Transport, Metabolism, and Hepatotoxicity of Flutamide, Drug–Drug Interaction with Acetaminophen Involving Phase I and Phase II Metabolites

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Treatment with flutamide has been associated with clinical hepatotoxicty. The toxicity, metabolism,and transport of flutamide were investigated using cultured human hepatocytes. Flutamide and its major metabolite, 2-hydroxyflutamide, caused an inhibition of taurocholate efflux in human hepatocytes with an $IC_{50} = 75\ \mu M$ and 110 μM, respectively. Treatment of hepatocytes with flutamide or 2-hydroxyflutamide for 24 h resulted in time- and concentration-dependent toxicity as assessed by inhibition of protein synthesis. Toxicity was greater after 1 h than after 24 h of treatment. Recovery in inhibition of protein synthesis by 24 h was attributed to the decreased presence of flutamide due to its metabolism. Flutamide was metabolized by hepatocytes to several metabolites, and formation of reactive intermediates of flutamide, as evidenced by the presence of glutathione-related adducts, was observed. Inhibition of flutamide metabolism by 1-aminobenzotriazole (ABT) resulted in enhancement of flutamide toxicity, which was associated with sustained levels of nonmetabolized drug. ABT also prevented the formation of reactive intermediates of flutamide. There was an additive toxicity when cells were treated with a combination of flutamide and 2-hydroxyflutamide. Simultaneous treatment with flutamide and acetaminophen (APAP) resulted in additive to synergistic toxic effects. Flutamide and APAP were found to have significant effects on each other's metabolism. Flutamide inhibited glucuronidation and sulfation of APAP, resulting in greater amounts of APAP available for bioactivation. APAP inhibited the hydroxylation of flutamide, and subsequent sulfation and acetylation of 4-nitro-3-(trifluoromethyl) aniline, a metabolite of flutamide. In summary, we suggest that inhibition of bile acid efflux by flutamide and its 2-hydroxy metabolite may play a role in flutamide-induced liver injury. Both flutamide and 2-hydroxyflutamide are responsible for cytotoxicity if not metabolized. The data also suggest a possible drug–drug interaction between flutamide and APAP, resulting in inhibition of flutamide metabolism and increased APAP bioactivation and toxicity.

Introduction

Flutamide, a nonsteroidal antiandrogen introduced in 1989, has been associated with severe hepatotoxicity. According to the FDA MedWatch Spontaneous Reporting System, the rate of flutamide-induced hepatotoxicity is about 3 per 10000 patients, which greatly exceeded the expected rate of hospitalization of 2.5 per 100000 men 65 years or older (*1*). The Spanish Pharmacovigilance System collected 88 reports of flutamide hepatotoxicity, mostly recorded as hepatitis and cholestatic hepatitis (for review of clinical cases, see refs (*2, 3*)). The common clinical symptoms of severe hepatotoxicity included an increase in serum activities of liver enzymes such as ALT (30X), AST (30X), and ALP (7X) and of total bilirubin concentrations (16×) with >60% detected as conjugated bilirubin, resulting in jaundice. The most common liver pathology was hepatic necrosis and cholestasis. Mild elevations of serum transaminases were also frequently detected without progression to drug-induced hepatitis (*4–7*). The characteristics of hepatotoxicity after a prescribed daily dose of 750 mg flutamide (250 mg three times daily) included a latency period before the onset of symptoms that ranged from 14 to 300 days (the mean of approximately 16 weeks) and a recovery period of approximately 9 weeks (*1–3*).

Several metabolites of flutamide have been previously characterized in human plasma and urine. 2-Hydroxyflutamide (M1) was found to be the major and most abundant pharmacologically active metabolite (*5, 6, 8*). The formation of M1 has been demonstrated to be mediated by CYP1A2 (*7, 8*). The steady state plasma concentrations of flutamide and M1 reported in humans are 113 ng/mL and 1629 μg/mL, respectively, with elimination half-lives of 7.8 and 9.6 h, respectively (*9*). Although original product label information suggested that flutamide and its metabolites were excreted mainly in the urine (*9*), later studies showed that only about 3% of flutamide and its metabolites, including conjugates, were eliminated in the urine collected for 3 h after oral intake, suggesting that biliary excretion plays a significant role in flutamide clearance (*6, 8*). Overall, clinical

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symptoms of hepatotoxicity associated with flutamide treatment were similar to those described previously for troglitazone and nefazodone, compounds that have produced idisyncratic liver toxicities. We have previously investigated the metabolism and inhibition of biliary transport as playing a role in adverse hepatic reactions produced by these compounds (*10, 11*). On the basis of the results of experiments described here, we propose that flutamide-mediated hepatoxicity is linked to the ability of the parent drug and its metabolite, 2-hydroxyflutamide, to inhibit bile acid transport and its own metabolism, resulting in sustained levels of bile acids and/or flutamide and 2-hydroxyflutamide in the liver.

Materials and Methods

Chemicals and Supplies. HMM (modified Williams E) culture medium and supplements were from BioWhittaker (Walkersville, MD). Pooled human liver microsomes (Lot HL-Mix-101, 20.4 mg/mL) were purchased from BD Gentest (Woburn, MA). 1-Aminobenzotriazole (ABT), 4-acetamidophenol (acetaminophen, APAP), NADPH, $MgCl_2$, and phosphate buffer (pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO). Flutamide and 2-hydroxyflutamide were obtained from LKT Laboratories (St. Paul, MN). HPLC-grade water, methanol, and acetonitrile were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). 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. Hepatocytes were isolated by three-step collagenase perfusion as described previously (*12*). The viability of cells obtained, as measured by trypan blue exclusion, ranged from 74 to 90%. Hepatocytes were plated in Williams E medium supplemented with 1×10^{-7} M dexamethasone, 1×10^{-7} M insulin, 100 units/mL penicillin G, 100 μg/mL 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. After 72 h in culture, cells were treated with flutamide for 1 or 24 h at concentrations shown in the figure legend. Where indicated, APAP and ABT were added simultaneously with flutamide. After 1 or 24 h incubation, media was collected and analyzed for flutamide and APAP metabolites.

These culture conditions maintain levels of phase I and phase II drug metabolizing activities in hepatocytes after 96 h in culture as demonstrated for several substrates (*13, 14*), and at the same time minimized differences among activities in control cells prepared from different donors (*15*). Toxicity was assessed by the measurement of total protein synthesis by pulse-labeling hepatocytes for 1 h with [^{14}C]leucine, as described previously (*10, 11*). All values were normalized per amount of cellular protein. Test compounds were added to cells in culture medium containing 0.1% DMSO.

Transport Assays. Inhibition of bile transport in cultured sandwich hepatocytes with developed canaliculi was conducted according to previously described protocols (*11, 16*). Briefly, 1 μM [^{3}H]taurocholic acid, with or without increasing concentrations of test compound, was added to hepatocytes for 15 min at 37 °C. After stopping transport by removing buffer and washing cells, taurocholate egress from canalicular spaces was initiated by adding standard or Ca/Mg^{2+}-free buffer for 10 min. Aliquots of media were harvested and counted in a liquid scintillation counter. The difference in amount of radioactivity between the two buffer conditions in the absence of tested compound was defined as 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 (*11, 16*). All values were normalized per amount of total cellular protein.

