of protein synthesis after 6 h and 24 h incubation, respectively. Toxicity was correlated with the amount of unmetabolized nefazodone. Partial recovery in toxicity by 24 h has been associated with metabolism of nefazodone to sulfate and glucuronide conjugates. The saturation of nefazodone metabolism resulted in sustained decrease in protein synthesis and cell death at $50 \mu\text{M}$. The toxicity was not observed with buspirone or trazodone. Addition of 1-aminobenzotriazole (ABT), an inhibitor of CYP450, resulted in enhancement of nefazodone toxicity at $10 \mu\text{M}$ and was associated with accumulation of unmetabolized nefazodone. In human liver microsomes, ABT also prevented metabolism of nefazodone and formation of glutathione conjugates. We suggest that inhibition of bile acid transport by nefazodone is an indicator of potential hepatotoxicity. Our findings are consistent with the clinical experience and suggest that described methodology can be applied in the selection of nonhepatotoxic drug candidates.

Key Words: BSEP; buspirone; trazodone; hepatocytes; metabolism.

With standard rodent and nonrodent test species, pharmaceutical industry scientists can only predict about 50% of clinical incidences of hepatotoxicity (Olson *et al.*, 2000). Hepatotoxicity includes unexpected increases in liver enzymes

after drug enters clinical trials or idiosyncratic toxicity usually recognized after several weeks or months of treatment. The purpose of this work is to investigate alternative *in vitro* models which may predict clinical hepatotoxicity for drugs that are expected to have a significant biliary clearance component.

Nefazodone is an antidepressant introduced in 1994. In June of 2004, nefazodone (Serzone) was withdrawn from the U.S. market following its previous withdrawal from European and Canadian markets. Analysis of data on adverse drug reaction from the World Health Organization found a statistically significant number of reports (91) for nefazodone-mediated hepatic injury (Spigset *et al.*, 2003). No significant hepatotoxicity was found for other serotonin reuptake inhibitors. Estimated incidence of hepatotoxicity based on reports collected by the Spanish Pharmacovigilance System was 29 cases per 100,000 patient years (Garcia-Pando *et al.*, 2002). Finally, a black-box warning on the nefazodone label estimated the rate of liver failure as 1 case per 250,000–300,000 patients per year, a rate of about 3–4 times the background rate of liver failure. This was considered as an underestimated number because of under-reporting (NDA, 2001). Most cases of liver failure were reported between 1 to 8 months after drug initiation (Aranda-Michel *et al.*, 1999; Conway *et al.*, 2004; Garcia-Pando *et al.*, 2002; Lucena *et al.*, 1999; Tzimas *et al.*, 2003) The common clinical symptoms of hepatotoxicity included jaundice and increases in ALT (10 \times), AST (10 \times), total bilirubin (mostly conjugated), and prothrombin time. Dark urine and clay-colored stool also were reported. Available histological evaluations of livers demonstrated centrilobular necrosis, bile-duct proliferation with cholestasis (Aranda-Michel *et al.*, 1999; Lucena *et al.*, 1999). About 60% of radiolabeled nefazodone administered intravenously to dogs was recovered in feces, suggesting that bile represents a significant route of drug elimination (Shukla *et al.*, 1993). In humans, after administration of a single 200-mg dose, the urinary excretion of radioactivity in the 24-h samples was 49%, suggesting that bile was the other major route of nefazodone clearance (Barbhaiya *et al.*, 1996). Therapeutic doses of nefazodone associated with liver failure were in the range of 200–400 mg/day. The total

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drug plasma concentration was at or above 1 $\mu\text{g/ml}$ (Barbhaiya *et al.*, 1995; Dockens *et al.*, 1996). In summary, clinical symptoms of hepatotoxicity, significant biliary drug clearance, high administered doses, and high plasma concentration of nefazodone were similar to those described previously for troglitazone, another drug withdrawn from the market where the role of biliary transport in hepatotoxicity was suggested (Funk *et al.*, 2001; Kostrubsky *et al.*, 2000; 2001). We hypothesized that the potential for clinical hepatotoxicity of nefazodone and its analogs could be evaluated by the potency of inhibition of bile acid transport and associated *in vitro* toxicity. An additional biomarker of hepatic effect thought to be linked to compromised biliary elimination was reported to be a transient increase in serum bile acids in the absence of frank liver toxicity in rodents. We compared results from nefazodone and its marketed structural analogs, buspirone and trazodone (Fig. 1), in specialized

in vitro and *in vivo* assays. Based on the results of these experiments we propose that: (1) clinical hepatotoxicity of nefazodone is linked to the ability of drug to inhibit bile acid transport, and (2) hepatotoxicity associated with nefazodone is based on the particular chemical structure, so that drugs with the same mechanism of action but slightly different structure will not be hepatotoxic. Therefore, when multiple compounds with a similar mechanism of action are available during pharmaceutical development, one can eliminate from the drug discovery process compounds with a higher potential for clinical hepatotoxicity.

MATERIALS AND METHODS

Chemicals. HMM (modified William's E) culture medium and supplements were from BioWhittaker (Walkersville, MD). Bile salt export pump (BSEP) recombinant transporter was expressed using baculovirus-infected Sf-9 insect cells with a final concentration of 5 mg/ml and purchased from Solvo Biotechnology (Hungary). Nefazodone hydrochloride, buspirone hydrochloride, trazodone hydrochloride, and 1-aminobenzotriazole were purchased from Sigma-Aldrich (St. Louis, MO). ^3H -Taurocholic acid (2 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). HPLC-grade water, methanol, and acetonitrile were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Human liver microsomes were purchased from BD Gentest (Woburn, MA). All general solvents and reagents were of the highest grade commercially available.

