Published in final edited form as:

Chem Res Toxicol. 2008 December ; 21(12): 2393–2406.

Comparison of in Vitro Bioactivation of Flutamide and Its Cyano Analogue: Evidence for Reductive Activation by Human NADPH:Cytochrome P450 Reductase

Bo Wen[†], Kevin J. Coe[‡], Peter Rademacher[‡], William L. Fitch[†], Mario Monshouwer[†], and Sidney D. Nelson^{*,‡}

[†] Department of Drug Metabolism and Pharmacokinetics, Roche Palo Alto, Palo Alto, California 94304

[‡] Department of Medicinal Chemistry, University of Washington, Seattle, Washington 98195

Abstract

Flutamide (FLU), a nonsteroidal antiandrogen drug widely used in the treatment of prostate cancer, has been associated with idiosyncratic hepatotoxicity in patients. It is proposed that bioactivation of FLU and subsequent binding of reactive metabolite(s) to cellular proteins play a causative role. A toxicogenomic study comparing FLU and its nitro to cyano analogue (CYA) showed that the nitroaromatic group of FLU enhanced cytotoxicity to hepatocytes, indicating that reduction of the nitroaromatic group may represent a potential route of FLU-induced hepatotoxicity [Coe et al. (2007) *Chem. Res. Toxicol.* 20, 1277–1290]. In the current study, we compared in vitro bioactivation of FLU and CYA in human liver microsomes and cryopreserved human hepatocytes. A nitroreduction metabolite FLU-6 was formed in liver microsomal incubations of FLU under atmospheric oxygen levels and, to a greater extent, under anaerobic conditions. Seven glutathione (GSH) adducts of FLU, FLU-G1–7, were tentatively identified in human liver microsomal incubations using liquid chromatography–tandem mass spectrometry (LC/MS/MS), while CYA formed only four corresponding GSH adducts, CYA-G1–4, under the same conditions. Of particular interest was the formation of FLU-G5–7 from FLU, where the nitroaromatic group of FLU was reduced to an amino group. A tentative pathway is that upon nitroreduction, the *para*-diamines undergo cytochrome P450 (P450)-catalyzed two-electron oxidations to form corresponding *para*-diimine intermediates that react with GSH to form GSH adducts FLU-G5–7, respectively. The identities of FLU-G5–7 were further confirmed by LC/MS/MS analyses of microsomal incubations of a synthesized standard FLU-6. In an attempt to identify enzymes involved in the nitroreduction of FLU, NADPH:cytochrome P450 reductase (CPR) was shown to reduce FLU to FLU-6 under both aerobic and anaerobic conditions. Furthermore, the formation of FLU-G5–7 was completely blocked by the addition of a reversible CPR inhibitor, α -lipoic acid, to the incubations of FLU under aerobic conditions. In summary, these results clearly demonstrate that nitroreduction of FLU by CPR contributes to bioactivation and potentially to hepatotoxicity of FLU.

*To whom correspondence should be addressed. Tel: 206-543-1419. Fax: 206-685-3252. sidnels@u.washington.edu.

Supporting Information Available: ESI-MS analyses of FLU-G1, FLU-G3, CYA-G1, and CYA-G3 in negative ion mode: MS/MS spectrum of FLU-G1 at m/z 596 ($[M - H]^-$) (Figure S1), MS/MS spectrum of CYA-G1 at m/z 576 ($[M - H]^-$) (Figure S2), MS/MS spectrum of FLU-G3 at m/z 596 ($[M - H]^-$) (Figure S3), and MS/MS spectrum of CYA-G3 at m/z 576 ($[M - H]^-$) (Figure S4). ESI-MS analyses of FLU in positive ion mode: MS/MS spectrum of FLU at m/z 277 ($[M + H]^+$) (Figure S5A) and MS³ spectrum of FLU (the fragment ion at m/z 207) (Figure S5B) in data-dependent scanning mode on an ion trap mass spectrometer. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Flutamide {2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide, FLU,¹ Scheme 1} is a nonsteroidal antiandrogen drug that is widely used for the treatment of prostate cancer. The combination of FLU with luteinizing hormone-releasing agonists or orchiectomy significantly increases the survival time of prostate cancer patients (1,2). Despite its therapeutic benefits, treatment with FLU has been overshadowed by rare but severe incidences of hepatic injury (3–10). Although the exact mechanism of FLU hepatotoxicity is not clearly understood, a probable causal link between FLU use and the onset of hepatic injury has been established (3).

In humans, FLU is rapidly absorbed after oral administration and undergoes extensive hepatic first-pass metabolism mainly by hydroxylation, hydrolysis, *N*-acetylation, and nitroreduction (11–14). The primary route of FLU metabolism is P450-catalyzed oxidation to 2-hydroxyflutamide (2-OH FLU), which is primarily catalyzed by CYP1A2 (15,16). It has been suggested that the antiandrogenic activity of FLU is largely associated with the metabolite 2-OH FLU (11). In addition to oxidative metabolism, another clearance pathway is the carboxyesterase-catalyzed hydrolysis to 4-nitro-3-(trifluoromethyl)-aniline (FLU-1, Scheme 1). FLU-1 was detected as a major metabolite in plasma (12), whereas 4-nitro-6-hydroxy-3-(trifluoromethyl)-aniline (FLU-3, Scheme 1) comprised 50–90% of the urinary metabolites of FLU (13).

Of particular interest in the biotransformation pathways of FLU in humans is the detection and characterization of several nitroreduction metabolites, namely, FLU-4, FLU-5, 2-methyl-*N*-(4'-amino-3'-(trifluoromethyl)phenyl)propanamide (FLU-6), and FLU-8 (Scheme 1) (13,14, 17–19). As depicted in Scheme 1, a substructure of FLU is a nitroaromatic group that has often been associated with toxicity due to its susceptibility to reduction that can yield reactive oxygen species, reactive nitrogen species, and/or electrophilic intermediates (20,21). Formation of these *para*-diamine metabolites FLU-4, FLU-5, FLU-6, and FLU-8 is presumably catalyzed by six-electron nitroreductive bioactivation that can produce nitroso and *N*-hydroxy metabolites as intermediates. In an effort to identify critical toxicophores and elucidate mechanisms of FLU-induced toxicity, our recent study (22) demonstrated that the nitroaromatic group of FLU enhances cytotoxicity to hepatocytes as compared to its cyano analogue. The nitro to cyano replacement in FLU prevented the possibility of reduction of the nitroaromatic group, while retaining a strong electron-withdrawing group at the *para*-position that preserved drug efficacy (22). These findings served as the first line of evidence to suggest that reduction of the nitroaromatic group may play an important role in FLU-induced hepatotoxicity.

Although several mechanistic studies have been performed to prove the involvement of metabolic activation in FLU-induced hepatotoxicity, the relationship between nitroreduction and FLU-induced toxicity has not been fully established. Recently, a glutathione (GSH) conjugate of hydroxylated FLU in human liver microsomal and hepatocyte incubations was identified (23,24). Similarly, a mercapturic acid conjugate of hydroxylated FLU was detected in the urine of prostate cancer patients (19). In addition, Kang and co-workers identified a novel N–S glutathionyl adduct (25), and recently, they also detected and characterized several other GSH adducts by direct incubations of FLU metabolites with human liver microsomes, including 1-hydroxyflutamide, a minor metabolite only observed in human liver microsomal

¹Abbreviations: P450, cytochrome P450; CPR, NADPH:cytochrome P450 reductase; FLU, flutamide; CYA, cyano analogue of flutamide or 2-methyl-*N*-(4'-cyano-3'-(trifluoromethyl)phenyl)propanamide; FLU-1, 4-nitro-3-(trifluoromethyl)aniline; FLU-6, 2-methyl-*N*-(4'-amino-3'-(trifluoromethyl)phenyl)propanamide; PI, precursor ion; NL, neutral loss; EPI, enhanced product ion; CID, collision-induced dissociation; GSH, glutathione.

incubations (26). Three regioisomers of GSH adducts were detected by direct incubation of the metabolite FLU-6 with human liver microsomes (26). However, to date, no reduced metabolites of FLU and their corresponding GSH adducts have been reported from in vitro incubations of FLU in human liver microsomes. Only oxidative bioactivation was implicated in the formation of reactive intermediates.