Analysis of Culture Media. The 1 and 24 h culture media were assayed for the presence of metabolites of flutamide and APAP. Aliquots of media (100 μL) were transferred to a clean polypropylene tube and internal standard (4-chloroacetanilide) was added. The samples were centrifuged and an aliquot (20 μL) injected onto an LC/MS without any further sample preparation. Peak area ratios for the analytes to the internal standard were obtained via via mass spectral analysis (see below) and comparisons made between various treatment groups. Full scan and MS/MS analyses were also performed on these samples to obtain the metabolite profiles and confirm the identities of metabolites.

Microsomal Metabolism of Flutamide and 2-Hydroxyflutamide. The metabolism of flutamide and its major metabolite, 2-hydroxyflutamide, was investigated using human liver microsomes. Flutamide or 2-hydroxyflutamide (50 μM) was incubated in the presence of human liver microsomes (1 mg/mL), $MgCl_2$ (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) or ABT (1 mM) was added to the incubation mixtures. The samples were preincubated for 5 min prior to initiating the reaction by adding substrate. Incubations were also performed without the addition of NADPH as a control for possible non-P450 degradation of flutamide. At the end of the incubation, the microsomal proteins were precipitated by adding 2 mL of ice-cold acetonitrile, followed by centrifugation at 3000 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: ammonium acetate (5:95 v/v; pH 3.5). An aliquot (40 μL) was injected onto LC/MS using the conditions described below.

Inhibition of APAP Glucuronidation Mediated by Various rUGTs in the Presence of Flutamide. The ability of flutamide to inhibit UGTs responsible for APAP glucuronidation was evaluated using the methods previously described using 1 mM APAP as substrate (*13*).

Liquid Chromatography–Mass Spectrometry (LC/MS) Analysis of APAP and Flutamide Metabolites. 1. Flutamide Metabolites. The microsomal incubation extracts or the media from the hepatocyte studies was injected onto a HPLC column (Synergi 4μ Hydro-RP C18, 150 × 2.0 mm, Phenomenex). The metabolites of flutamide were separated on the HPLC column using a gradient solvent system consisting of acetonitrile and 10 mM ammonium acetate (pH 3.5) with the flow rate set at 0.27 mL/min. The initial mobile phase consisting of a mixture of acetonitrile and ammonium acetate (5:95 v/v) was maintained for 2 min after the sample was injected. The percentage of acetonitrile was increased linearly from 5 to 80% in the next 18 min. After an additional 5 min at 80% acetonitrile, the column was re-equilibrated with the initial mobile phase for 10 min before the next injection.

The HPLC system was coupled to a LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), equipped with an electrospray source. The capillary temperature and voltage were set at 370 °C and −29 V, respectively. The mass spectrometer was operated in the negative ion mode with the electrospray needle kept at −4600 V. Ultrapure nitrogen was used as the nebulizer and curtain gas. The mass spectrometer was operated either in the full scan or multiple reaction monitoring (MRM) modes for qualitative and semiquantitative analyses, respectively. MRM analysis was carried out using nitrogen as the collision gas. The relative collision energy was kept at 30 eV. Other parameter settings for the MRM analyses included arbitrary values of 55, 20, and 10 for sheath, auxiliary, and sweep gases (ultrapure nitrogen), respectively. The mass transitions for the metabolites and the internal standard were: 168 → 126 (4-chloroacetanilide, internal standard), 205 → 175 (Flu-1 or M3), 247 → 205 (Flu-2 or M4), 275 → 205 (flutamide), 291 → 205 (OH-Flu or M1 or M2), and 301 → 221 (Flu-3 sulphate or M6). The peak areas from each of these transitions were obtained and ratios of the analyte to the internal standard obtained for each sample. The identities of these flutamide metabolites were based on previously reported data (*5, 17*). Additionally, the GSH-related adducts of flutamide were monitored by full scan mass spectral analyses for characterization purposes. MRM analyses of these GSH-related adducts were also conducted to obtain semiquantitative information on the relative levels of these metabolites under

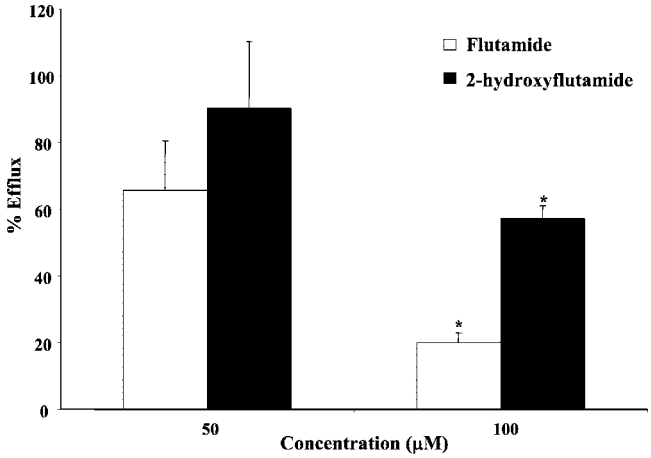

Figure 1. Effect of flutamide and 2-hydroxyflutamide on taurocholate efflux in sandwich cultured human hepatocytes. Hepatocytes from three human donors were treated with 1 μM [^{3}H]taurocholic acid alone or in combination with increasing concentrations of flutamide or 2-hydroxyflutamide for 15 min. 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 SD indicated by the vertical bars. Treatments also were conducted at 10 and 25 μM and used in calculation of IC_{50}. These treatments produced no decrease in taurocholate efflux. * = Significantly different from corresponding 50 μM flutamide and 2-OH flutamide with $P < 0.01$.

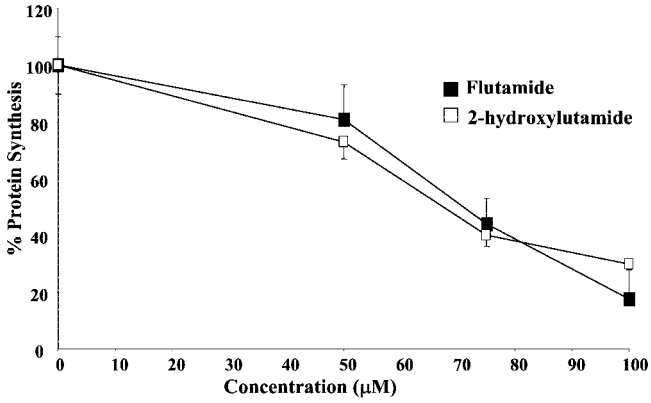

Figure 2. Effect of flutamide and 2-hydroxyflutamide on protein synthesis in human hepatocytes. Hepatocytes from two donors were treated for 24 h with 50, 75, and 100 μM of fluatmide or 2-hydroxyflutamide. The protein synthesis was determined by pulse labeling with [^{14}C]leucine for 1 h, as described in Materials and Methods. Each value represents the mean of duplicate treatments of hepatocytes from each of two donors, with the range indicated by the vertical bars.

different incubation conditions. The mass transitions for these metabolites were: 394 → 273 (cysteine), 451 → 307 (cysteinylglycine), and 580 → 307 (glutathione). The identities of the GSH adduct (MH^+ at *m/z* 580) and its cleaved product, cysteinylglycine conjugate (MH^+ at *m/z* 451), have recently been described in detail (*18*). The MS/MS data obtained for the GSH and cysteinylglycine adducts in this study were found to be identical to those reported by these investigators and are reported as supplementary data. The proposed GSH-related adducts of flutamide identified by mass spectral analyses are shown in Figure 5.