Hepatocyte cultures and treatment protocol. Human hepatocytes were isolated from livers not used for whole-organ transplant within 24 h of procurement. Hepatocytes were isolated by three-step collagenase perfusion as described previously (Strom *et al.*, 1996). The viability of cells obtained, as measured by trypan blue exclusion test, ranged from 74 to 90%. Hepatocytes were plated in Williams E medium supplemented with 10^{-7} M dexamethasone, 10^{-7} M insulin, 100 Units/ml of penicillin G, 100 $\mu\text{g/ml}$ of streptomycin, and 10% bovine calf serum. Hepatocytes (2×10^6 /well) were plated on six-well culture plates previously coated with type I (rat-tail) collagen. Cells were allowed to attach for 4–6 h at 37°C , at which time the medium was replaced with serum-free medium with the supplements listed above and changed every 24 h thereafter. Following 24 h in culture, medium was removed, and the cells overlaid with a neutralized preparation of collagen at a final concentration of 1.5 mg/ml for transport studies, as described previously for cultured sandwich hepatocytes (Kostrubsky *et al.*, 2003). After 72 h in culture, cells were treated with nefazodone, buspirone, or trazodone at concentrations indicated in the figure legend for 1 h, 6 h, or 24 h, and toxicity was determined by the measurement of total protein synthesis by pulse-labeling hepatocytes for 1 h with ^{14}C -leucine, as described previously (Kostrubsky *et al.*, 1997, 2000). This culture condition maintains a sufficient level of functional Phase I and Phase II drug, metabolizing activities in hepatocytes after 96 h in culture, as we demonstrated for several substrates including testosterone 6β -hydroxylation (CYP3A), ethoxyresorufin- and methoxyresorufin O-dealkylation (CYP1A), glucuronidation and sulfation of acetaminophen, 4-methylumbelliferon, and troglitazone (Kostrubsky *et al.*, 1999, 2000, 2005, Wen *et al.*, 2002). At the same time, incubation for 96 h allows minimizing the difference in basal enzyme activities in control cells prepared from different donors (Kostrubsky *et al.*, 1999).

Transport Assays

Cultured sandwich hepatocytes with developed canaliculi. Inhibition of bile transport in cultured sandwich hepatocytes with developed canaliculi was conducted according to previously described protocol (Kostrubsky *et al.*, 2003). Briefly, 1 μM ^3H -taurocholic acid, with or without increasing concentrations of test compound, was added to hepatocytes for 15 min at 37°C . After stopping

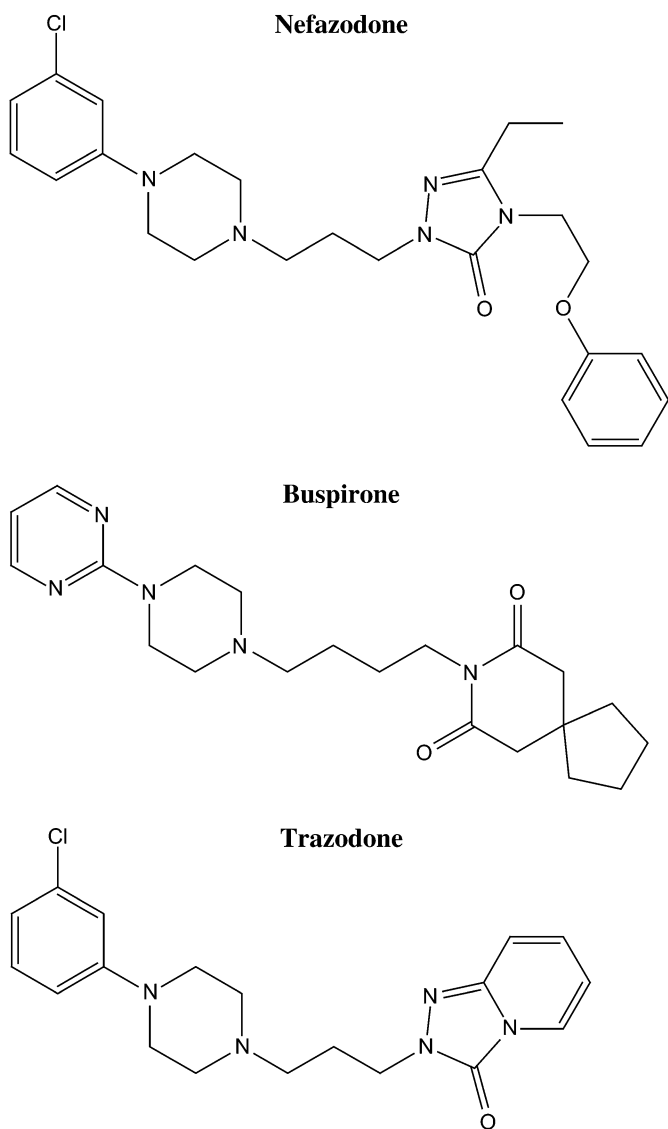


FIG. 1. Structures of nefazodone, buspirone, and trazodone.

transport by removing buffer and washing cells, taurocholate egress from canalicular spaces was initiated by adding standard or Ca/Mg²⁺-free buffer for 10 min. Aliquots of media were harvested and counted in a liquid scintillation counter. The difference in amount of radioactivity between two buffer conditions in the absence of tested compound was defined as a 100% taurocholic acid efflux in canaliculi. In the presence of compound, this difference became smaller and was used to calculate the percent inhibition of bile acid efflux (Kostrubsky *et al.*, 2003). All values were normalized per amount of total cellular protein.

Bile salt export pump. Inhibition of bile acid transport via BSEP was studied in membrane vesicles prepared from recombinant baculovirus infected Sf9 cells expressing human BSEP. Membrane preparation had inside-out orientation vesicles transporting substrates via BSEP, in the presence of ATP, into the vesicles. Assay was performed according to manufacturer's protocol (SOLVO Biotechnology) with some modifications using glyburide as a positive control. Specifically, test compounds were dissolved in dimethylsulfoxide as a 1000× stock. Vesicles (50 µg) were added to the assay mix containing 20% 50 mM HEPES-Tris (pH 7.4), 10% 1 M KNO₃, 10% 0.1 M Mg(NO₃)₂ and 2 µM of ³H-taurocholate in the presence or absence of tested drugs and preincubated for 5 min at 37°C. Transport was initiated with 4 mM of ATP or AMP (background subtraction), and the mixtures were incubated for another 5 min at 37°C. Reactions were stopped with ice-cold wash buffer (10% 100 mM Tris-HCl (pH 7.4) and 10% 1 M KNO₃). Membrane suspension was then filtered and washed twice with wash buffer to remove substrate on the outside of the vesicles. After rapid filtration to separate the vesicles from the incubation solution, filters were counted in scintillation counter. The results were expressed as percent inhibition of taurocholate transport in the presence of inhibitors relative to untreated control.