In the present study, we compare the in vitro bioactivation of FLU and its cyano analogue CYA and herein report several reactive metabolites formed only in incubations with FLU but not with CYA. The identities of these reactive metabolites were confirmed to be derived from the reduced metabolite of FLU, FLU-6. Microsomal enzymes involved in nitroreduction of FLU were also investigated. These findings provide direct evidence that nitroreduction of FLU by NADPH:cytochrome P450 reductase (CPR) contributes to bioactivation and potentially hepatotoxicity of FLU and affords a possible explanation for the enhanced cytotoxicity of FLU as compared to CYA.

Experimental Procedures

Materials

Reagents and solvents used in the current study were of the highest grade commercially available. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): FLU, GSH, trichloroacetic acid, and NADPH. Pooled human liver microsomes, purified recombinant human NADPH:P450 reductase, and Supersomes containing cDNA-baculovirus-insect cell-expressed P450s (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) were obtained from BD Gentest (Woburn, MA). Purified recombinant human cytochrome *b*₅ and purified cytochrome P450 (P450) enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) were purchased from Invitrogen (Carlsbad, CA). Pooled human cryopreserved hepatocytes were purchased from In Vitro Technologies (Baltimore, MD). L- α -Dilaurylphosphatidylcholine was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Formic acid, methanol, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ).

Synthesis of FLU-6 and CYA

FLU (100 mg, 0.362 mmol) and Na₂S₂O₄ (690 mg, 3.96 mmol) were dissolved in 15 mL of 35% methanol. The reaction turned to a cloudy darker yellow color over 1 h at room temperature. At termination, a saturated solution of NaCl (1 mL) and ethyl acetate (5 mL) was added to the reaction. The mixture was vortexed, and the ethyl acetate layer was extracted, dried over Na₂SO₄, and evaporated under a stream of N₂. The resulting crude product weighed 99 mg and consisted of 61% of product based on HPLC/UV analysis (214 nm), a crude product yield of 68%. The product was dissolved in 50% methanol and purified using an Econosphere C18 prep column (10 μ m, 250 mm $\times$ 10 mm, Alltech, Part #28085, Deerfield, IL) and a Hewlett-Packard 1100 HPLC-UV system. A methanol gradient at a flow rate of 3 mL/min was employed as follows: (1) a linear gradient from 50 to 90% for 23 min, (2) a hold for 1 min at 90%, (3) a decline to 50% for 1 min, and (4) a re-equilibration for 5 min at 50%. After 100 μ L injections, product was collected from 6.7 to 8 min before it was concentrated using a Rotavapor-R (Brinkmann Instruments, Westbury, NY), extracted with ethyl acetate, and dried. Purified product was a faint yellow solid and estimated to be >98% pure via HPLC/UV analysis (260 nm). ¹H NMR (CDCl₃): 1.25 ppm (6 H, d, CH₃, *J* = 6.8 Hz), 2.48 ppm (1H, m, CH, *J* = 6.8 Hz), 3.9–4.2 ppm (2 H, m, NH₂), 6.71 ppm (1H, d, aromatic 5'-H, *J* = 8.8 Hz), 7.00 ppm (1H, s, NH), 7.49 ppm (1H, dd, aromatic 6'-H, *J* = 8.8 Hz, *J* = 2.3 Hz), and 7.56 ppm (1H, d, aromatic 2'-H, *J* = 2.3 Hz). HRMS (*m/z*) 269.0868 (M + Na), 285.0606 (M + K), 291.0688 (M - H + 2Na). Synthesis of CYA was carried out according to the procedure previously described (22).

Microsomal Incubations

All incubations were performed at 37 °C in a water bath. Stock solutions of the test compounds were prepared in methanol. The final concentration of methanol in the incubation was 0.05% (v/v). Pooled human liver microsomes and the human cDNA-expressed P450 isozymes were carefully thawed on ice prior to the experiment. FLU, CYA, or FLU-6 (10 μ M) was individually mixed with human liver microsomal proteins (1 mg/mL) in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 1 mM GSH. The total incubation volume was 1 mL. After 3 min of preincubation at 37 °C, the incubation reactions were initiated by the addition of 1 mM NADPH. Reactions were terminated by the addition of 150 μ L of trichloroacetic acid (10%) after 60 min of incubation. Incubations with the recombinant cDNA-expressed P450 isozymes were performed similarly except that liver microsomes were substituted by Supersomes (100 nM). Control samples containing no NADPH or substrates were included. Samples were centrifuged at 10000g for 15 min at 4 °C to pellet the precipitated proteins, and supernatants were subjected to LC/MS/MS analysis of GSH adducts. For human liver microsomal incubations, supernatants (20 μ L) were concentrated by solid-phase extraction (SPE) as described below, prior to LC/MS/MS analyses.

For anaerobic conditions, incubation mixtures were purged by argon and deaerated for 10 min before reactions were initiated by 1 mM NADPH. For the study of enzyme kinetics of FLU-6 formation, incubations containing FLU (1, 2.5, 4, 8, 15, 25, 50, 100, 250, 500, 1000, and 2000 μ M) were deaerated by argon and carefully sealed before the reactions were started by addition of 1 mM NADPH. The effect of α -lipoic acid, a selective inhibitor of CPR (27), was tested using 2.0–5.0 mM α -lipoic acid. The P450-specific inhibitors α -naphthoflavone (1 μ M), sulfaphenazole (5 μ M), tranilcypromine (15 μ M), quinidine (2 μ M), and ketoconazole (1 μ M) were used to investigate the involvement of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively. Incubations containing FLU were initiated with the addition of 1 mM NADPH, and reactions were terminated after 30 min by trichloroacetic acid. Controls containing no chemical inhibitors were included. The effectiveness of individual P450 inhibitors was evaluated using P450 marker substrates 50 μ M phenacetin (CYP1A2), 150 μ M tolbutamide (CYP2C9), 100 μ M (*S*)-mephenytoin (CYP2C19), 10 μ M dextromethorphan (CYP2D6), and 100 μ M testosterone (CYP3A4) in human liver microsomes as described previously (28). A comparison was made relative to the controls without inhibitors, and P450 activity was expressed as the percentage of control activity. To investigate the role of cytochrome *b*₅ reductase in the FLU nitroreduction, NADPH was replaced by 1 mM NADH in the human liver microsomal incubations. Quantitation of FLU-6 was conducted using simultaneous MRM transitions monitored for *m/z* 247→227 and 247→177, using a standard calibration curve of FLU-6 over a concentration range of 10.0–1000.0 ng/mL. Each incubation was performed in triplicate.