2. APAP Metabolites. Aliquots of reconstituted microsomal incubation extracts or hepatocyte media were injected directly onto an HPLC column (Aqua C18, 150 × 2.0 mm, Phenomenex) and the metabolites separated chromatographically using the previously described method (*13*). The eluent from the HPLC system was introduced directly to a LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), equipped with an electrospray source. The capillary temperature and voltage were set at 370 °C and 16

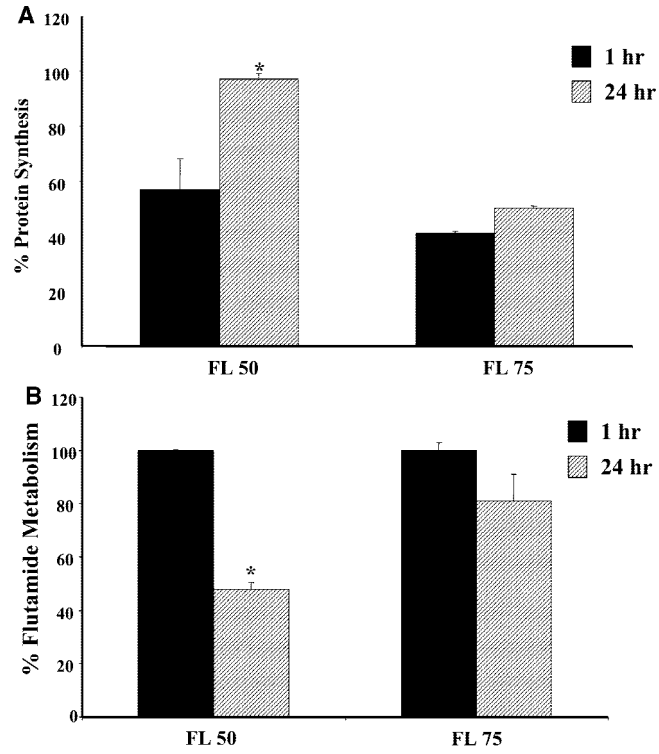

Figure 3. Effect of flutamide on protein synthesis (A) and metabolism of flutamide (B) after 1 or 24 h incubation in human hepatocytes. Hepatocytes from two donors were treated for 1 or 24 h with 50 or 75 μM flutamide. The protein synthesis was determined by pulse labeling with [^{14}C]leucine for one incubation hour, as described in Materials and Methods. Aliquots of the medium were removed after 1 or 24 h incubation, and the concentration of flutamide was determined as described in Materials and Methods. Results are shown as a percent of remaining flutamide detected in the culture media after 1 or 24 h incubation. 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 from 1 h with $P < 0.001$.

V, respectively. The mass spectrometer was operated in the positive ion mode with the electrospray needle maintained at 4500 V. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode for semiquantitative analyses. MRM analysis was carried out using nitrogen as the collision gas. The relative collision energy was kept at 30 eV. Other parameter settings for the MRM analyses included arbitrary values of 55, 20, and 10 for sheath, auxiliary, and sweep gases (ultrapure nitrogen), respectively. The mass transitions for the APAP metabolites and the internal standard were: 170 → 128 (4-chloroacetanilide, internal standard), 232 → 152 (APAP sulfate), 271 → 140 (cysteine conjugate), 328 → 152 (APAP glucuronide), 328 → 182 (cysteinylglycine), and 457 → 328 (APAP glutathione). The peak areas from each of these transitions were obtained and ratios of the analyte to the internal standard obtained for each sample. The identities of APAP metabolites were based on previously reported data (*20*).

Statistical Analysis and IC_{50} Determination. Results were analyzed by a two-factor ANOVA. A $p < 0.05$ was interpreted as the level of statistical significance.

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

Results

Effect of Flutamide and 2-Hydroxyflutamide on Bile Acid Efflux in Sandwich Human Hepatocytes. Inhibition of bile acid eflux in vitro is associated with clinical hepatotoxicity

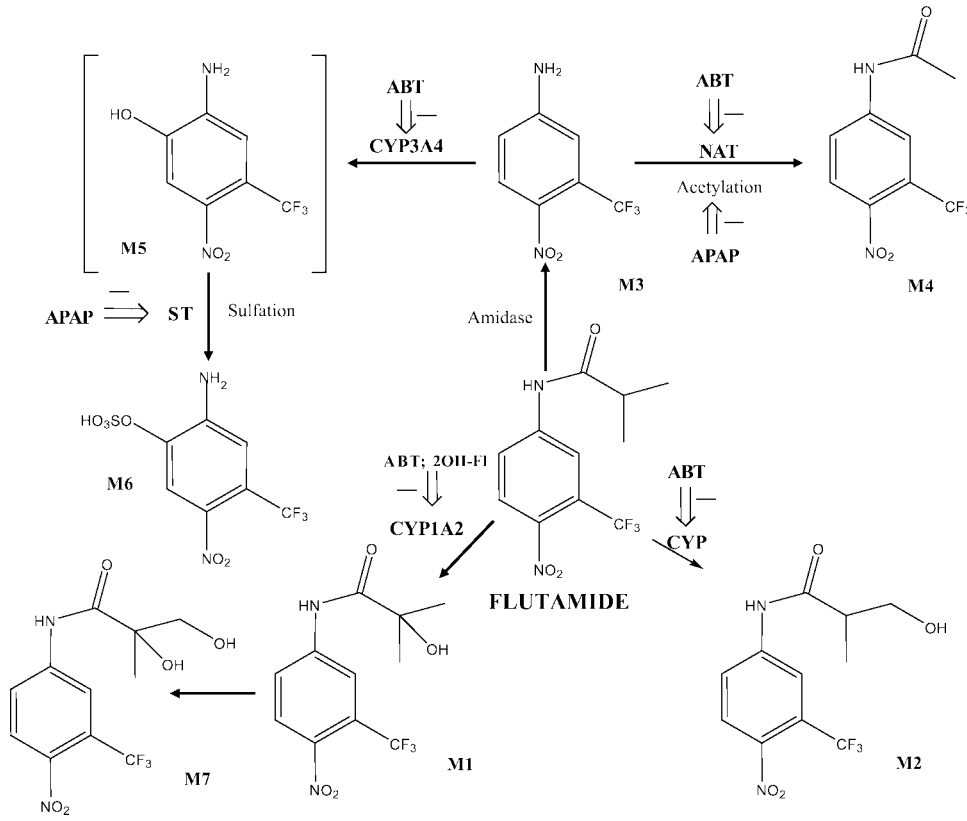

Figure 4. Flutamide metabolism. CYP, cytochrome P450; ST, sulfotransferase; NAT, *N*-acetyltransferase. Inhibitors of metabolism are shown as ABT, 1-aminobenzotriazole; APAP, acetaminophen; 2OH-Fl, 2-hydroxyflutamide.

(*11, 16*). This assay does not measure the cytotoxicity in cultured cells but suggests the clinical hepatotoxic potential of tested compounds. Therefore, we investigated whether flutamide and 2-hydroxyflutamide inhibit efflux of exogenously added bile acid into canaliculi. Incubation of cultured human hepatocytes prepared from three different donors with flutamide resulted in concentration-dependent inhibition of taurocholic acid efflux in canaliculi with an $IC_{50} = 75$ μM (Fig. 1). Treatment of hepatocytes with 2-hydroxyflutamide also caused inhibition of taurocholic acid transport, but this metabolite was less potent with $IC_{50} = 110$ μM. These data suggest that bile acids and flutamide share the transport system responsible for biliary clearance. Inhibition of this transport mechanism may result in their mutual hepatic accumulation.