MRP2-mediated efflux of calcein AM. Inhibition of multidrug resistant associated protein-2 (MRP2)-mediated efflux of calcein AM was studied in MDCK cells transfected with MRP2 obtained from Prof. Piet Borst, Netherlands Cancer Institute. Wild type MDCK cells and MDCK cells stably transfected with human MRP2 were grown and maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in 5% CO₂ for 24 h before the study. Each cell line (50,000 cells per well) was plated into Costar 3904 black 96-well plates (Packard Instrument Co., Meridian, CT) with 100 µl medium supplemented with 1% FBS and allowed to become confluent overnight. Test compounds were added to monolayers in 10 µl of culture medium containing 1% DMSO as solvent. Plates were incubated at 37°C for 30 min. Calcein AM was added in 10 µl of Hank's Balanced Salt Solution (HBSS) to yield a final concentration of 0.1 µM, and plates were incubated for another 60 min. Cells were then washed three times with ice-cold phosphate buffered saline (PBS), and fluorescence was measured using a Victor fluorometer (Perkin Elmer, Downers Grove, IL) at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Inhibition of bile acid transport in intact rats. *In vivo* experiments to measure the response of serum bile acids to drug administration were conducted in Sprague-Dawley rats weighing approximately 300 g. Animals received powdered rodent chow (Purina certified chow; Purina, St. Louis, MO) and tap water by bottle *ad libitum*. All procedures involving animals were conducted in accordance with Guide for the Care and Use of Laboratory Animals. Three rats were assigned to either nefazodone or buspirone experimental groups. A single oral dose of 75 mg/kg nefazodone or buspirone was given by gavage as a solution in sterile water at a dose volume of 10 ml/kg to nonfasted animals. Prior to each blood collection, animals were anesthetized with isoflurane. Blood was collected for evaluation of pretest serum bile acids 1 day before drug administration. Blood samples were collected from the jugular vein 1 h and 24 h after dosing, and serum was analyzed for the total bile acids using a Hitachi 911 analyzer.

Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis of nefazodone metabolites in media from cultured hepatocytes. Aliquots (200 µl) of media were spiked with the internal standard (buspirone) and

analyzed by LC/MS/MS without any further sample preparation. An aliquot (20 µl) from each sample was injected directly onto an HPLC column (Discovery C18, 150 × 2.1 mm, Supelco) coupled to an API 4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada), equipped with a TurbolonSpray® source held at 275°C. The electrospray needle was maintained at 4600 V with the declustering and exit potentials set at 46 and 10 V, respectively. Ultrapure nitrogen was used as the nebulizer and curtain gas. The mass spectrometer was operated in full scan (100–1000 amu) or multiple reaction monitoring (MRM) modes for qualitative and quantitative analyses, respectively. MRM analysis was carried out using nitrogen as the collision gas. The collision energy was kept at 25 eV. Other parameter settings for the MRM analyses included arbitrary values of 6, 25, 40, and 40 for collision (CAD), curtain, nebulizer, and turbo gases, respectively. The mass transitions for the metabolites and the internal standard were 386 → 122 (buspirone, internal standard), 566 → 486 (sulfate conjugate), and 662 → 486 (glucuronide conjugate). The peak areas from each of these transitions were obtained, and ratios of the analytes to the internal standard were obtained for each sample. The metabolites of nefazodone were separated on the HPLC column using a gradient solvent system consisting of acetonitrile and 0.1% formic acid with the flow rate set at 0.3 ml/min. The initial conditions consisted of a mixture of acetonitrile and 0.1% formic acid (4:96 v/v) and were maintained for 3 min after the sample was injected. The percentage of acetonitrile was increased linearly from 3% to 90% in the next 17 min. After an additional 5 min at 90% acetonitrile, the column was reequilibrated with the initial mobile phase for 10 min before the next injection.

Metabolism of nefazodone by human liver microsomes. The metabolism of nefazodone was investigated using human liver microsomes. Nefazodone (25 µM) was incubated in the presence of human liver microsomes (1 mg/ml), MgCl₂ (3 mM), and NADPH (2 mM) in 0.1 M phosphate buffer (pH 7.4) at 37°C for 30 min. Where indicated, glutathione (2 mM) and 1-aminobenzotriazole (ABT, 1 mM) were added to the incubation mixtures. The samples were preincubated for 15 min prior to initiating the reaction by adding substrate. Incubations were also performed without the addition of NADPH as a control for possible nonenzymatic degradation of nefazodone. At the end of the incubation, the microsomal proteins were precipitated by adding 2 ml of ice-cold acetonitrile, followed by centrifugation at 3,000 rpm using an Allegra™ X-22R centrifuge equipped with SX4250 rotor. The supernatant was removed, dried under a stream of nitrogen, and reconstituted in 400 µl of ACN:0.1% formic acid (4:96 v/v). An aliquot (40 µl) was injected onto LC/MS using the conditions described earlier.

Statistical analysis and IC₅₀ determination. Results were analyzed by a two-factor ANOVA. A *p* < 0.05 was interpreted as the level of statistical significance.

The IC₅₀ estimates were determined by nonlinear curve fitting using the WinNonLin software (Pharsight, Mountain View, CA) and were defined as the concentration of compound required to inhibit taurocholate transport or cause inhibition of protein synthesis by 50%.