Hepatocyte Incubations

Pooled human cryopreserved hepatocytes suspended in 10% fetal bovine serum-supplemented William's Medium E were purified by Percoll gradient centrifugation. Cell viability was determined as 81% using the trypan blue exclusion method. For metabolite profiling, hepatocyte incubations were conducted in a 24 well polystyrene plate (1 $\times$ 10⁶ viable hepatocytes/mL). For each test compound, 900 μ L of prewarmed William's Medium E was pipetted into two wells of a 24 well plate. Control incubations contained an additional 500 μ L of William's Medium E, whereas 500 μ L of human hepatocytes was placed into experimental incubations. After 5 min of preincubation at 37 °C, 100 μ L of a 15 $\times$ solution of FLU or CYA was added to the control well and the experimental well containing hepatocytes. The total incubation volume was 1.5 mL, and the final concentration of test compound was 20 μ M. The incubations were allowed to proceed for 120 min at 37 °C in an atmosphere containing 5% CO₂ and 95% relative humidity. At the end of incubation, 700 μ L was taken from each well,

and the reaction was quenched by adding 700 μ L of ice-cold acetonitrile. Samples were then centrifuged at 10000g for 15 min at 4 $^{\circ}$ C, and supernatants (40 μ L) were subjected to LC/MS/MS analysis.

Incubations with Purified P450s, CPR, and Cytochrome b_5

Purified P450 enzymes (Invitrogen, Carlsbad, CA) were initially reconstituted with 0.5 mg/mL Chaps and 100 μ g/mL liposomes (L- α -dilaurylphosphatidylcholine). FLU (10 μ M) was incubated for 1 h at 37 $^{\circ}$ C in an incubation system consisting of 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 0.1 mg/mL Chaps, 20 μ g/mL liposomes, and individual purified P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, 100 nM) in a final volume of 1 mL. After 3 min of preincubation at 37 $^{\circ}$ C, the incubation reactions were initiated by the addition of 1 mM NADPH. Reactions were terminated by the addition of 150 μ L of trichloroacetic acid (10%). Incubations with the recombinant human NADPH:P450 reductase and human cytochrome b_5 were performed similarly except that P450 enzymes were substituted by reductase (100 nM) or b_5 (100 nM). The reaction of b_5 incubations was initiated by 1 mM NADH, instead of NADPH. Control samples containing no NADPH or substrates were included. Samples were centrifuged at 10000g for 15 min at 4 $^{\circ}$ C to pellet the precipitated proteins, and supernatants were subjected to LC/MS/MS analysis of FLU-6. Quantitation of FLU-6 was achieved through LC/MRM analyses as described above. Data were analyzed using Analyst 4.1 version software (Applied Biosystems, Foster City, CA). Each incubation was performed in duplicate under both aerobic and anaerobic conditions.

SPE

Samples resulting from incubations were desalted and concentrated by SPE, prior to the negative precursor ion (PI) scan MS/MS analyses. SPE was performed using Oasis SPE cartridges packed with 60 mg of sorbent C_{18} (Waters, Milford, MA). Cartridges were first washed with 2 mL of methanol and then conditioned with 2 mL of water. Supernatants resulting from centrifugation were loaded onto the cartridges, and cartridges were washed with 2 mL of water and then eluted with 2 mL of methanol. The methanol fractions were dried by nitrogen gas and reconstituted with 100 μ L of a water-methanol (70:30) mixture. Aliquots (20 μ L) of the reconstituted solutions were subjected to LC/MS/MS analysis.

Instrumentation

LC/MS/MS analyses were performed on an API 4000 Q-Trap hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) interfaced online with a Shimadzu HPLC system (Columbia, MD). Complete profiling of reactive metabolites was carried out using the PI-enhanced product ion (EPI) method previously described (29). Briefly, the PI scan of m/z 272 was run in the negative mode with 0.2 Da step size, 5 ms pause between mass ranges, and 2 s scan rate or 50 ms dwell. The TurboIonSpray ion source conditions were optimized and set as follows: curtain gas = 35, collision gas = medium, ionspray voltage = -4500, and temperature = 500. Information-dependent acquisition was used to trigger acquisition of EPI spectra. The EPI scans were run in the positive mode at a scan range for daughter ions from m/z 100 to 1000. The instrument parameters of the positive EPI scans were set as follows: ionspray voltage = 5000, temperature = 500, collision energy = 40, and collision energy spread = 20. Upon a positive peak was detected in the negative PI scanning over the range m/z 270–1000, a collision-induced dissociation (CID) MS/MS spectrum was simultaneously obtained to further elucidate the structure of the GSH adduct. Data were processed using Analyst 4.1 software (Applied Biosystems). The Shimadzu HPLC system was coupled with an Agilent Eclipse XDB-Phenyl C_{18} column (3.0 mm $\times$ 150 mm, 3.5 μ m, Agilent Technologies, Palo Alto, CA). The HPLC mobile phase A was 10 mM ammonium acetate in water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. A Shimadzu LC-20AD solvent delivery

module (Shimadzu Scientific Instruments) was used to produce the following gradient elution profile: 5% solvent B for 2 min, followed by 5–70% B in 20 min, and 70–90% B in 2 min. The HPLC flow rate was 0.3 mL/min. At 26 min, the column was flushed with 90% acetonitrile for 3 min before re-equilibration at initial conditions. LC/MS/MS analyses were performed on 20 μ L aliquots of cleaned samples. For relative comparison of GSH adduct levels, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions were simultaneously monitored for detecting FLU-G1: m/z 598 $\rightarrow$ 469 and 598 $\rightarrow$ 323. Data were analyzed using Analyst 4.1 version software (Applied Biosystems).

Metabolite profiling was performed on a Finnigan LTQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Agilent 1100 HPLC system (Agilent Technologies). Separation was achieved using a Polaris C18 column (5 μ m, 250 mm $\times$ 2.1 mm, Varian Inc., Palo Alto, CA) at a flow rate of 0.3 mL/min. A gradient of solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) was as follows: 5% solvent B for 5 min, followed by 5–70% B in 30 min, and 70–90% B in 2 min. Major operating parameters for the ion trap ESI-MS method were set as follows: capillary temperature, 300 $^{\circ}$ C; spray voltage, 5.0 kV; capillary voltage, 15 V; sheath gas flow rate, 90 (arbitrary value); and auxiliary gas flow rate, 30 (arbitrary value). For a full scan, the automatic gain control was set at 5.0×10^8 , maximum ion time was 100 ms, and the number of microscans was set at 3. For MSⁿ scanning, the automatic gain control was set at 1.0×10^8 , the maximum ion time was 400 ms, and the number of microscans was set at 2. For data-dependent scanning, the default charge state was 1, the default isolation width was 3.0, and the normalized collision energy was 30.

Results

In Vitro Formation of FLU-6

The potential in vitro nitroreduction of FLU to FLU-6 was demonstrated by conducting studies with human liver microsomes and cryopreserved human hepatocytes. The presence of the reduced metabolite FLU-6, with $[M + H]^+$ at m/z 247, was demonstrated by LC/MS analysis of incubation extracts of human liver microsomes and hepatocytes. The identity of the reduced metabolite was confirmed by comparing its HPLC retention time and mass spectral data with those of a synthetic standard (Figure 1). On the basis of the UV adsorption at 250 nm, approximately 1.8% of FLU-6 was formed in human liver micorsomes, and the total turnover of FLU was calculated as 65%. The results provide direct evidence that FLU undergoes nitroreductive metabolism to form FLU-6 not only in vivo but also in vitro.

Characterization of GSH Adducts of FLU and CYA

For the LC/MS/MS analysis of GSH adducts, samples generated from incubations with human liver microsomes were desalted and concentrated by SPEs, and resulting samples were subjected to the both PI-EPI experiments and data-dependent scanning. MS detection of the PI-EPI experiments was carried out using the negative PI scanning of m/z 272, corresponding to deprotonated γ -glutamyl-dehydroalanyl-glycine, originating from the glutathionyl moiety. MS/MS spectra were acquired in positive ion mode using information-dependent data acquisition (29). As shown in Figure 2A, a total of seven major components were detected in a human liver microsomal incubation of FLU, and they were arbitrarily designated as FLU-G1 (16.4 min), FLU-G2 (18.6 min), FLU-G3 (17.8 min), FLU-G4 (15.7 min), FLU-G5 (14.2 min), FLU-G6 (12.9 min), and FLU-G7 (11.8 min), respectively. In contrast, a total of four components were detected in a human liver microsomal incubation of CYA (Figure 2B), and they were designated as CYA-G1 (14.8 min), CYA-G2 (17.2 min), CYA-G3 (16.1 min), and CYA-G4 (13.9 min) accordingly. None of these peaks was detected when either FLU, CYA, or NADPH was absent from the incubations.