Effect of Flutamide and 2-Hydroxyflutamide on Toxicity in Human Hepatocytes. Metabolism of Flutamide. Because there is a potential for flutamide and its metabolite to accumulate in the liver, creating conditions for hepatotoxicity, we next investigated the effect of flutamide and 2-hydroxyflutamide on cellular toxicity in cultured human hepatocytes by measuring total protein synthesis. Effects on these parameters were observed as early as 1 h after drug treatment, and preceded enzyme release and morphological changes as reported previously (*10*). Cultured hepatocytes were treated with flutamide or 2-hydroxyflutamide at 50, 75, and 100 μM concentrations for 24 h, and the effects on total protein synthesis were determined (Fig. 2). Both flutamide and 2-hydroxyflutamide produced similar decreases in protein synthesis with $IC_{50} = 75$ μM. Morphological evidence for cell death was observed microscopically at a 100 μM concentration of flutamide or 2-hydroxyflutamide. We hypothesized that inhibition of protein synthesis may recover after a longer incubation with flutamide because of metabolism of the parent drug. As shown in Figure 3A, flutamide at 50 and 75 μM produced 43 and 60% decreases in protein synthesis after 1 h of treatment, respectively. In contrast, continued incubation for 24 h resulted in full recovery of cells treated with 50 μM flutamide, whereas there was little recovery in cells exposed to 75 μM (Figure 3A) ,suggesting the possible role of the parent drug in toxicity. Culture media from cells treated with flutamide for 1 or 24 h was analyzed for parent drug and metabolites. There was a 50% decrease in the concentration of parent flutamide after 24 h treatment compared to 1 h treatment with 50 μM flutamide (Figure 3B). In contrast, there was no significant difference in metabolism of 75 uM flutamide over these times. Thus, toxicity at 50 μM correlated with the high level of flutamide at the early time point (1 h) with recovery of cells after significant clearance of flutamide at 24 h. In contrast, flutamide at 75 μM exceeded the threshold of toxicity, and the compromised cellular metabolism resulted in a lack of flutamide clearance and sustained toxicity at 24 h.

Analysis of metabolites in the media indicated that flutamide underwent oxidative metabolism with the formation of M1 (major) and M2 (minor) metabolites. Flutamide underwent hydrolysis, catalyzed by an amidase(s) to M3, which in turn was hydroxylated and conjugated to form a sulfate conjugate (M6). Alternatively, M3 was further acetylated to form M4 (Figure 4). Other metabolites that were detected in the hepatocyte media included dihydroxylated flutamide (M7), GSH-related adducts (Figure 5), and trace levels of glucuronide conjugates of hydroxylated flutamide. Many of these metabolites have been previously described in the literature (*5, 6, 17, 18*), and their detailed characterization will not be described in this report. Treatment of hepatocytes for 24 h with 50 or 75 μM flutamide demonstrated saturation of metabolism at 50 μM since the level of metabolites produced at 75 μM was similar to or less than those produced at 50 μM flutamide. Thus, metabolism of 50 μM flutamide over 24 h resulted in recovery of protein

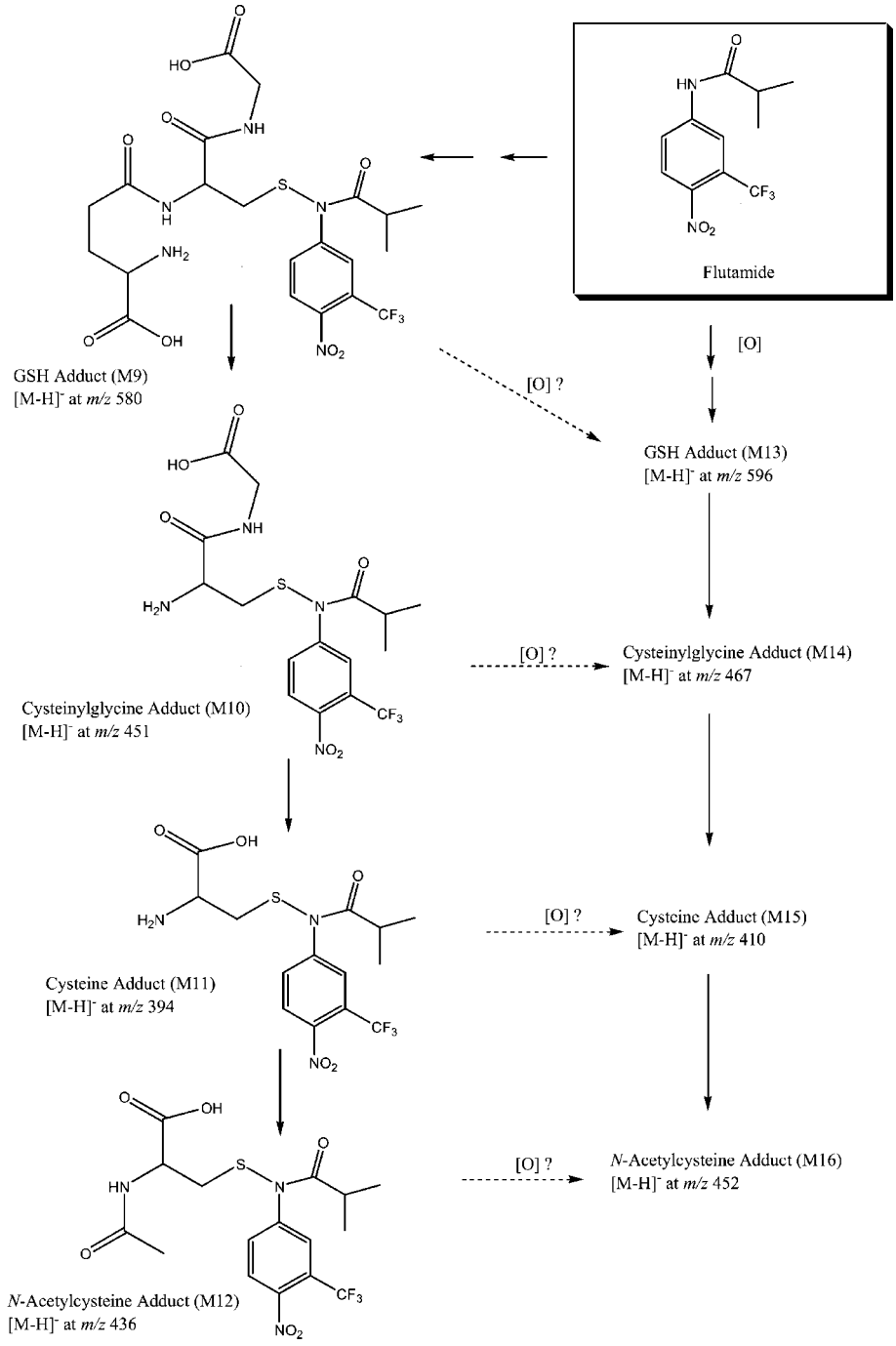

Figure 5. Bioactivation of flutamide leading to GSH-related adducts in human hepatocytes. The structures of metabolites M9 and M10 are based on comparison with previously published data in literature (*19*). The identities of M9–M16 are based on the observed pseudomolecular ions, MS/MS data, and the known catabolic pathway for GSH adducts.

synthesis. In contrast, insufficient metabolism at ≥75 μM resulted in a sustained decrease in protein synthesis and cell death by 24 h.