RESULTS

Effect of Nefazodone, Buspirone, and Trazodone on Bile Acid Efflux in Sandwich Human Hepatocytes, Membrane Vesicles, and Intact Rats

Incubation of cultured human hepatocytes (prepared from two different donors) with the nefazodone resulted in concentration-dependent inhibition of taurocholic acid efflux in canaliculi with IC₅₀ = 14 µM (Fig. 2). In contrast, neither buspirone nor trazodone inhibited canalicular transport. Hepatocytes were fully capable of metabolizing nefazodone (see Figs. 8 and 9), and hence the inhibitory effect on bile transport may have

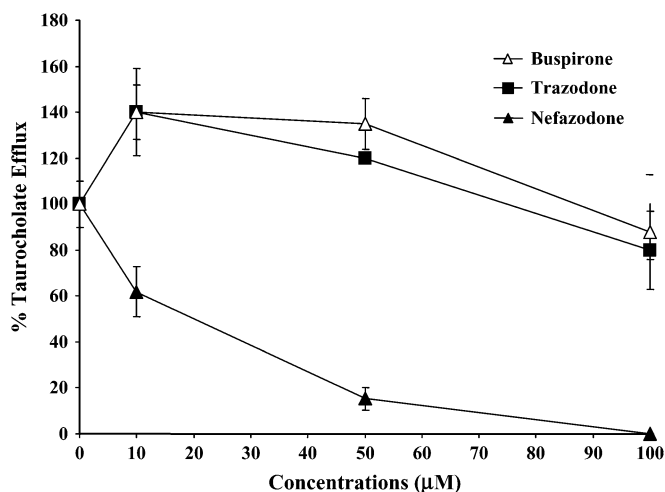


FIG. 2. Effect of nefazodone, buspirone, and trazodone on taurocholate efflux in sandwich cultured human hepatocytes. Hepatocytes from two human donors were treated with $1 \mu\text{M}$ ^3H -taurocholic acid alone or in combination with increasing concentrations of antidepressants for 15 min. Cells were washed, and standard or Ca/Mg^{2+} -free buffer was added. After 10 min incubation, aliquots of media were harvested and counted in a liquid scintillation counter, and percent inhibition of taurocholate efflux was calculated. Each value represents the mean of duplicate treatments of hepatocytes from each of two donors, with the range indicated by the vertical bars.

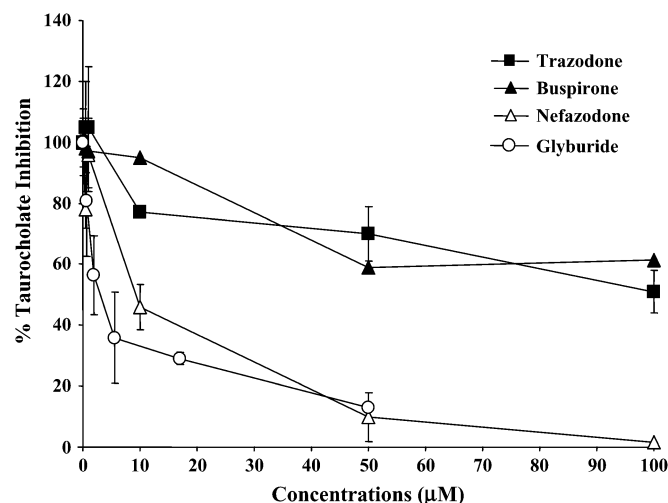


FIG. 3. Inhibition of BSEP-mediated transport of taurocholic acid in membrane vesicles. Inhibition of BSEP-mediated transport of ^3H -taurocholate by nefazodone, glyburide (positive control), buspirone, and trazodone was measured in membrane vesicles prepared from recombinant baculovirus-infected Sf9 cells expressing human BSEP as described in Materials and Methods. Each value represents the mean of duplicate vesicle incubations, with the range indicated by the vertical bars.

resulted from either parent compound or metabolites. In addition, inhibition of transporter activity may be a consequence of toxicity. Therefore, we investigated the effect of drugs on BSEP-mediated transport activity in membrane vesicles. Incubation of nefazodone with BSEP-expressed vesicles resulted in a concentration-dependent inhibition of taurocholic acid transport with $\text{IC}_{50} = 9 \mu\text{M}$ (Fig. 3), a result which was similar to that observed with human hepatocytes (Fig. 2), suggesting that the parent nefazodone directly inhibited BSEP-mediated bile acid transport. In the same experiment, glyburide was used as a positive control, which displayed an $\text{IC}_{50} = 2 \mu\text{M}$. We have previously shown the potent inhibitory effect of glyburide on taurocholate efflux in human hepatocytes, where glyburide at $10 \mu\text{M}$ inhibited taurocholate efflux by 70% (Kostrubsky *et al.*, 2003). In contrast, buspirone and trazodone produced no apparent inhibition of BSEP-mediated bile acid transport, when tested at concentrations as high as $100 \mu\text{M}$ (Fig. 3). Since increases in conjugated bilirubin were observed clinically, we tested whether nefazodone inhibited multidrug resistant associated protein-2 (MRP2), which is responsible for the canalicular excretion of conjugates including bilirubin, glutathione, bile salts, leukotrienes, and others. Nefazodone inhibited MRP2 with approximate $\text{IC}_{50} = 113 \mu\text{M}$. Buspirone and trazodone showed no inhibition up to the top concentration of $200 \mu\text{M}$. Previously, we and others have shown a transient increase in rat serum bile acids following iv administration of clinically hepatotoxic drugs (troglitazone, bosentan, CI-1034) that also inhibited bile efflux transport (Fattinger *et al.*, 2001; Funk *et al.*, 2001; Kostrubsky *et al.*, 2003). Often, rats are resistant to liver

