In Vitro Metabolism, Permeability and Efflux of Bazedoxifene in Humans

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Running title: In vitro glucuronidation of bazedoxifene

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Document Statistics

Text pages: 28
Tables: 2
Figures: 5
References: 26
Word count: 5556
Abstract word count: 242
Introduction word count: 535
Results and Discussion word count: 2071

Abbreviations: SERM, selective estrogen receptor modulator; UGT, uridine 5′-diphosphoglucuronic acid transferase; Glu, glucuronide; m/z, mass-to-charge ratio; BZA, bazedoxifene.
Abstract:
Bazedoxifene acetate (BZA), a novel estrogen receptor modulator being developed for the prevention and treatment of post-menopausal osteoporosis, undergoes extensive metabolism in women following oral administration. In this study, the in vitro metabolism of [14C]BZA was determined in human hepatocytes and hepatic and intestinal microsomes, and the UGT isozymes involved in the glucuronidation of BZA were identified. In addition, BZA was evaluated for its potential as a substrate of P-glycoprotein (P-gp) transporter in Caco-2 cell monolayers. BZA was metabolized to two mono-glucuronides, BZA-4′-glucuronide and BZA-5-glucuronide in hepatocytes and in liver and intestinal microsomes including jejunum, duodenum and ileum. Both BZA-4′-glucuronide and BZA-5-glucuronide were major metabolites in the intestinal microsomes, while BZA-4′-glucuronide was the predominant metabolite in liver microsomes and hepatocytes. The kinetic parameters of BZA-4′-glucuronide formation were determined in liver, duodenum, and jejunum microsomes and with UGT 1A1, 1A8, 1A10, the most active UGT isoforms involved in the glucuronidation of BZA, while those of BZA-5-glucuronide were determined with all the enzyme systems except in liver microsomes and in UGT 1A1 as the formation of the BZA-5-glucuronide was too low. \( K_m \) values in liver, duodenum, and jejunum microsomes and UGT 1A1, 1A8, 1A10, were similar and ranged from 5.1 to 33.1 µM for BZA-4′-glucuronide formation and 2.5 to 11.1 µM for BZA-5-glucuronide formation. \( V_{max} \) values ranged from 0.8 to 2.9 nmol/min/mg protein for BZA-4′-glucuronide and from 0.1 to 1.2 nmol/min/mg protein for BZA-5-glucuronide. In Caco-2 cells, BZA appeared to be a P-gp substrate.
DMD # 30999

Introduction

Osteoporosis is a chronic and progressive skeletal disorder that is common in the elderly (Gennari et al., 2008), characterized by low bone mass and weak bone strength leading to increased risk of fractures. Osteoporosis affects about 40% of post-menopausal women due to declining levels of estrogen. Selective estrogen receptor modulators (SERMs) are a class of agents that provide favorable therapeutic effects on the bone while minimizing undesirable effects of estrogens on other tissues by acting differently at the various estrogen receptors throughout the body.

Bazedoxifene acetate (BZA), a novel indole-based third generation selective estrogen receptor modulator (SERM), was selected for development for the prevention and treatment of post-menopausal osteoporosis using stringent preclinical screening parameters, including requirements for favorable effects on the bone and lipid profiles (Komm et al., 2005, Gruber et al., 2004, Ullrich et al., 2006). In clinical trials, BZA has been shown to prevent bone loss, increase bone mineral density and reduce bone turnover in a two year study in post-menopausal women (Kanis 2009 and Miller et al., 2008). Treatment with BZA significantly reduced the incidence of new vertebral fractures in a three year study in osteoporotic post-menopausal women (Silverman et al., 2008). BZA did not stimulate the uterine endometrium in women at dosages up to 40 mg, correlating well with the preclinical pharmacology (Miller et al., 2002; Lewiecki, et al., 2007; Ronkin et al., 2005). BZA represents a promising pharmacotherapy against osteoporosis with a potentially enhanced safety profile (Stump et al., 2007). Both preclinical and clinical data indicated that BZA has a unique combination of attributes, making it an
attractive option for the treatment and prevention of osteoporosis (Gennari, 2007 and Gennari et al., 2008).

BZA displayed straightforward pharmacokinetic properties following a single oral dose of \([^{14}\text{C}]\text{BZA}\) (Chandrasekaran et al., 2009). BZA was rapidly absorbed and the mean plasma elimination half-life of unchanged drug was long (about 33 hours). BZA was extensively metabolized in post-menopausal women through the glucuronidation pathway with few P450-mediated metabolites formed. Although conjugation takes place at both the phenyl (4') and indole (5) moieties of the molecule, the major circulating metabolite was the indole glucuronide (BZA-5-glucuronide). Unchanged drug and BZA-4'-glucuronide were minor components in plasma. Extensive metabolism of BZA was consistent with the reported low bioavailability and high oral clearance (Ermer et al., 2003 and Patat et al., 2003) indicating high first-pass effect. A long elimination half-life value and secondary peaks in the concentration-versus-time profiles also suggested that BZA underwent enterohepatic recirculation. The majority of the radioactive dose (85%) was excreted via the feces as unchanged drug, representing both unabsorbed drug and/or hydrolyzed glucuronides.