Reactive Metabolites of Flutamide. Metabolites (M1–M6) detected in culture media (Figure 4) were consistent with what has been detected in human subjects dosed with flutamide (*5, 6, 18*). However, some reactive metabolites biologically trapped and identified as GSH-related adducts of flutamide were found in all human cultures (Figure 5). A GSH adduct of flutamide showed an $[M - H]^-$ at *m/z* 580 and had an HPLC retention time and mass spectral fragmentation pattern identical to those an adduct identified in human liver microsomes. In addition, cysteinylglycine, M10 (*m/z* 451), cysteine, M11 (*m/z* 394), and *N*-acetylcysteine, M12 (*m/z* 436) conjugates were also present in the culture media. Two of these metabolites, the GSH ($[M - H]^-$ at *m/z* 580) and the cysteinylglycine ($[M - H]^-$ at *m/z* 451) adducts have recently been reported in the literature in which the GSH adduct was isolated from human liver microsomes and characterized by mass spectral and NMR analysis (*18*). The MS/MS data obtained for both of these two conjugates found in human liver microsomes and hepatocytes in our study matched with those reported by these investigators (see the Supporting Information). The structures of adducts based on these data are shown in Figure 5. Other GSH adducts, probably derived from hydroxylated flutamide, were also observed in all hepatocyte cultures (M13–M16, Figure 5). Microsomal or hepatocyte incubations with 2-hydroxyflutamide (M1) did not produce any GSH adduct with the same molecular

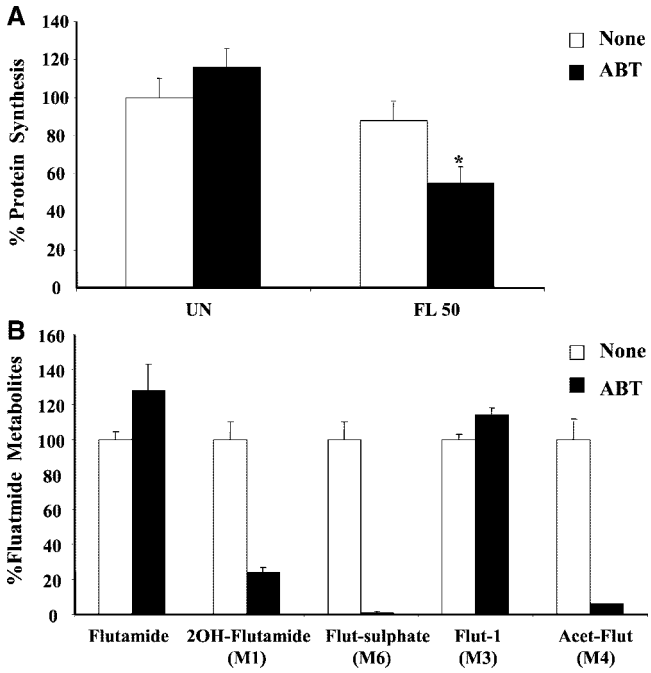

Figure 6. Effect of ABT on toxicity (A) and metabolism (B) of flutamide in human hepatocytes. Human hepatocytes from three donors were treated for 24 h with 50 μM flutamide alone or in combination with 1 mM ABT. The protein synthesis was determined by a pulse labeling with [^{14}C]leucine for one incubation hour, as described in Materials and Methods. Aliquots of the medium were removed, and concentrations of parent drug and metabolites were determined as described in Materials and Methods. Results are shown as a percent of flutamide or flutamide metabolites detected in the culture media in the absence or presence of ABT. Each value represents the mean of duplicate treatments of hepatocytes with the SD indicated by the vertical bars. Untreated cells (UN). * = Significantly different from flutamide alone treated cells, with $P < 0.01$.

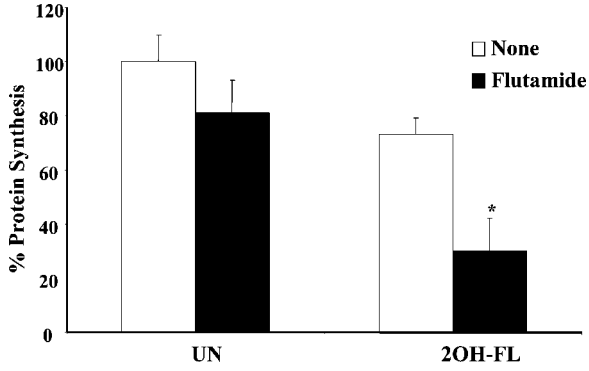

Figure 7. Effect of combined treatment with flutamide and 2-OH flutamide on protein synthesis in human hepatocytes. Hepatocytes from three donors were treated for 24 h with 50 μM fluatmide or 2-OH flutamide alone or in combination. The protein synthesis was determined by pulse labeling with [^{14}C]leucine for 1 h, as described in Materials and Methods. Each value represents the mean of duplicate treatments of hepatocytes from each of two donors, with the SD indicated by the vertical bars. * = Significantly different from either flutamide or 2-OH flutamide treated cells with $P < 0.001$.

weight as M9 ([M – H]$^-$ at *m/z* 580). However GSH-related adducts with [M – H]$^-$ at 596 (GSH adduct), 467 (cysteinylglycine), 410 (cysteine), and 452 (*N*-acetylcysteine) were detected in these incubations (Figure 5). Treatment with 50 or 75 μM flutamide for 24 h resulted in the formation of similar amounts of metabolites despite the fact that there was toxicity only in cells treated with 75 μM flutamide

Effect of ABT and 2-Hydroxyflutamide on Flutamide Toxicity and Metabolism. To further assess if metabolism

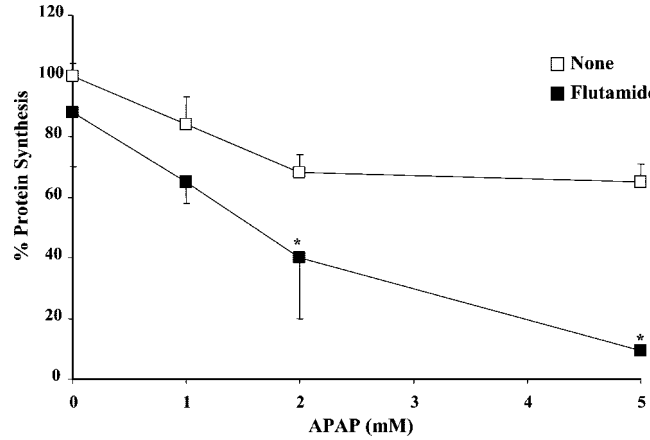

Figure 8. Effect of flutamide and acetaminophen on protein synthesis in human hepatocytes. Hepatocytes from two donors were treated for 24 h with either APAP alone or in the combination with 50 μM of flutamide. The protein synthesis was determined by pulse labeling with [^{14}C]leucine for 1 h, as described in Materials and Methods. Each value represents the mean of duplicate treatments with the range indicated by the vertical bars. * = Significantly different from APAP alone, with $P < 0.01$.

protects hepatocytes from toxicity, we treated hepatocytes with a combination of 50 μM flutamide and 1 mM ABT, a nonspecific inhibitor of CYP enzymes that has been shown previously to inhibit P450 activity in primary hepatocytes (*11, 20*). Simultaneous treatment with the combination of ABT and flutamide for 24 h resulted in a 35% decrease in total protein synthesis as compared to either treatment alone (Figure 6A). In contrast, there was almost full recovery in hepatocytes treated with 50 μM flutamide alone (Figure 6A). Analysis of culture media from cells treated with ABT showed an increase in the relative concentration of unmetabolized flutamide by about 30%. ABT inhibited all the CYP-mediated pathways of flutamide metabolism (Figure 4, 5). The levels of M1, M2, and M4–M16 were reduced significantly. ABT almost totally eliminated the formation of M6 by reducing the formation of its precursor, M5. ABT also inhibited acetylation of M3 to M4 (Figure 6B), resulting in an increased amount of M3 (Figure 6B). Thus, inhibition of flutamide metabolism enhanced toxicity and was associated with an accumulation of parent drug and M3. ABT treatment also eliminated the formation of flutamide–GSH metabolites.