toxicity, which is characterized by traditional markers including an increase in serum hepatic enzymes and histological changes. Assuming that a drug has a high affinity for transporter-mediated biliary elimination, serum bile acids could be increased due to inhibition of hepatic bile acid efflux into canaliculi by the drug. This may occur when the drug reaches the maximum plasma concentration, which might parallel the peak liver concentration. This increase could be transient and diminish with drug clearance. However, it provides a potential biomarker for predicting hepatic effects in humans as well. In contrast to previous studies that monitored serum bile acids after iv administration of test drugs, in the present work, nefazodone and buspirone were administered orally to mimic the standard preclinical toxicity study. Blood was collected at the approximate T_{max} time for both nefazodone and buspirone (Barbhैया *et al.*, 1995; Dockens *et al.*, 1996; Salazar *et al.*, 2001) and 24 h after dosing. As shown in Figure 4A, nefazodone increased serum bile acids by 60% after 1 h, and bile acids returned to control values by 24 h. No such response was detected for buspirone (Fig. 4B). Since there was a large animal-to-animal variation, as well as the difference in the pretest values, we repeated this experiment two more times. In the nefazodone group, the overall change in the pattern of serum bile acids characterized by an increase after 1 h followed by a decrease by 24 h was observed in all experiments. Specifically, the mean increases in serum bile acids after 1 h were 60%, 400%, and 40% in three separate experiments. This pattern was not found in the buspirone group. Insufficient amounts of trazodone prevented us from testing it along with nefazodone and buspirone. In these normal rats, we did not anticipate increase in serum conjugated

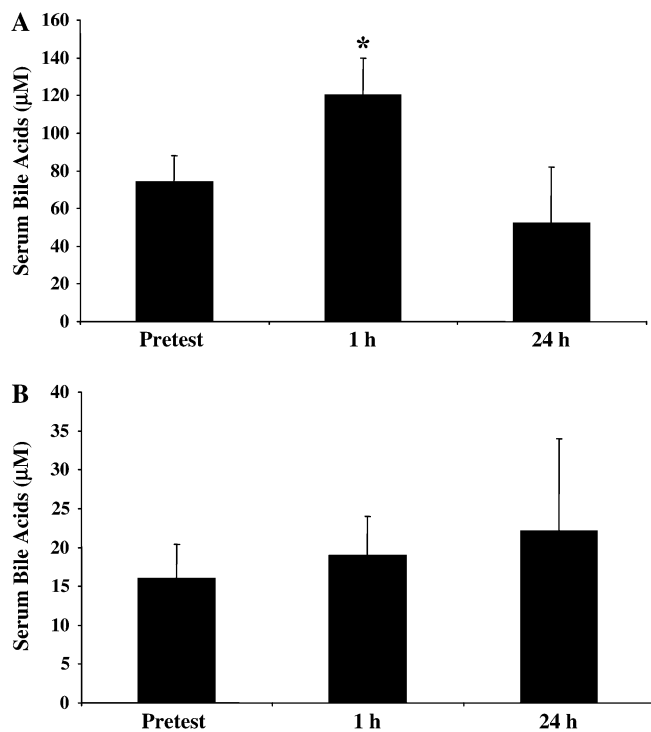


FIG. 4. Effect of nefazodone (A) and buspirone (B) on rat serum bile acids. A single oral dose of nefazodone (75 mg/kg) or buspirone (75 mg/kg) was administered by gavage to male Sprague-Dawley rats. Blood samples were collected at predose and 1 h and 24 h from the jugular vein, and serum was analyzed for the total bile acids using a Hitachi 911 analyzer. Each value represents the mean of three rats \pm SD. *Significantly different from predose and 24 h, with $p < 0.01$.

bilirubin, since rats appear to have high transport clearance rate of MRP2 substrates compared to other species (Ishizuka *et al.*, 1999) and do not always respond to treatment with cholestatic compounds. However, it is likely that MRP2-deficient (TR⁻) rats will experience a transient increase in bilirubin in response to nefazodone, similar to the increase we observed with troglitazone when it was administered by gavage as a single dose of 200 mg/kg (Kostrubsky *et al.*, 2001).

Thus, all three systems, sandwich human hepatocytes, expressed BSEP, and rat serum bile acids, demonstrated that nefazodone inhibits bile acid transport.

Effect of Nefazodone, Buspirone, and Trazodone on Toxicity in Human Hepatocytes

Next, we compared the effect of three drugs on cellular toxicity in cultured human hepatocytes by measuring total protein synthesis. This sensitive method allowed the detection of toxicity as early as 1 h after drug treatment, preceding enzyme release and morphological changes as we reported previously (Kostrubsky *et al.*, 2000). Hepatocytes were treated with nefazodone, buspirone, or trazodone at 10, 50, and 100 μ M for 6 h, and total protein synthesis was determined (Fig. 5). Nefazodone produced a concentration-dependent decrease in

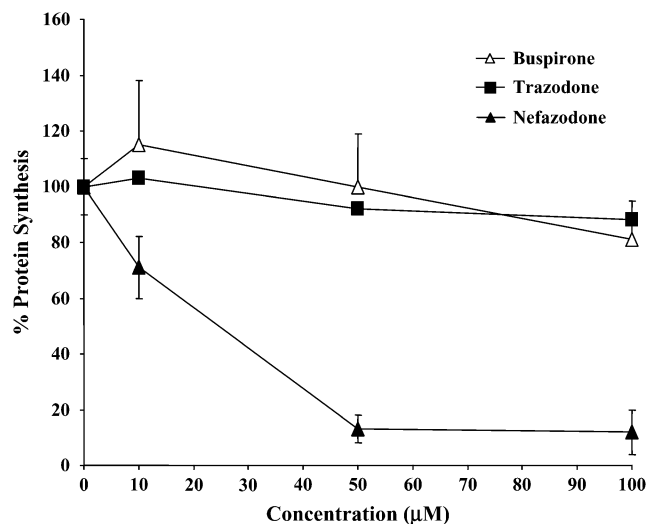


FIG. 5. Effect of nefazodone, buspirone, and trazodone on protein synthesis in human hepatocytes. Hepatocytes from a single donor were treated for 6 h with 10, 50, and 100 μ M of antidepressants. The protein synthesis was determined by pulse labeling with 14 C-leucine for the last incubation hour, as described in Materials and Methods. Each value represents the mean of duplicate treatments, with the range indicated by the vertical bars.