In this study, the metabolite profiles of \([^{14}\text{C}]\text{BZA}\) were determined in cryopreserved human hepatocytes, and in hepatic and intestinal microsomes, and the UGT isozymes involved in the glucuronidation of BZA were identified by using cDNA expressed human UGT enzymes, including UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17. Kinetic parameters (\(K_m, V_{\text{max}}\)) for the formation of BZA glucuronides were also determined in human hepatic, duodenum and jejunum microsomes, as well as
in three UGT (1A1, 1A8 and 1A10) isoforms. In addition, BZA was evaluated for its potential as a substrate of P-glycoprotein (P-gp) transporter in Caco-2 cell monolayers.
Methods

Chemicals and Reagents

\[^{14}\text{C}]\text{Bazedoxifene acetate (BZA)}\] was obtained from ViTrax Co. (Placentia, CA). The specific activity of \[^{14}\text{C}]\text{BZA}\] was 47 mCi/mmol. Radiochemical purity and chemical purity were > 98%. Unlabeled BZA and two BZA metabolites, BZA-4'-glucuronide and BZA-5-glucuronide were synthesized by Wyeth Research, Pearl River, NY. The structures of \[^{14}\text{C}]\text{BZA}\] and its glucuronide metabolites are shown in Figure 1. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP\(^+\)), uridine 5'-diphosphogluconuronic acid trisodium salt (UDPGA), alamethicin, 4-nitrophenol, testosterone, trifluoperazine, naphthol, dihydrotestosterone, saccharolactone, and \[^{14}\text{C}]4\text{-nitrophenol}\] (Lot # 124K9402, 8.5 mCi/mm, purity 98%) were obtained from Sigma Chemical Co. (St. Louis, MO). \[^{14}\text{C}]\text{Testosterone}\] (Lot # 3379-017, 53.6 mCi/mm, purity 98%) was purchased from New England Nuclear (Boston, MA). \[^{3}\text{H}]\text{Digoxin}\] (37 Ci/mm) was purchased from Perkin Elmer (Boston, MA). Ultima Gold and Ultima Flo M scintillation fluids were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Glusulase\(^\text{®}\) (125,000 units of glucuronidase per mL and 10,600 units of sulfatase per mL) was obtained from DuPont (Lot # 093149, Boston, MA). Pooled female human liver microsomes were purchased from Xenotech LLC (Lenexa, KS). Pooled female duodenum, jejunum and ileum microsomes, UGT 1A1, 1A3, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 were obtained from BD Biosciences (Woburn, MA). UGT 1A4, 1A6 and 1A7 were purchased from Invitrogen (Carlsbad, CA). The cryopreserved human hepatocytes, hepatocyte thawing and incubation media were purchased from In Vitro
Technologies Inc. (Baltimore, MD). Ammonium acetate, magnesium chloride, and all solvents used for extraction and for chromatographic analysis were obtained from EMD Chemicals (Gibbstown, NJ) and were HPLC grade or ACS reagent grade. Cell culture medium and reagents were purchased from Invitrogen (Grand Island, NY). All other reagents were analytical grade or better.

**Incubation of $[^{14}\text{C}]$BZA in Microsomes and Expressed Human UGTs**

$[^{14}\text{C}]$BZA was mixed with non-radiolabeled BZA (1:1) for the incubations. $[^{14}\text{C}]$BZA (1, 10 or 50 $\mu$M) was combined with pooled female human liver, jejunum, duodenum or ileum microsomes (1.0 mg/mL), or cDNA expressed human UGT isozymes (0.25 mg/mL), and magnesium chloride (10 mM) in 1.0 mL of potassium phosphate buffer (0.1 M, pH 7.4). The mixture was pre-incubated at 37°C for 3 min and the reaction was initiated by the addition of an NADPH regenerating system or UDPGA or both. The NADPH regenerating system consisted of glucose-6-phosphate (3.6 mM, final concentration), glucose-6-phosphate dehydrogenase (0.4 units/mL) and NADP$^{+}$ (1.3 mM). UDPGA was added to incubations as a 50 $\mu$L aliquot of an 80 mM solution in water, to give a final concentration of 4 mM. Alamethicin (25 $\mu$g/mL) was mixed with liver microsomes for incubations containing UDPGA only. Incubations were carried out at 37°C for up to 60 min. Incubations without any cofactor were also conducted under the same conditions. Positive control incubations were performed in female human liver microsomes with $[^{14}\text{C}]$testosterone (100 $\mu$M) as the substrate in the presence of the NADPH regenerating system, or with $[^{14}\text{C}]$4-nitrophenol (100 $\mu$M) as the substrate in the presence of UDPGA under the same conditions, except for only a 15 min incubation time. Positive control incubations were also performed in selected UGT1A4, 1A6, and
2B17 isozymes using trifluoperazine, naphthol, and dihydrotestosterone, respectively as the substrates at final concentrations of 100 µM, in the presence of UDPGA under similar conditions, except for only a 30 min incubation time. All incubations were performed in duplicate. Incubations were stopped by the addition of 2 mL of cold methanol. Samples were vortexed and centrifuged at 1,800 g, 4°C for 10 min (Sorvall centrifuge) to separate the denatured proteins. Supernatants were transferred to clean tubes and evaporated to about 1 mL under a nitrogen stream in a TurboVap LV evaporator (Caliper Life Sciences, Hopkinton, MA). Concentrated samples were centrifuged again at 1,800 g, 4°C for 15 min and aliquots of supernatants were radioassayed using a Tri-Carb Model 2900 liquid scintillation counter (PerkinElmer Life and Analytical Sciences) and 5 mL of Ultima Gold as the scintillant. Samples were also analyzed by HPLC with radioactivity detection for metabolite profiles, and by LC/MS for metabolite identification.