The major plasma metabolite of flutamide is 2-hydroxyflutamide, which accounts for 23% of the plasma tritium-labeled drug 1 h after flutamide administration (*9*). We found that 2-hydroxyflutamide inhibited bile acid transport and caused toxicity equivalent to that of flutamide in human hepatocytes. Therefore, we investigated whether there was a potentiation of toxicity if 2-hydroxyflutamide and flutamide were present together. As shown in Figure 7, treatment of cultured hepatocytes prepared from three donors with the combination of both compounds at 50 μM for 24 h resulted in an average 65% decrease in protein synthesis, which suggests that flutamide and M1 potentiate the toxic effect of each other. This decrease in protein synthesis paralleled a respective 40 and 30% increase in unmetabolized flutamide and 2-hydroflutamide compared to their extents of metabolism when applied individually. Thus, hepatic accumulation of flutamide may also result from 2-hydroxyfluatmide-mediated inhibition of flutamide metabolism by CYP1A2 (*7*).

Flutamide–Acetaminophen Drug–Drug Interaction. 1. Toxicity. Because flutamide is metabolized via CYP1A2 and CYP3A4 and by sulfation (Figure 4), we investigated the

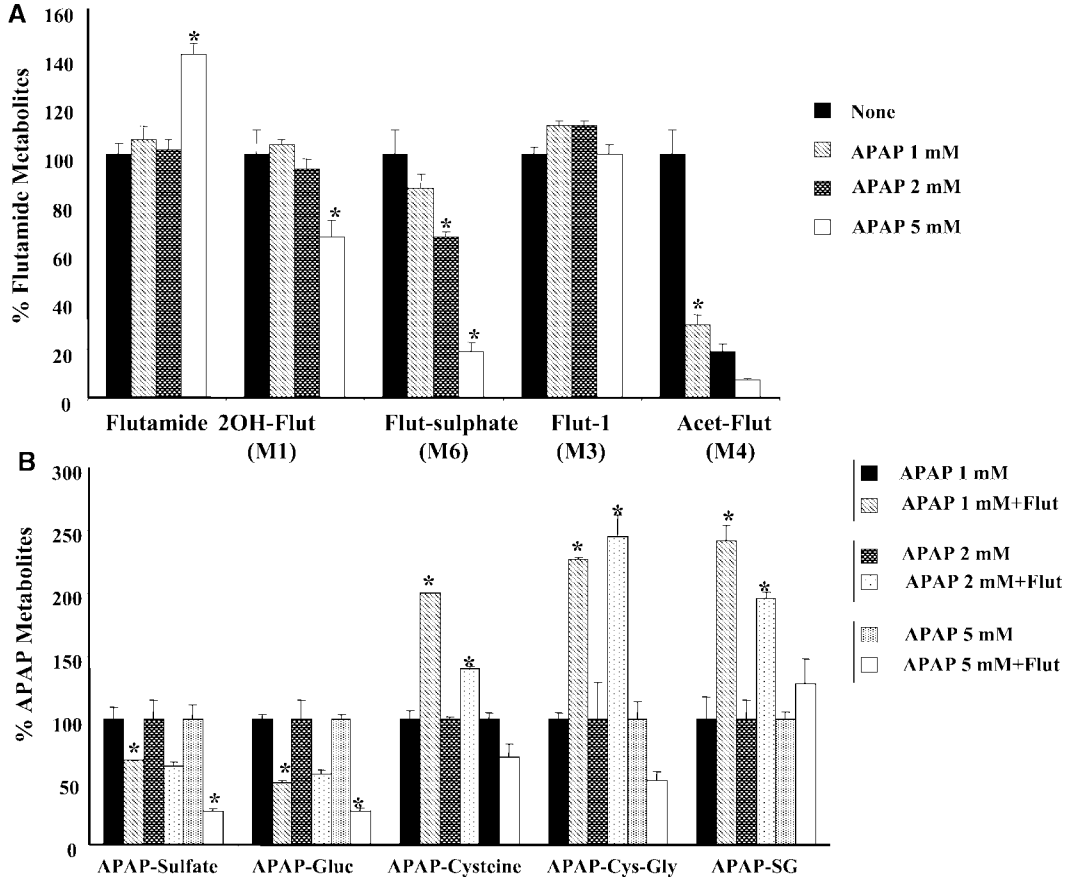

Figure 9. Metabolism of flutamide (A) and acetaminophen (B) in human hepatocytes. Hepatocytes were treated for 24 h as shown in Figure 8. Aliquots of the medium were removed, and the concentration of flutamide, metabolites of flutamide, APAP, and metabolites of APAP were determined as described in Materials and Methods. Results are shown as a percent of flutamide and flutamide metabolites (A) detected in the culture media in the absence or presence of APAP or APAP metabolites and (B) detected in the culture media in the presence or absence of flutamide. 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 from correspondent controls with $P < 0.001$. APAP-Gluc, APAP-glucuronide; APAP-Cys-Gly, APAP-cysteine-glycine; APAP-SG, APAP-glutathione.

Table 1. GSH-Related Adducts of Flutamide Found in Human Hepatocyte Incubation Extracts

metabolite	$[M - H]^-$	MS/MS fragment ions
M9 (GSH conjugate)[a]	580	562(B), 451, 307, 306, 288, 272, 254, 210, 205, 179
M10 (cysteinylglycine)[a]	451	307 (B), 273, 205
M11 (cysteine)	394	307, 273 (B)
M12 (*N*-acetylcysteine)	436	307 (B), 273, 205, 162
M13 (GSH conjugate)	596	578, 467, 323, 306 (B), 289, 288, 272, 254, 210, 179
M14 (cysteinylglycine)	467	323, 289 (B), 259, 205
M15 (cysteine)	410	323, 289 (B)
M16 (*N*-acetylcysteine)	452	416, 323, 289 (B), 259, 205, 162

[a] MS/MS data for metabolites M9 and M10 were identical to those reported recently (*19*). Individual MS/MS data provided as Supporting Information, Figures 1–6.

possible interaction with APAP as an inhibitor of these metabolic pathways. Hepatocytes were treated with the combination of 50 μM flutamide and increasing concentrations of APAP of 1, 2, and 5 mM for 24 h, and total protein synthesis was determined (Figure 8). There was a concentration-dependent decrease in protein synthesis with an additive effect at 2 mM (60% decrease) and synergistic toxicity at 5 mM APAP (90% decrease). The combination of flutamide and 5 mM APAP also resulted in morphological cell death at 24 h as determined microscopically. There was only a 30% decrease in protein synthesis in hepatocytes treated with 5 mM APAP alone (Figure 8).