protein synthesis with an $IC_{50} = 18 \mu$ M. Concentrations equal to or exceeding 50 μ M resulted in morphological cell death observed microscopically. Buspirone and trazodone did not decrease protein synthesis at the concentration range tested. We hypothesized that inhibition of protein synthesis may recover after a longer incubation with nefazodone, due to metabolism of the parent drug. As shown in Fig. 6, nefazodone at 10 μ M produced 45%, 30%, and 5% decrease in protein synthesis after 1, 6, and 24 h incubation, respectively, indicating hepatocytes

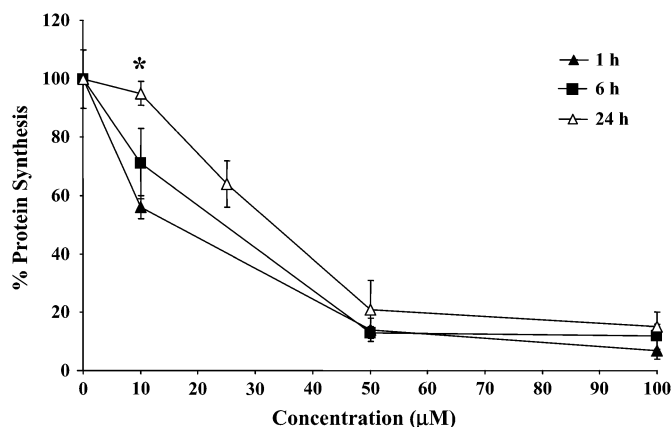


FIG. 6. Effect of nefazodone, buspirone, and trazodone on protein synthesis in human hepatocytes after 1-, 6-, or 24-h incubation. Hepatocytes from three donors were treated for 1, 6, or 24 h with 10, 50, and 100 μ M of antidepressants. The protein synthesis was determined by pulse labeling with 14 C-leucine for the last incubation hour, as described in Materials and Methods. Each value represents the mean of duplicate treatments, with the range indicated by the vertical bars. *Significantly different from 6 h, with $p < 0.01$.

recovery after 24 h. In contrast, nefazodone at 50 and 100 μM produced sustained inhibition of protein synthesis, resulting in cell death.

Metabolism of Nefazodone in Human Hepatocytes

To confirm the effect of parent drug on toxicity, we analyzed culture media from cells treated with 10 μM nefazodone for 6 and 24 h. There was a 90% decrease in the concentration of parent nefazodone by 24 h compared to 6 h incubations (Fig. 7). In contrast, there were no significant differences for 50 and 100 μM nefazodone between 6 and 24 h, suggesting that cellular metabolism was compromised, resulting in the lack of nefazodone clearance at these toxic concentrations. Analysis of metabolites in the media indicated that nefazodone underwent oxidative metabolism followed by conjugation with sulfate and glucuronide, with only trace amounts of unconjugated hydroxylated metabolites detected in the culture media (Fig. 8). Collision-induced dissociation of the molecular ions at $\text{MH}^+ = 566$ and $\text{MH}^+ = 662$, which corresponded to the sulfate and glucuronide conjugates of mono-hydroxynefazodone, indicated that conjugation had occurred on the hydroxyl group on the 3-chlorophenyl ring in nefazodone. Incubation for 24 h with 10, 25, 50, and 100 μM nefazodone demonstrated the saturation of metabolism, as evident from the diminished capacity of the hepatocytes to produce conjugated metabolites at 25 μM . Furthermore, there was a decrease in the amount of conjugates produced at 50 and 100 μM , most likely due to cell toxicity resulting in compromised drug metabolizing enzyme activities (Figs. 5 and 8). Thus, metabolism of nefazodone to conjugated products at 10 μM resulted in recovery of protein

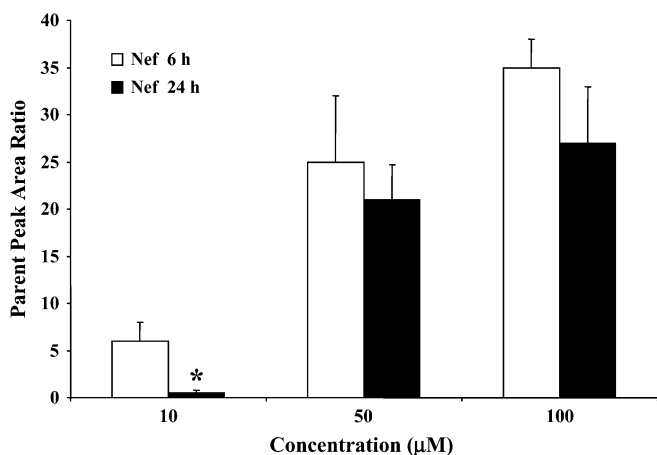


FIG. 7. Metabolism of nefazodone in human hepatocytes. Human hepatocytes from two donors (6 h) were treated for 6 or 24 h (one out of two used at 6 h) with 10, 50, and 100 μM nefazodone. Aliquots of the medium were removed, and the concentration of parent drug was determined as described in Materials and Methods. Results are shown as a peak area ratio of nefazodone to the internal standard. Each value represents the mean of duplicate treatments of hepatocytes from each of two donors, with the range indicated by the vertical bars. *Significantly different nefazodone 6 h, with $p < 0.001$.