Structures of these detected components were identified based on positive MS/MS spectra acquired from Qtrap and data-dependent MS scanning obtained from an ion trap instrument. The predominant adduct FLU-G1 displayed a molecular ion $[M + H]^+$ of m/z 598, suggesting that this component was a GSH conjugate of mono-oxygenated FLU. Fragmentation of FLU-G1 molecular ions resulted in neutral loss (NL) of 129 and 75, corresponding to elimination of the pyroglutamate and glycine of GSH, respectively (Figure 3A). The ion at m/z 323 was formed via cleavage of sulfur–carbon bond of the glutathionyl moiety. Further fragmentation of the ion at m/z 323 afforded several fragment ions including ions at m/z 207 and 187 (Figure 3B). These data suggested that FLU-G1 is a GSH adduct with attachment of the glutathionyl moiety to the isobutyramide instead of the aromatic group previously identified based on negative MS spectra and NMR analysis (26). This was confirmed by the negative MS/MS spectrum of FLU-G1 (Supporting Information, Figure S1), which was essentially identical to the negative MS spectra reported previously (26). In parallel, the predominant adduct CYA-G1 of CYA showed an $[M + H]^+$ ion at m/z 578, with product ions at m/z 503, 449, 432, and 303 (Figure 3C). Further fragmentation of the ion at m/z 303 afforded several fragment ions including the ion at m/z 187 (Figure 3D). These data suggested that CYA-G1 is a GSH adduct of mono-oxygenated CYA, corresponding to FLU-G1 of FLU. This assignment was supported by the negative MS/MS spectrum of CYA-G1 (Supporting Information, Figure S2), which showed an intense fragment ion at m/z 306 corresponding to the molecular ion $[M - H]^-$ of GSH.

The PI-directed positive MS/MS spectrum of FLU-G2 showed an $[M + H]^+$ ion at m/z 582, with product ions at m/z 507, 453, 436, 350, 307, and 144 (Figure 4A). This component was subsequently confirmed as the N–S glutathionyl adduct previously reported (25). The adduct CYA-G2 showed an $[M + H]^+$ ion at m/z 562 with product ions at m/z 487, 433, 416, and 287 (Figure 4B). This component was confirmed as the N–S glutathionyl adduct of CYA corresponding to FLU-G2.

The component FLU-G3 displayed a molecular ion $[M + H]^+$ of m/z 598, suggesting that this component was a GSH conjugate of mono-oxygenated FLU. Fragmentation of FLU-G3 molecular ions resulted in NL of 129 and 75, corresponding to elimination of the pyroglutamate and glycine of GSH, respectively (Figure 5A). The ion at m/z 323 was formed via cleavage of sulfur–carbon bond of the glutathionyl moiety. Further fragmentation of the ion at m/z 323 afforded several fragment ions including an intense ion at m/z 257 (Figure 5B), distinct from that of FLU-G1 (Figure 3B). The fragment ion at m/z 277 was formed via loss of the elements of NO_2 , a common fragmentation observed in MS spectra of FLU (Supporting Information, Figure S5). Further loss of the elements of isobutyl carbonyl moiety from the ion at m/z 277 afforded the fragment ion at m/z 207 (Figure 5B). These data suggested that although the MS/MS spectrum of FLU-G3 was essentially identical to that of FLU-G1, FLU-G3 was a GSH conjugate with attachment of the glutathionyl moiety to the aromatic group, previously detected as a quaternary ammonium GSH conjugate of hydroxylated FLU (23). This structural assignment was further supported by the negative MS/MS spectrum of FLU-G3 (Supporting Information, Figure S3), which showed two intense fragment ions at m/z 272 and 323. Most informative was the lack of a fragment at m/z 306, which was a base peak of the negative MS/MS spectrum of FLU-G1 (Supporting Information, Figure S1) corresponding to the molecular ion $[M - H]^-$ of GSH. The lack of the ions at m/z 306 and 289 was consistent with the presence of an aromatic thioether motif in the GSH adduct FLU-G3 (30). Similarly, CYA-G3 showed an $[M + H]^+$ ion at m/z 578, with product ions at m/z 503, 449, 432, and 303 (Figure 5C). Further fragmentation of the ion at m/z 303 afforded several fragment ions at m/z 233, 283, and 285 (Figure 5D). These data suggested that CYA-G3 is a GSH adduct of mono-oxygenated CYA, corresponding to FLU-G3 of FLU. This assignment was supported by the negative MS/MS spectrum of CYA-G3 (Supporting Information, Figure S4), which showed two intense

fragment ions at m/z 272 and 303, but lack of the ion at m/z 306, which was a base peak in the negative MS/MS spectrum of CYA-G1 (Supporting Information, Figure S2).

There are four other components, FLU-G4–7, detected in the microsomal incubations of FLU (Figure 2A). The MS/MS spectrum of $[M + H]^+$ ion of FLU-G4 at m/z 528 provided characteristic product ions at m/z 453 and 399, resulting from NLs of glycine (75 Da) and pyroglutamate (129 Da), respectively (Figure 6A). The molecular ion $[M + H]^+$ at m/z 528 was consistent with the addition of one molecule of GSH to FLU-3 (Scheme 1), a FLU metabolite identified in human serum and urine (14, 18). These data confirmed that FLU-G4 was a GSH adduct formed in the incubation of FLU detected previously (26). A proposed structure for FLU-G4, which is consistent with the CID cleavage, is shown in Figure 6A. Corresponding to FLU-G4, CYA-G4 was formed in incubations of CYA with a $[M + H]^+$ at m/z 508 (Figure 6B). These results suggested that FLU and CYA undergo similar bioactivation pathways to form FLU-G1–4 and CYA-G1–4, respectively.

Three GSH adducts, FLU-G5–7, were generated in incubations of FLU, but not CYA. The deprotonated molecular ion of FLU-G5 eluted at 14.2 min was m/z 550 in the negative ion mode (Figure 7A). The MS/MS spectrum of $[M + H]^+$ ion at m/z 552 afforded the diagnostic product ions at m/z 477 and m/z 423, resulting from NLs of glycine and pyroglutamate, respectively (Figure 7B). The molecular ion $[M + H]^+$ at m/z 552 was consistent with the addition of one molecule of GSH to FLU-6 (Scheme 1), a reduced FLU metabolite identified in human serum and urine (14, 18), as well as in *in vitro* incubations of FLU described above. The MS/MS spectrum of FLU-G5 provided the product ion at m/z 207, presumably resulting from cleavage of the amide bond of the product ion at m/z 277. The occurrence of the product ion at m/z 277 was consistent with the presence of an aromatic thioether motif in this GSH adduct (30). FLU-G5 was identified as a GSH adduct of the reduced metabolite FLU-6, previously only detected in direct incubations of FLU-6 with human liver microsomes (26). These data provide direct evidence that FLU undergoes nitroreductive metabolism to form FLU-6, which is subsequently bioactivated to form FLU-G5 in human liver microsomal incubations.