2. Effect of APAP on Flutamide Metabolism. The 24 h culture media from hepatocytes treated with the combination of flutamide (50 μM) and APAP (various concentrations) were analyzed for metabolites of both flutamide (Figure 9A) and APAP (Figure 9B). As expected, APAP inhibited sulfation of flutamide at all concentrations and inhibited the formation of 2-hydroxyflutamide (M1) and M2 (not shown) at 5 mM APAP. Interestingly, the acetylation of metabolite M3 to M4 was strongly inhibited by APAP at all concentrations with 70% inhibition at 1 mM APAP. A 40% increase in the level of parent flutamide was found in 5 mM APAP-treated hepatocytes, which experienced the most cytotoxicity.

3. Effect of Flutamide on APAP Metabolism. In the same hepatocyte cultures, it was observed that flutamide (50 μM) produced a strong inhibition of glucuronidation and sulfation of APAP at all concentrations studied (Figure 9B). The glucuronidation of APAP (at >1 mM) was reduced to more than 50% in the presence of flutamide (50 μM). The ability of flutamide to inhibit the glucuronidation of APAP by various UGTs was further demonstrated by assessing the IC_{50} values. Flutamide was found to inhibit recombinant UGT1A1, 1A6, 1A9, and 2B15 with IC_{50} values of 160, 170, 31, and 89 μM, respectively (data not shown). Similarly, APAP sulfate formation was reduced by more than 30% in the presence of flutamide (Figure 9B). The decrease in phase II sulfate and glucuronide conjugates in the presence of flutamide was accompanied by an increase in bioactivaton of APAP, as evidenced by the much higher levels of GSH-related adducts in the hepatocytes treated with flutamide (Figure 9B). The formation of GSH adducts

doubled in the presence of flutamide and 1 or 2 mM APAP. There was only an increase in APAP-glutathione in hepatocytes treated with a combination of 5 mM APAP and 50 μM flutamide, likely because of toxicity that prevented the formation of other GSH adducts. Microsomal studies showed that the relative levels of APAP-GSH adduct was similar, both in the presence and the absence of flutamide (data not shown), suggesting the lack of stimulation of APAP-SG formation. Thus, both flutamide and APAP inhibited several pathways of metabolism common to each drug, yet potentiated toxicity.

Discussion

In this study, we investigated whether an adverse hepatic effect of flutamide is mediated through inhibition of bile transport and its own metabolism, leading to potential sustained levels of drug in liver. Flutamide and its pharmacologically active and abundant metabolite, 2-hydroxyflutamide, inhibited taurocholate efflux (Figure 1), suggesting inhibition of the bile salt export pump (BSEP). As a consequence, it may result in hepatic accumulation of bile acids and accumulation of flutamide and 2-hydroxyflutamide, and may also have a regulatory effect on the expression of other hepatic transporters through increased level of bile acids. Using plasma membrane vesicles prepared from Sf9 cells expressing human BSEP, Hirano et al. have shown taurocholate-dependent saturation of BSEP kinetics with a K_m value of 10.2 μM (5.5 μg/mL) (*21*). We have previously suggested that for medications that inhibit bile acid transport in vitro, clinical hepatotoxicity is associated with a maximum plasma concentration >1 μg/mL (*11, 16*). However, often the plasma concentrations of drugs do not necessarily reflect the actual level of drug in the liver. The hepatic drug concentrations may be sufficient to saturate BSEP, resulting in subsequent accumulation of bile acids and/or drug as well as changes in bile composition. In vesicles containing BSEP, IC_{50} values for inhibition of BSEP-mediated taurocholate transport by nefazodone, troglitazone, and glibenclamide (inhibition of bile transport by these drugs was implicated in hepatic toxicity) were 9, 25, and 60 μM, respectively (*11, 21*). Recently, flutamide was shown to inhibit taurocholate transport in BSEP vesicles with an IC_{50} value of about 50 μM (*22*). In our study, using intact hepatocytes, IC_{50} values were 75 and 110 μM for flutamide and 2-hydroxyfluatmide, respectively. These concentrations exceed reported total plasma levels for parent (0.4 μM) and the metabolite (5.6 μM) after the administration of a standard 750 mg dose of flutamide. However, concentrations of flutamide or 2-hydroxyflutamide could be significantly greater in the liver and could inhibit bile acid transport as well as its own elimination, resulting in progressively increasing concentrations in the liver. One needs to take a conservative approach and not assume that the actual IC_{50s} for inhibition of bile transport reflects the probability of toxicity. It is rather determined by a concentration-dependent inhibition of bile acid efflux, suggesting hepatotoxicity in susceptible individuals.

We found that toxicity, as measured by inhibition of protein synthesis, was similar for both flutamide and 2-hydroxyflutamide, suggesting that both parent and metabolite could equally contribute to toxicity if not cleared (Figure 2). Thus, the formation of 2-hydroxyflutamide does not represent a detoxification pathway. However, the formation of other minor metabolites and especially secondary metabolism of 2-hydroxyflutamide to M7 may play an important role in the detoxification of flutamide.

Flutamide was metabolized to at least five metabolites, including 2-hydroxyflutamide (Figure 4). This metabolic profile was similar to what has been described previously on the basis of metabolites identified in human serum and urine (*5, 6, 17*). The HPLC/UV/MS profile of flutamide metabolites produced by freshly cultured hepatocyte from a single donor is provided as Supporting Information, Figure 7. The major metabolites identified were M1 (2-hydroxyflutamide), M2 (hydroxyflutamide), M4 (acetylated aniline), and M7 (dihydroxylated flutamide). Small quantities of metabolite M6 (sulfate conjugate), GSH-related adducts, and glucuronide conjugates of hydroxylated flutamide were also found. The current study was designed to investigate the bioactivation of flutamide and whether reactive metabolites play any role in cytotoxicity. It was found that flutamide was capable of being bioactivated in the presence of human liver microsomes and hepatocytes. The MS/MS data, which were obtained from our study for M9 and M10, were found to match with those reported in the literature (*18*). In addition to these metabolites, we identified a number of other GSH-related adducts whose structures are yet to be determined (Figure 5, Table 1). A metabolite that showed similar MS/MS data as M16 has been found in urine of subjects dosed with flutamide (*18*). The structure of this metabolite was based on the mass spectral data and hence not fully elucidated. Interestingly, the authors did not find its analogue, M12 (Figure 5) in urine, suggesting that the initially formed GSH adduct (M9) may have been further oxidized. Studies conducted in our laboratory have suggested that metabolite M1 was not bioactivated at all in the presence of human liver microsomes and hepatocytes and hence was demonstrated not to be a precursor to M13–M16. A list of all GSH-related adducts found in human hepatocytes is given in Table 1. The MS/MS spectral data for these conjugates are listed in Table 1 and provided as Supporting Information (Figures 1–6).

Flutamide at 75 μM caused a sustained decrease in protein synthesis, saturation of metabolism, and lack of parent drug disappearance (Figure 3). In addition, there was no recovery in protein synthesis when oxidative metabolism was prevented with ABT, resulting in a 35% increase in parent flutamide concentration (Figure 6). Surprisingly, ABT also had a strong inhibitory effect on acetylation of M3 to M4, resulting in about a 15% increase in M3. However, the following two findings argue against the role of M3 in toxicity. First, the recovery in toxicity by 24 h in the absence of ABT was associated with an increased formation of M3, and second, toxicity at 75 μM flutamide was not accompanied by a higher level of M3 as compared to the concentration of M3 found in hepatocytes treated with 50 μM flutamide .