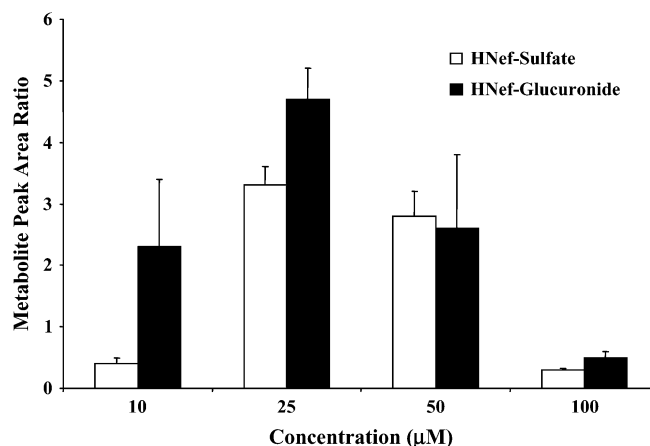


FIG. 8. Metabolism of nefazodone in human hepatocytes. Human hepatocytes from a single donor (used for 24 h in Fig. 7) were treated for 24 h with 10, 25, 50, and 100 μM nefazodone. Aliquots of the medium were removed, and concentrations of the sulfate and glucuronide conjugates of nefazodone were determined as described in Materials and Methods. Results are shown as a peak area ratio of nefazodone-conjugates to the internal standard. Each value represents the mean of quadruplicate treatments of hepatocytes, with the SD indicated by the vertical bars. HNef-sulfate, hydroxynefazodone-sulfate; HNef-glucuronide, hydroxynefazodone-glucuronide.

synthesis. In contrast, insufficient metabolism at ≥ 25 μM resulted in a sustained decrease in protein synthesis (40% at 25 μM) and cell death at 50 and 100 μM by 24 h.

To further characterize the importance of metabolism in preventing nefazodone toxicity, we treated hepatocytes with the combination of 10 μM nefazodone and 1 mM ABT, a non-specific inhibitor of P450 enzymes that has been shown previously to inhibit P450 activity in primary hepatocytes (Harrel *et al.*, 1994). Treatment with ABT and nefazodone (10 μM) for 24 h resulted in a 45% decrease in total protein synthesis (Fig. 9A). In contrast, there was almost full recovery in hepatocytes treated with 10 μM nefazodone alone (Fig. 9A). Analysis of culture media from cells treated with ABT showed an increase in the concentration of unmetabolized nefazodone associated with a decrease in formation of sulfate and glucuronide conjugates (Fig. 9B). Thus, inhibition of nefazodone metabolism enhanced toxicity and was associated with an accumulation of parent drug.

Metabolism of Nefazodone in Human Liver Microsomes

Incubation of human liver microsomes with nefazodone and ABT resulted in inhibition of nefazodone metabolism by 90% with a corresponding decrease in the formation of hydroxylated metabolites, thus confirming the role of ABT as a metabolic inhibitor observed in human hepatocytes. Since bioactivation of drugs to reactive metabolites is considered as one of the major risk factors for developing toxicity, and bioactivation can be modeled *in vitro* by the formation of drug-glutathione products, we also investigated whether nefazodone was capable of being metabolized to glutathione conjugates. Previously,

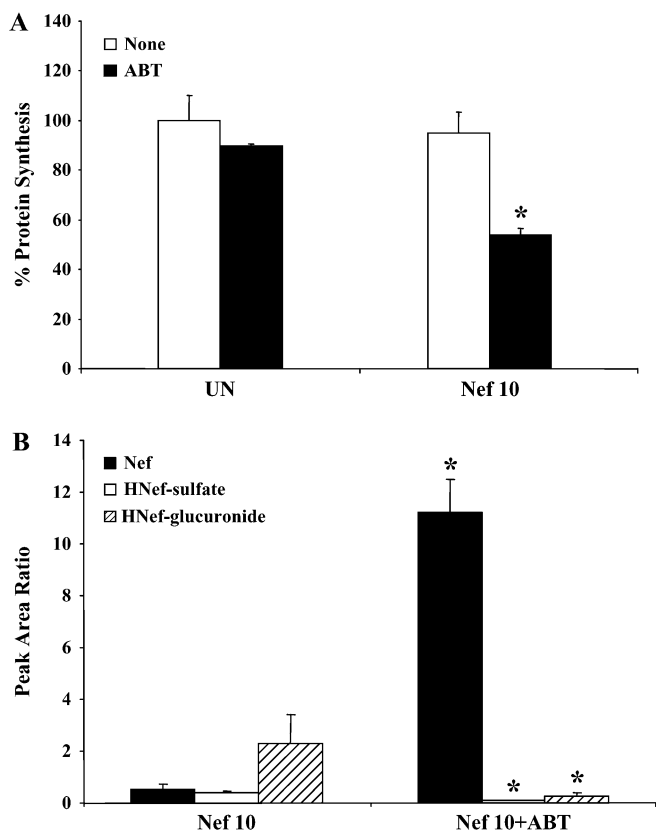


FIG. 9. Effect of ABT on toxicity (A) and metabolism (B) of nefazodone in human hepatocytes. Human hepatocytes from a single donor (shown in Fig. 8) were treated for 24 h with 10 μ M nefazodone alone or in combination with 1 mM ABT. The protein synthesis was determined by a pulse labeling with 14 C-leucine for the last incubation hour, as described in Materials and Methods. Aliquots of the medium were removed, and concentration of parent drug was determined as described in Materials and Methods. Each value represents the mean of quadruplicate treatments of hepatocytes, with the SD indicated by the vertical bars. Untreated cells (UN). *Significantly different from nefazodone treated cells, with $p < 0.001$. HNef-sulfate, hydroxynefazodone-sulfate; HNef-glucuronide, hydroxynefazodone-glucuronide.

bioactivation of nefazodone was demonstrated in human liver microsomes and recombinant CYP3A (Kalgutkar *et al.*, 2005). Incubation of nefazodone (25 μ M) in liver microsomes with glutathione resulted in the formation of a glutathione adduct of a hydroxylated nefazodone. This reaction was completely abolished by ABT. As we demonstrated with human hepatocytes, treatment with ABT and nefazodone potentiated toxicity due to accumulation of unmetabolized nefazodone, suggesting that metabolic bioactivation is not the mechanism responsible for nefazodone toxicity.