The parent ion of a newly detected GSH adduct FLU-G6, eluted at 12.9 min, was m/z 566 in the negative ion mode (Figure 8A). The positive MS/MS spectrum of $[M + H]^+$ ion at m/z 568 provided characteristic product ions at m/z 493 and 439, resulting from NLs of glycine and pyroglutamate, respectively (Figure 8B). The molecular ion $[M + H]^+$ of m/z 568 is consistent with the addition of one molecule of GSH to FLU-5 (Scheme 1), presumably resulting from nitroreduction of the major circulating metabolite 2-OH FLU. FLU-5 is a circulating metabolite identified in human serum (14). The occurrence of the product ion at m/z 293 was consistent with the presence of an aromatic thioether motif in this GSH adduct (30). Cleavage of the amide bond from the product ion at m/z 293 resulted in the fragment ion at m/z 207, consistent with hydroxylation of the isopropyl moiety.

Another GSH adduct involving FLU nitroreduction was FLU-G7 (Figure 9). The MS/MS spectrum of $[M + H]^+$ ion at m/z 482 provided characteristic product ions at m/z 407 and 353, resulting from NLs of glycine and pyroglutamate, respectively (Figure 9B). This confirmed that FLU-G7 was a new GSH adduct formed in the incubation of FLU. The molecular ion $[M + H]^+$ at m/z 482 was consistent with the addition of one molecule of GSH to FLU-8 (Scheme 1), another nitroreduced metabolite detected in humans (13). Double NLs of glycine and pyroglutamate formed the product ion at m/z 278. The occurrence of the product ion at m/z 208 was consistent with the presence of an aromatic thioether motif in this GSH adduct (30). A proposed structure for FLU-G7, which is consistent with the CID cleavage, is shown in Figure 9B. These results clearly demonstrated that three GSH adducts FLU-G5–7 derived from nitroreduced metabolites of FLU were formed only in human liver microsomal incubations of

FLU but not in those of CYA where the nitro to cyano replacement prevents the possibility of reduction of the nitroaromatic group.

In addition, similar to FLU, CYA was mainly metabolized via hydroxylation and hydrolysis, except for nitroreduction in human liver microsomes. Similar biotransformation pathways of FLU and CYA were also observed in human hepatocyte incubations (data not shown). It is noteworthy that no additional phase II metabolites, such as glucuronidation, sulfation, or *N*-acetylation, of the reduced aniline metabolite of FLU (FLU-6) was detected in the hepatocyte incubations.

Bioactivation of FLU-6

Characterization of GSH adducts FLU-G5–7 formed from incubations of FLU suggested bioactivation of the reduced metabolite FLU-6. To investigate the mechanisms of bioactivation and further confirm the identities of FLU-G5–7 generated from incubations of FLU, FLU-6 was synthesized and incubated with human liver microsomes. As shown in Figure 2C, FLU-G5–7 were detected at the same HPLC retention times as the corresponding components from the incubation of FLU (Figure 2A). The MS/MS spectra of these components were essentially identical to those of the corresponding GSH adducts FLU-G5–7 from the FLU incubation (Figures 79). These data clearly demonstrated that FLU-G5–7 can be formed from incubations of the nitroreduction metabolite FLU-6.

Formation of FLU-G1 and CYA-G1 with Recombinant P450s

To investigate the roles of individual human P450 isozymes in the bioactivation of FLU and CYA to the GSH adducts, incubations were carried out with insect cell-expressed recombinant P450s. After normalization for the relative hepatic abundance of P450 isozymes (31), CYP1A2 was the predominant enzyme in the formation of FLU-G1 in incubations of FLU (Figure 10). CYP2C19 and CYP1A1 also catalyzed FLU-G1 formation, and the levels were approximately 20 and 10% of those formed by CYP1A2, respectively. Only trace amounts or no FLU-G1 were detected in incubations with other P450 enzymes including CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. Similarly, CYA-G1 formation was predominantly carried out by CYP1A2 (Figure 10). These results suggested that CYP1A2 is the major P450 enzyme involved in the formation of FLU-G1 and CYA-G1 presumably via an *ortho*-quinone imine intermediate after hydroxylation followed by two-electron oxidations (Scheme 2).

Anaerobic Nitroreduction of FLU by Human Liver Microsomes

Microsomal nitroreduction enzymes have differing sensitivities to oxygen (21). To investigate the mechanism of nitroreduction, FLU was incubated with human liver microsomes under anaerobic conditions. As shown in Figure 11, the rate of FLU-6 formation under anaerobic conditions was calculated as 74 pmol/min/mg protein, as compared to 1.8 pmol/min/mg protein under atmospheric oxygen levels. The deaerated and argon-purged incubation conditions dramatically increased the rate of FLU-6 formation by approximately 41-fold as compared to atmospheric oxygen conditions. These results suggest that although nitroreduction of FLU is observed at atmospheric oxygen concentrations, it is significantly enhanced under anaerobic conditions. Thus, nitroreduction of FLU appears to be highly sensitive to the presence of oxygen.

Characterization of Microsomal Enzymes Responsible for Nitroreduction

The dramatic increase of nitroreduction of FLU under anaerobic conditions suggested that at least some microsomal enzymes involved in FLU nitroreduction in human liver microsomes are oxygen-sensitive. To characterize microsomal enzymes responsible for nitroreduction,

individual purified P450 enzymes, CPR, or cytochrome *b*₅ were incubated separately with FLU under both anaerobic and aerobic conditions. Among all purified enzymes tested including liposome-reconstituted P450 enzymes (Invitrogen, Carlsbad, CA), only CPR was shown to reduce FLU to FLU-6. No formation of FLU-6 was detected when individual P450 enzymes or cytochrome *b*₅ were incubated with FLU under either anaerobic or aerobic conditions (data not shown). The rate of FLU-6 formation by CPR under atmospheric oxygen levels was calculated as 1.7 pmol/min/mg protein, and the rate increased dramatically by approximately 129-fold to 220 pmol/min/mg protein when the incubation mixtures were purged with argon before reactions were initiated by NADPH (Figure 12). These data clearly demonstrated that CPR alone reduced FLU to FLU-6 under both anaerobic and aerobic conditions, and this nitroreduction by CPR was greatly inhibited by air. Furthermore, under anaerobic conditions, the rate of FLU-6 formation by CPR was decreased to 13 pmol/min/mg protein with addition of α -lipoic acid, a reversible and selective CPR inhibitor (27), while formation of FLU-6 by CPR was totally blocked in the presence of α -lipoic acid under aerobic conditions (Figure 12). In parallel, very similar inhibitory effects of α -lipoic acid were also observed in human liver microsomal incubations of FLU. The rate of FLU-6 formation in human liver microsomal incubations was decreased from 74 to 7.8 pmol/min/mg protein in the presence of α -lipoic acid under anaerobic conditions, while the nitroreduction was completely blocked by α -lipoic acid under atmospheric oxygen levels (Figure 11). These data suggest a major role of CPR in the nitroreductive metabolism of FLU under both anaerobic and aerobic conditions.

The inhibitory effects of P450 isozyme-specific inhibitors on the formation of FLU-6 were also examined using pooled human liver microsomes. Inhibitory activity was confirmed using P450 marker substrates as previously described (28). In all incubations of FLU, the inhibitory effects on the formation of FLU-6 were minimal (<10%) for P450-specific inhibitors including α -naphthoflavone (CYP1A2), sulfaphenazole (CYP2C9), tranlylcypromine (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4/3A5) (Table 1). These data are consistent with the observations that no formation of FLU-6 was detected when individual P450 enzymes were incubated alone with FLU. No FLU-6 formation was detected when NADH, instead of NADPH, was used in human liver microsomal incubations, suggesting that microsomal cytochrome *b*₅ reductase is not involved in nitroreduction of FLU (data not shown).