Treatment with equimolar concentrations of flutamide and 2-hydroxyflutamide resulted in a potentiation of toxicity. 2-Hydroxyflutamide inhibited the metabolism of flutamide, resulting in a 40% increase in parent drug and 30% increase in 2-hydroxyflutamide. The inhibitory effect of 2-hydroxyflutamide on CYP1A2-mediated metabolism of flutamide has been previously reported (*7*). The formation of 2-hydroxyflutamide is mediated by CYP1A2 (*7, 8*) suggesting the product-based inhibition of flutamide metabolism to its 2-hydroxy form. The formation of other metabolites was decreased, as well. It is possible that inhibition of flutamide metabolism was secondary to toxicity due to a decrease in enzymatic activities. In any case, the accumulation of flutamide and/or 2-hydroxyflutamide would potentiate toxicity and cause a further inhibitory effect on flutamide metabolism, resulting in progressive accumulation of even more nonmetabolized drug and eventually toxicity. In isolated rat liver mitochondria, 50 μM flutamide strongly inhibited respiration and caused an uncoupling effect (*23*). Thus,

the toxicity may result from compromised hepatic elimination followed up by drug accumulation and mitochondrial toxicity.

As APAP is frequently used as a nonprescription painkiller and can be used in conjunction with flutamide, we investigated the potential for any drug–drug interaction between flutamide and APAP. Treatment with the combination of APAP and flutamide led to a dramatic potentiation of cytotoxicity as a result of an inhibitory effect of both drugs on each other's metabolism (Figures 8 and 9). As expected, APAP blocked the formation of M6, most likely by competing for sulfation and/or inhibiting CYP3A, and inhibited the formation of 2-hydroxyflutamide, most likely through competitive inhibition of CYP1A2 metabolism of flutamide. In addition, APAP inhibited the acetylation of 4-nitro-3-(trifluoromethyl)phenylamine (M3) to M4 (Figure 4). Acetaminophen has been previously reported to cause inhibition of sulfamethazine acetylation in human liver homogenates and in healthy volunteers, as shown by a decrease in the ratio of acetylated caffeine metabolite to 1-methylxanthine (*24*). Reversible acetylation of APAP has also been studied in intact rats dosed with *p*-aminophenol, which may be responsible for APAP-induced nephrotoxicity (*25*). Thus, inhibition of acetylation of M3 is likely to be mediated by the deacetylated form of APAP. As a result of overall metabolic inhibition by APAP, the parent flutamide increased by 40%.

Flutamide also caused inhibition of APAP metabolism, resulting in about 30–50% inhibition of glucuronidation and sulfation at each concentration of APAP tested (Figure 9B). To investigate the mechanism responsible for inhibition of APAP glucuronidation, we used recombinant UGT1A1, 1A6, 1A9, and 2B15. These UGTs are known to metabolize APAP (*27*). Flutamide inhibited APAP glucuronidation by these isoforms with IC_{50} values of 160 (1A1), 170 (1A6), 31 (1A9), and 89 μM (2B15). We have previously characterized the role of UGTs in APAP glucuronidation and the potential for inhibition of this detoxification pathway by phenobarbital and phenytoin (*13, 26*). Phenobarbital and phenytoin inhibited preferentially UGT2B15 and UGT1A9, respectively (*13*), resulting in potentiation of APAP toxicity. Similarly, in this work, flutamide was shown to be an inhibitor of UGT1A9 and 2B15 at concentrations that could be pharmacologically relevant in susceptible individuals considering the ability of flutamide to accumulate. Therefore, there is a potential for interaction of flutamide with APAP and other medications that rely principally on glucuronidation or sulfation for their metabolism and elimination.

It appears that both bioactivation of APAP and sustained high concentrations of flutamide in the presence of 5 mM APAP contributed to toxicity. Bioactivation products of APAP metabolism were increased at 1 and 2 mM APAP in the presence of 50 μM flutamide. The observed decrease in APAP-cysteinylglycine and APAP-cysteine conjugates at 5 mM APAP could be attributed to compromised enzymatic activities of γ-glutamyltranspeptidase and dipeptidase mediating the formation of these metabolites. There still remained a 30% increase in APAP-glutathione product in the presence of flutamide and 5 mM APAP (Figure 9B).

Flutamide does not produce liver toxicity in normal or CYP1A2 knockout mice after 28 days of oral treatment at 800 mg/kg (*27*). Only a 2-fold increase in alanine aminotransferase (ALT) and minor histological changes were found in CYP1A2 knockout mice (ALT) fed with an amino-deficient diet for 2 weeks prior to and during a 4 week treatment with flutamide. Similarly, rats treated with 100 mg/kg for 15 days did not develop hepatotoxicity (*28*). The lack of hepatotoxicity in rodents in response to flutamide treatment is not a surprising finding. This has been previously observed with other drugs that are substrates for active biliary elimination and known to have adverse clinical hepatic effects (*10, 11*). Treatment of dogs for 2, 3, and 4 years with flutamide up to 40 mg/kg daily resulted in a slight increase in ALT (up to 135%) and alkaline phosphatase activities (up to 125%). Histopathologic findings included centrilobular hepatocyte pigmentation, cytoplasmaic vacuolation, hepatic bile duct proliferation, and excessive mucus in gallbladder (*29*). Bicalutamide, which is another nonsteroidal antiandrogen and chemical analog of flutamide, produced no liver findings in preclinical toxicology studies in rats treated with bicalutamide up to 500 mg/kg for 1 month or up to 75 mg/kg for 12 months (*30*). In contrast, treatment of dogs with 50 mg/kg for 12 months resulted in small ALT and ALP increases (*30*). Our preliminary data in primary cultures of human hepatocytes showed that the effects of bicalutamide and flutamide on inhibition of bile acid transport and toxicity were similar (data not shown). These data suggests that the clinical hepatic effect of both medications will be similar, as well. In addition, the steady-state plasma concentration of bicalutimide is 8.9 μg/mL and parent and metabolites are excreted in bile (*30, 31*). In support of our in vitro data, clinical record of bicalutamide indicates that elevated liver enzymes occurred in 7% of patients vs. 11% treated with flutamide (*30*). Hepatitis or marked increases in liver enzymes leading to drug discontinuation occurred in approximately 1% of bicalutamide patients in controlled clinical trials (*30*). Thus, the clinical record for both drugs eliciting increases in liver enzymes is similar. However, the difference appears in the lower frequency of bicalutamide-induced severe idiosyncratic hepatitis compare to flutamide (*1, 3*).

In conclusion, we found that both flutamide and its major metabolite inhibit bile acid transport. In addition, both have the similar toxic effect on human hepatocytes. Flutamide is an inhibitor of glucuronidation and sulfation of APAP and 2-hydroxyflutamide is an inhibitor of flutamide metabolism. Taken together, these data suggest that an effective hepatic elimination mechanism must be present to avoid a drug–metabolite accumulation and hepatotoxicity. It may not be the case in patients with genetic deficiencies in biliary clearance or hepatic disorders or on multiple medications, thus increasing the probability of adverse liver effects.

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Supporting Information Available: MS/MS spectral data and LC/UV chromatogram (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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