DISCUSSION

The critical link between biliary elimination and hepatotoxicity has been demonstrated in the bile duct-ligated rodent

models, where the toxicity of xenobiotics was significantly enhanced in animals with obstructed bile flow (Klaassen, 1973). In this study, our hypothesis was based on the major principle of toxicology stating that “the right dose differentiates a poison from a remedy” as was postulated by Paracelsus in 1541 AD. The initiating event for nefazodone hepatotoxicity may be an accumulation of drug and bile in the liver of certain individuals. Previously, we have shown that drugs that have strong inhibitory effects on bile acid transport also demonstrate clinical hepatotoxicity which is frequently not recognized in animal studies (Kostrubsky *et al.*, 2003). Now, with the greater number of compounds we analyzed, it is becoming clear that a combination of three factors are associated with clinical hepatotoxicity: high-molecular-weight compounds with expected biliary excretion of parent and metabolites, affinity for biliary transporters as shown by a concentration-dependent inhibition of bile acid transport *in vitro*, and projected human drug plasma concentration at or above 1 μ g/ml. Nefazodone meets all these criteria. In contrast, buspirone and trazodone did not have an inhibitory effect on bile acid efflux, suggesting a structure-specific interaction with transporters. The inhibitory effect of nefazodone on bile transport was mediated via BSEP in expressed system. However, in cultured hepatocytes and in humans other transporters may also contribute. In membrane vesicles, human MRP2 was inhibited by parent nefazodone, but at concentrations that are not pharmacologically relevant. Since nefazodone is metabolized to sulfate and glucuronide conjugates, the effect on MRP2 via metabolites could be much stronger compared to that found in membrane vesicles. It should be pointed out that the purpose of the screen proposed in this work is not to identify the individual transporters but to demonstrate the functional assay which is able to discriminate the effect of drugs on bile acid efflux. By screening the ability of drugs to inhibit bile transport at concentrations that will saturate active transporters (1–100 μ M), we can potentially identify hepatotoxic compounds including those that could cause an unexpected increase in liver transaminases or an idiosyncratic response. As we described here and as was previously reported for troglitazone (Funk *et al.*, 2001; Kostrubsky *et al.*, 2001) it appears that the common initiating event for these drugs is the inhibition of bile acid transport. Much work, however, needs yet to be done, particularly in conducting structure-toxicity studies to validate the predictive power of this methodology.

Concentrations of 2–4 μ M (1–2 μ g/ml) were reported as the maximum plasma concentrations in humans taking nefazodone at doses of 200–400 mg/day (Barbhaiya *et al.*, 1995; Dockens *et al.*, 1996). These concentrations could go disproportionately higher with increasing dose (Barbhaiya *et al.*, 1995). There is no information on plasma:liver ratio of nefazodone. However, it is likely that level of total drug including metabolites in the liver could be higher compared to plasma. In BSEP-expressed membranes and human hepatocytes, we found inhibition of bile acid transport at $IC_{50} = 9$ and 14 μ M, respectively, suggesting that nefazodone, if it accumulates in the liver, could

inhibit bile acid transport and its own elimination, resulting in progressively increasing drug and bile acid concentrations in hepatocytes of certain individuals. When administered for only 2 days, nefazodone did not cause hepatic changes in most patients, even at high doses with reported plasma concentration up to 9.6 $\mu\text{g/ml}$ (20.4 μM) (Barbhaiya *et al.*, 1995), suggesting that unique patient-specific factors including hepatic drug and bile acid accumulation over a longer time, genetic, and individual tissue repair response may play a role in a progression of toxicity in idiosyncratic cases. We also found that inhibition of bile transport occurred at concentrations lower than those where sustained toxicity in human hepatocytes was observed ($\text{IC}_{50} = 30 \mu\text{M}$). In addition, parent nefazodone inhibited bile acid transport in BSEP-expressed membranes at $\text{IC}_{50} = 9 \mu\text{M}$. These data would suggest that inhibition of bile transport occurs prior to cytotoxicity.

We found that nefazodone was metabolized to sulfate and glucuronide conjugates of mono-hydroxylated nefazodone. Conjugates were previously identified in human plasma but have not been characterized (Mayol *et al.*, 1994). Nefazodone conjugation was saturated at 25 μM and showed apparent decrease in metabolites produced at 50 and 100 μM (Fig. 8), concentrations that caused cell death. This suggests that the dose at which saturation of conjugation occurs represents a threshold of toxicity and perhaps could be monitored in humans by the level of conjugates.

Toxicity was associated with accumulation of unmetabolized nefazodone (Fig. 7). Inhibition of nefazodone metabolism by ABT also resulted in toxicity (Fig. 9), supporting the critical role of parent drug. Furthermore, incubation of human liver microsomes with nefazodone and glutathione produced an adduct, suggesting that nefazodone can be metabolically bioactivated to form reactive metabolites, as previously reported (Kalgutkar *et al.*, 2005). The formation of this adduct was fully prevented by ABT. However, this adduct was not observed in cultured human hepatocytes. This is not surprising, because the precursor (hydroxylated nefazodone) to the reactive intermediates appears to be rapidly glucuronidated or sulfated, hence preventing its further bioactivation. In addition, there was no evidence of reactive metabolites (GSH-related products) at 10–100 μM in either cell lysates or culture media, suggesting that neither recovery in protein synthesis nor toxicity at 50 μM and 100 μM was due to the formation of reactive metabolites. The total absence of any glutathione-related adduct of nefazodone in human hepatocytes, combined with the results from ABT study showing decreased metabolism, high parent concentration and enhanced toxicity, suggests that unmetabolized nefazodone, and not the products of bioactivation, is responsible for hepatotoxicity.

Serum bile acids were increased at T_{max} time in rats administered a single oral dose of nefazodone. Subsequently, the bile acids returned to control levels after 24 h. This transient increase in serum total bile acids can be used as another signal for a compound's potential to cause human hepatotoxicity.

In conclusion, we ranked three drugs according to their ability to inhibit bile acid transport and cause *in vitro* toxicity. Nefazodone was a potent inhibitor of bile transport. Nefazodone also caused toxicity in human hepatocytes associated with saturation of conjugation and accumulation of parent drug. We propose that the testing approach described in this work, for compounds with a substantial portion of biliary clearance, can be used to predict the propensity of a drug to cause human hepatotoxicity. Such an approach should also be beneficial in rank-ordering potential new drug candidates for further advancement in development.

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