Enzyme Kinetics of FLU-6 Formation

The kinetics of FLU-6 formation from FLU was studied in human liver microsomes under anaerobic conditions. As shown in Figure 13, the substrate-velocity curve of FLU-6 formation is hyperbolic when the data (rates of FLU-6 formation vs FLU concentrations) were fitted to a single-enzyme Michaelis-Menten equation. The Eadie-Hofstee plot exhibits a monophasic profile (inset in Figure 13), indicating apparent single enzyme kinetics. The apparent K_m and V_{max} were estimated to be 86 μ M and 188 pmol/min/mg, respectively. As a result, the turnover number (V_{max}/K_m) in human liver microsomes under anaerobic conditions was 2.2 μ L/min/mg for the corresponding enzyme. These data suggest that a single enzyme is involved in the formation of FLU-6 via FLU nitroreduction.

Discussion

Nitroreductive metabolism to nitroanion radicals, nitroso derivatives, and *N*-hydroxy intermediates is often associated with the toxicity of nitroaromatic compounds (20,21). Numerous drugs that bear a nitroaromatic group cause idiosyncratic hepatotoxicity, including nimesulide (32), tolcapone (33), nilutamide (34), and nitrofurantoin (35). FLU possesses a nitroaromatic group that may be a contributor to its mechanism of toxicity. In a previous study, we demonstrated that the nitroaromatic group of FLU enhanced cytotoxicity to hepatocytes as compared to its nitro to cyano analogue CYA (22). To date, oxidative bioactivation has been

implicated in the formation of reactive intermediates of FLU (19,23,25,26). No direct evidence for nitroreductive bioactivation has been presented. Although several nitroreduction metabolites have been identified in humans, little is known about the contribution of these metabolites to the idiosyncratic hepatotoxicity associated with FLU.

In this study, we investigated the bioactivation profiles of FLU and CYA in human liver microsomes and hepatocytes. The current study showed that FLU and CYA shared similar oxidative bioactivation pathways to generate reactive metabolites resulting in the formation of FLU-G1–4 and CYA-G1–4, respectively. This is in parallel with the previous observation that although less toxic, the mechanism of CYA toxicity is apparently similar to that of FLU in some aspects (22). Of significance was the detection and characterization of several GSH adducts resulting from nitroreductive metabolism of FLU. The reduced metabolite FLU-6 and its corresponding reactive metabolites were identified for the first time in human liver microsomal incubations of FLU, which is consistent with the detection of several reduced metabolites of FLU in human serum and urine (13,14,17–19). Direct evidence for the bioactivation of FLU-6 comes from the incubation of FLU-6 in human liver microsomes, which resulted in formation of the same GSH adducts FLU-G5–7 observed for FLU. Taken together, these data provide direct evidence that FLU undergoes nitroreductive metabolism in human liver microsomes to form reduced metabolites that can be subsequently bioactivated to form GSH adducts, presumably via diimine intermediates (Scheme 2). Similar involvement of nitroreduction in the formation of reactive intermediates was also observed in the case of tolcapone (36), in which an *ortho*-quinone imine intermediate was proposed. Formation of FLU-G5–7 was blocked in human liver microsomal incubations of CYA because the nitro to cyano replacement prevents the possibility of reduction of the nitroaromatic group, eliminating an additional route of toxicity. Inhibition of FLU-G5–7 formation may contribute to the attenuated cytotoxicity of CYA previously observed (22). However, it is noteworthy that apart from the bioactivation pathways presented here, formation of FLU-6 from FLU itself suggests the possibility of redox cycling, which may also contribute to the cytotoxicity of FLU (21). Although no additional phase II metabolites of the reduced aniline metabolite of FLU (FLU-6) were detected in the hepatocyte incubations, an *N*-acetyl metabolite FLU-4 with the reduced aniline group has been detected in human urine (Scheme 1,17). The role of phase II metabolism of the reduced aniline metabolites of FLU in vivo remains to be elucidated. Further studies in P450 overexpressing cell lines to elucidate the relationships of FLU and CYA metabolism and their cytotoxicity are currently underway.

The results from the current investigation also constitute the first report of CPR-mediated nitroreduction of FLU. Nitroreductive metabolism is primarily catalyzed by P450 and/or P450 reductase (37–40), although other enzymes may be involved, such as xanthine oxidoreductase (XO) (41), NADH:quinone oxidoreductase (NQO) (42), nitric oxide synthase (NOS) (43), and cytochrome *b*₅ reductase (44). While XO, NQO, and NOS are primarily present in cytosol and/or mitochondria, P450, CPR, and cytochrome *b*₅ reductase are the main known “nitroreductases” in human liver microsomes. Our results demonstrated that only CPR reduced FLU to FLU-6 under both aerobic and anaerobic conditions. A dramatic increase in the rate of FLU-6 formation under anaerobic conditions was also observed, supporting the fact that CPR is highly sensitive to oxygen. It is noteworthy that the liver contains functional units/lobules with regions close to the central vein, which can be physiologically hypoxic (45). However, the relative contribution of CPR to the formation of reduced FLU metabolites in humans remains to be investigated. Lack of P450 involvement in FLU nitroreduction was confirmed by studies using P450-specific inhibitors, which showed no significant effects on FLU-6 formation in human liver microsomal incubations of FLU. The role of cytochrome *b*₅ reductase was ruled out primarily because this enzyme only utilizes NADH. When NADH, instead of NADPH, was used in human liver microsomal incubations, no FLU-6 formation was detected. The role of CPR in nitroreduction of FLU was further confirmed by addition of α -lipoic acid,

a specific and reversible CPR inhibitor. Under anaerobic conditions, FLU-6 formation decreased by approximately 90% with addition of α -lipoic acid, while the CPR inhibitor completely blocked FLU-6 formation under aerobic conditions. Taken together, these data clearly demonstrate a major role of CPR in the nitroreductive metabolism of FLU, although other “nitroreductases” cannot be absolutely ruled out.

In conclusion, we found that FLU undergoes nitroreductive metabolism to form FLU-6, which is subsequently bioactivated to form GSH adducts in human liver microsomes. Our data clearly demonstrate that the nitroaromatic group of FLU contributes to FLU bioactivation under atmospheric concentrations of oxygen. These results provide a possible explanation for the difference in cytotoxicity between FLU and CYA. Nitroreduction of FLU is primarily mediated by CPR, although other hepatic enzymes in cytosol and mitochondria may also be involved in vivo. Such nitroreductive metabolism is dramatically elevated under anaerobic conditions. In summary, findings from the current study are important to a better understanding of the bioactivation pathways of FLU and their potential link to mechanisms of toxicity of FLU.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

- McLeod DG. Antiandrogenic drugs. *Cancer* 1993;71:1046–1049. [PubMed: 8428326]
- Schmitt B, Wilt TJ, Schellhammer PF, DeMasi V, Sartor O, Crawford ED, Bennett CL. Combined androgen blockade with nonsteroidal antiandrogens for advanced prostate cancer: A systematic review. *Urology* 2001;57:727–732. [PubMed: 11306391]
- Gomez JL, DuPont A, Cusan L, Tremblay M, Suburu R, Lemay M, Labrie F. Incidence of liver toxicity associated with the use of flutamide in prostate cancer patients. *Am J Med* 1992;92:465–470. [PubMed: 1349790]
- Wysowski DK, Fourcroy JL. Flutamide hepatotoxicity. *J Urol* 1996;155:209–212. [PubMed: 7490837]
- Crownover RL, Holland J, Chen A, Krieg R, Young BK, Roach M, Fu KK. Flutamide-induced liver toxicity including fatal hepatic necrosis. *Int J Radiat Oncol Biol Phys* 1996;34:911–915. [PubMed: 8598370]
- Pontiroli L, Sartori M, Pittau S, Morelli S, Boldorini R, Albano E. Flutamide-induced acute hepatitis: Investigation on the role of immunoallergic mechanisms. *Ital J Gastroenterol Hepatol* 1998;30:310–314. [PubMed: 9759603]
- Cetin M, Demirci D, Unal A, Altinbas M, Guven M, Unluhizarci K. Frequency of flutamide induced hepatotoxicity in patients with prostate carcinoma. *Hum Exp Toxicol* 1999;18:137–140. [PubMed: 10215102]
- Nakagawa Y, Koyama M, Matsumoto M. Flutamide-induced hepatic disorder and serum concentrations of flutamide and its metabolites in patients with prostate cancer. *Acta Urol Jpn* 1999;45:821–826.
- Kraus I, Vitezic D, Oguic R. Flutamide-induced acute hepatitis in advanced prostate cancer patients. *Int J Clin Pharmacol Ther* 2001;39:395–399. [PubMed: 11563686]
- Osculati A, Castiglioni C. Fatal liver complications with flutamide. *Lancet* 2006;367:1140–1141. [PubMed: 16616550]
- Katchen B, Buxbaum S. Disposition of a new, nonsteroid, antiandrogen, alpha, alpha, alpha-trifluoro-2-methyl-4'-nitro-m-propionolide (flutamide), in men following a single oral 200 mg dose. *J Clin Endocrinol Metab* 1975;41:373–379. [PubMed: 1159048]
- Schulz M, Schmold A, Donn F, Becker H. The pharmacokinetics of flutamide and its major metabolites. *Eur J Clin Pharmacol* 1988;34:633–636. [PubMed: 3169114]
- Asakawa N, Yamashita K. Studies on the metabolic fate of flutamide. *Antibiot Chemother* 1995;11:1418–1427.

14. Takashima E, Iguchi K, Usui S, Yamamoto H, Hirano K. Metabolite profiles of flutamide in serum from patients with flutamide-induced hepatic dysfunction. *Biol Pharm Bull* 2003;26:1455–1460. [PubMed: 14519954]
15. Shet MS, McPhaul M, Fisher CW, Stallings NR, Estabrook RW. Metabolism of the antiandrogenic drug (Flutamide) by human CYP1A2. *Drug Metab Dispos* 1997;25:1298–1303. [PubMed: 9351907]
16. Watanabe N, Goda R, Irie T, Yamashita K. In vitro effects of fluoroquinolone anti-bacterial agents on flutamide metabolism in human liver microsomes. *Jpn J Clin Pharmacol Ther* 2001;32:65–71.
17. Aizawa Y, Ikemoto I, Kishimoto K, Wada T, Yamazaki H, Ohishi Y, Kiyota H, Furuta N, Suzuki H, Ueda M. Flutamide-induced hepatic dysfunction in relation to steady-state plasma concentrations of flutamide and its metabolites. *Mol Cell Biochem* 2003;252:149–156. [PubMed: 14577588]
18. Goda R, Nagai D, Akiyama Y, Nishikawa K, Ikemoto I, Aizawa Y, Nagata K, Yamazoe Y. Detection of a new N-oxidized metabolite of flutamide, N-[4-nitro-3-(trifluoromethyl)phenyl]hydroxylamine, in human liver microsomes and urine of prostate cancer patients. *Drug Metab Dispos* 2006;34:828–835. [PubMed: 16507648]
19. Tevell A, Lennernäs H, Jönsson M, Norlin M, Lennernäs B, Bondesson U, Hedeland M. Flutamide metabolism in four different species *in vitro* and identification of flutamide metabolites in human patient urine by high performance liquid chromatography/tandem mass spectrometry. *Drug Metab Dispos* 2006;34:984–992. [PubMed: 16540588]
20. Purohit V, Basu AK. Mutagenicity of nitroaromatic compounds. *Chem Res Toxicol* 2000;13:673–692. [PubMed: 10956054]
21. Boelsterli UA, Ho HK, Zhou S, Leow KY. Bioactivation and hepatotoxicity of nitroaromatic drugs. *Curr Drug Metab* 2006;7:715–727. [PubMed: 17073576]
22. Coe KJ, Jia Y, Ho HK, Rademacher P, Bammler TK, Beyer RP, Farin FM, Woodke L, Plymate SR, Fausto N, Nelson SD. Comparison of the cytotoxicity of the nitroaromatic drug flutamide to its cyano analogue in the hepatocyte cell line TAMH: Evidence for complex I inhibition and mitochondrial dysfunction using toxicogenomic screening. *Chem Res Toxicol* 2007;20:1277–1290. [PubMed: 17702527]
23. Soglia JR, Contillo LG, Kalgutkar AS, Zhao S, Hop CE, Boyd JG, Cole MJ. A semiquantitative method for the determination of reactive metabolite conjugate levels in vitro utilizing liquid chromatography-tandem mass spectrometry and novel quaternary ammonium glutathione analogues. *Chem Res Toxicol* 2006;19:480–490. [PubMed: 16544956]
24. Kostrubsky SE, Strom SC, Ellis E, Nelson SD, Mutlib AE. Transport, metabolism, and hepatotoxicity of flutamide, drug–drug interaction with acetaminophen involving phase I and phase II metabolites. *Chem Res Toxicol* 2007;20:1503–1512. [PubMed: 17900172]
25. Kang P, Dalvie D, Smith E, Zhou S, Deese A. Identification of a novel glutathione conjugate of flutamide in incubations with human liver microsomes. *Drug Metab Dispos* 2007;35:1081–1088. [PubMed: 17403914]
26. Kang P, Dalvie D, Smith E, Zhou S, Deese A, Nieman JA. Bioactivation of flutamide metabolites by human liver microsomes. *Drug Metab Dispos* 2008;36:1425–1437. [PubMed: 18411402]
27. Slepneva IA, Sergeeva SV, Khramtsov VV. Reversible inhibition of NADPH-cytochrome P450 reductase by α -lipoic acid. *Biochem Biophys Res Commun* 1995;214:1246–1253. [PubMed: 7575537]
28. Wen B, Ma Li, Rodrigues AD, Zhu M. Detection of novel reactive metabolites of trazodone: Evidence for CYP2D6-mediated bioactivation of m-chlorophenylpiperazine. *Drug Metab Dispos* 2008;36:841–850. [PubMed: 18238857]
29. Wen B, Ma L, Nelson SD, Zhu M. High-throughput screening and characterization of reactive metabolites using polarity switching of hybrid triple quadrupole linear ion trap mass spectrometry. *Anal Chem* 2008;80:1788–1799. [PubMed: 18251522]
30. Baillie TA, Davis MR. Mass spectrometry in the analysis of glutathione conjugates. *Biol Mass Spectrom* 1993;22:319–325. [PubMed: 8329460]
31. Rodrigues AD. Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem Pharmacol* 1999;57:465–480. [PubMed: 9952310]

32. Boelsterli UA. Mechanisms of NSAID-induced hepatotoxicity: focus on nimesulide. *Drug Saf* 2002;25:633–648. [PubMed: 12137558]
33. Borges N. Tolcapone in Parkinson's disease: Liver toxicity and clinical efficacy. *Exp Opin Drug Saf* 2005;4:69–73.
34. Ask K, Dijols S, Giroud C, Casse L, Frapart YM, Sari MA, Kim KS, Stuehr DJ, Mansuy D, Camus P, Boucher JL. Reduction of nilutamide by NO synthases: Implications for the adverse effects of this nitroaromatic antiandrogen drug. *Chem Res Toxicol* 2003;16:1547–1554. [PubMed: 14680368]
35. Núñez-Vergara LJ, Sturm JC, Olea-Azar C, Navarrete-Encina P, Bollo S, Squella JA. Electrochemical, UV–visible and EPR studies on nitrofurantoin: nitro radical anion generation and its interaction with glutathione. *Free Radical Res* 2000;32:399–409. [PubMed: 10766408]
36. Smith KS, Smith PL, Heady TN, Trugman JM, Harman WD, Macdonald TL. In vitro metabolism of tolcapone to reactive intermediates: relevance to tolcapone liver toxicity. *Chem Res Toxicol* 2003;16:123–8. [PubMed: 12588182]
37. Saito K, Kamataki T, Kato R. Participation of cytochrome P-450 in reductive metabolism of 1-nitropyrene by rat liver microsomes. *Cancer Res* 1984;44:3169–73. [PubMed: 6430544]
38. Arlt VM, Stiborova M, Hewer A, Schmeiser HH, Phillips DH. Human enzymes involved in the metabolic activation of the environmental contaminant 3-nitrobenzanthrone: Evidence for reductive activation by human NADPH:cytochrome p450 reductase. *Cancer Res* 2003;63:2752–61. [PubMed: 12782579]
39. Walton MI, Workman P. Nitroimidazole bioreductive metabolism. Quantitation and characterisation of mouse tissue benznidazole nitroreductases in vivo and in vitro. *Biochem Pharmacol* 1987;36:887–896. [PubMed: 3105539]
40. Walton MI, Wolf CR, Workman P. The role of cytochrome P450 and cytochrome P450 reductase in the reductive bioactivation of the novel benzotriazine di-N-oxide hypoxic cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233, WIN 59075) by mouse liver. *Biochem Pharmacol* 1992;44:251–259. [PubMed: 1642640]
41. Pritsos CA. Cellular distribution, metabolism and regulation of the xanthine oxidoreductase enzyme system. *Chem-Biol Interact* 2000;129:195–208. [PubMed: 11154741]
42. Siegel D, Gibson NW, Preusch PC, Ross D. Metabolism of diaziquone by NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase): Role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* 1990;50:7293–7300. [PubMed: 2121335]
43. Ask K, Décologne N, Asare N, Holme JA, Artur Y, Pelczar H, Camus P. Distribution of nitroreductive activity toward nilutamide in rat. *Toxicol Appl Pharmacol* 2004;201:1–9. [PubMed: 15519603]
44. Kurian JR, Bajad SU, Miller JL, Chin NA, Trepanier LA. NADH cytochrome *b5* reductase and cytochrome *b5* catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther* 2004;311:1171–1178. [PubMed: 15302896]
45. Noll T, Hugo-Wissemann D, Littauer A, De Sagara RM, De Groot H. The decisive pO₂-levels in haloalkane-mediated liver cell injury. *Free Radical Res Commun* 1987;3:293–298. [PubMed: 3508440]

Figure 1. LC/MRM analyses of FLU-6 formed in human liver microsomal incubations of FLU: (A) incubation extracts and (B) synthesized standard of FLU-6 (10.0 ng/mL) spiked in the incubation medium. (C) MS/MS spectrum of FLU-6 formed in human liver microsomal incubations. (D) MS/MS spectrum of synthesized standard FLU-6.

Figure 2.
LC/MS/MS detection of GSH adducts using negative PI scanning at m/z 272 in human liver microsomal incubations of FLU (A), CYA (B), and FLU-6 (C).

Figure 3.

(A) MS/MS spectrum of FLU-G1 at m/z 598 ($[M + H]^+$), (B) MS³ mass spectrum of the fragment ion of FLU-G1 at m/z 323, (C) MS/MS spectrum of CYA-G1 at m/z 578 ($[M + H]^+$), and (D) MS³ mass spectrum of the fragment ion of CYA-G1 at m/z 303.

Figure 4.
MS/MS spectra of GSH adducts formed in human liver microsomes: (A) FLU-G2 and (B) CYA-G2.

Figure 5.

(A) MS/MS spectrum of FLU-G3 at m/z 598 ($[M + H]^+$), (B) MS³ mass spectrum of the fragment ion of FLU-G3 at m/z 323, (C) MS/MS spectrum of CYA-G3 at m/z 578 ($[M + H]^+$), and (D) MS³ mass spectrum of the fragment ion of CYA-G3 at m/z 303.

Figure 6.
MS/MS spectra of GSH adducts formed in human liver microsomes: (A) FLU-G4 and (B) CYA-G4.

Figure 7. LC/MS/MS analysis of FLU-G5. (A) Extracted ion chromatogram of $[M - H]^-$ ion at m/z 550 in the negative ion mode. (B) MS/MS spectrum of FLU-G5 at m/z 552 in the positive ion mode.

Figure 8. LC/MS/MS analysis of FLU-G6. (A) Extracted ion chromatogram of $[M - H]^-$ ion at m/z 566 in the negative ion mode. (B) MS/MS spectrum of FLU-G6 at m/z 568 in the positive ion mode.

Figure 9. LC/MS/MS analysis of FLU-G7. (A) Extracted ion chromatogram of $[M - H]^-$ ion at m/z 480 in the negative ion mode. (B) MS/MS spectrum of FLU-G7 at m/z 482 in the positive ion mode.

Figure 10.

Formation of FLU-G1 and CYA-G1 in incubations with cDNA-expressed recombinant P450 isozymes. The enzyme activities were expressed as the percentage of CYP1A2 activity and shown as an average of three measurements.

Figure 11.

Formation of FLU-6 in incubations of FLU with human liver microsomes under anaerobic and aerobic conditions and in the presence of α -lipoic acid, a reversible CPR inhibitor.

Figure 12.

Formation of FLU-6 in incubations of FLU with CPR alone under anaerobic and aerobic conditions and in the presence of α -lipoic acid, a reversible CPR inhibitor.

Figure 13. Substrate-velocity curve of FLU-6 formation from FLU in human liver microsomes under anaerobic conditions.

Scheme 1.

Structures of FLU, the Nitro to Cyano Analogue of FLU (CYA), and the Metabolites of FLU 2-OH FLU, FLU-1, -3-6, and -8

^a Note that hydroxylation of the isobutyl moiety in FLU-G6 can be on C1 or C2.

Scheme 2.
Proposed Mechanisms for the Formation of GSH Adducts of FLU in Human Liver Microsomes^a

Table 1

Effects of P450 Isoform-Specific Inhibitors on the Formation of FLU-6 in Human Liver Microsomal Incubations of FLU^a

P450 inhibitor	FLU-6 formation	P450 activity ^b
α -naphthoflavone (CYP1A2)	95 ($\pm$ 7)	15 ($\pm$ 4)
sulfaphenazole (CYP2C9)	98 ($\pm$ 4)	29 ($\pm$ 8)
tranylcypromine (CYP2C19)	97 ($\pm$ 6)	25 ($\pm$ 6)
quinidine (CYP2D6)	95 ($\pm$ 5)	12 ($\pm$ 8)
ketoconazole (CYP3A4/3A5)	93 ($\pm$ 5)	22 ($\pm$ 9)

^aValues are the means ($\pm$ SDs) of triplicates and expressed as a percentage relative to the control without inhibitors.

^bP450 activities were determined using known P450 substrates. P450 activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/3A5 without inhibitors were calculated as 322 ± 17 (phenacetin *O*-deethylation), 129 ± 15 (tolbutamide hydroxylation), 37 ± 4 [(*S*)-mephenytoin-4'-hydroxylation], 118 ± 16 (dextromethorphan *O*-demethylation), and 2378 ± 124 pmol/mg/min (testosterone 6 β -hydroxylation), respectively.