**Incubation of [14C]BZA in Cryopreserved Human Hepatocytes**

[14C]BZA was incubated with cryopreserved human hepatocytes in a hepatocyte incubation medium at three concentrations (1, 10 and 50 µM). Hepatocytes from 3 individuals were quickly thawed at 37°C, then immediately washed with hepatocyte thawing media (50 mL/vial). The cells were centrifuged at 50 x g at room temperature for 5 min, and resuspended in 10 mL of incubation media. The number of viable cells was determined by using the Trypan Blue exclusion method. Aliquots (1mL/well) of the cell suspension (approximately 1 million viable cells per well) were preincubated at 37°C with 5% CO₂ : 95% O₂ for 5 min and then [14C]BZA was added. Incubations were performed in duplicates at 37°C with 5% CO₂ : 95% O₂ for 2 hr. Positive controls
containing 50 µM [14C]7-ethoxycoumarin were incubated under the same conditions but only for 0.5 hr. Negative controls without hepatocytes were also run under the same conditions as those containing [14C]BZA. Reactions were stopped by adding 1 mL of cold methanol. Samples were extracted and proteins were removed by centrifugation as described in microsomal incubations. Supernatants were analyzed by HPLC with radioactivity detection for metabolite profiles, and by LC/MS for metabolite identification.

**Determination of Kinetic Parameters (Km and Vmax)**

Incubations for the determination of kinetic parameters for the formation of BZA glucuronides by pooled female human liver, duodenum, and jejunum microsomes, and cDNA expressed human UGT1A1, 1A8 and 1A10 isoforms were performed under predetermined linear conditions with respect to time and protein concentrations. [14C]BZA (10 µM) was incubated with human liver microsomes (0.6 mg protein/mL) or UGT1A10 (0.25 mg protein/mL) in the presence of UDPGA (4 mM) at 37°C for up to 60 min in duplicates to determine the optimum incubation time. To optimize protein concentrations, [14C]BZA (10 µM) was also incubated with human liver microsomes (0.05 to 1.5 mg/mL) or UGT1A10 (0.02 to 1 mg/mL) in the presence of UDPGA (4 mM) at 37°C for 15 or 30 min, respectively. The formation of the major glucuronide of BZA was measured by HPLC with radioactivity detection, and it was linear up to 30 min and up to 1 mg/mL of liver microsomal protein concentrations or up to 0.5 mg/mL of UGT 1A10 (data not shown). To determine kinetic parameters, Km and Vmax values, [14C]BZA at concentrations of 0.31, 0.62, 1.25, 2.5, 5.0, 10, 20, 50, 100 and 150 µM was incubated
with liver (0.5 mg/mL) or intestinal (0.2 mg/mL) microsomes, or cDNA expressed UGT1A1, 1A8 and 1A10 isoforms (0.2 mg/mL) in 1.0 mL of potassium phosphate buffer (0.1 M, pH 7.4) containing 10 mM magnesium chloride and alamethicin (25 µg/mL) at 37°C up to 30 min. Due to the solubility limit in the incubation buffer, the highest concentration of BZA that could be used for the determination of kinetics was 150µM. Reactions were initiated by the addition of UDPGA (4 mM) and were stopped by adding 1 mL of cold methanol. Proteins were removed by centrifugation and an aliquot of the extract was analyzed by HPLC. $K_m$ and $V_{max}$ values were calculated by a nonlinear regression method using WinNonlin software (version 5.1, Pharsight, Mountain View, CA).

**Transport Experiments Across Caco-2 Cell Monolayers**

Twelve-well plates with cell culture inserts (BD Biosciences, Franklin Lakes, NJ) were used for growing the cells. The cells were routinely cultured on a flask at 37°C in a humidified atmosphere of 5% CO$_2$. Near confluent Caco-2 cell cultures were harvested by trypsinization with 0.25% trypsin at 37°C for 5 min and resuspended in culture medium (Dulbecco’s Modified Eagle Medium (DMEM) containing 1% non-essential amino acids (NEAA), 100 unit/mL of streptomycin, 100 µg/mL of penicillin, and 10% Fetal bovine serum). Caco-2 cells were seeded onto inserts at a density of 140,000 cells/cm$^2$. Cell culture medium was changed every 2-3 days. After 21 days culture on the inserts, the cells were rinsed with incubation medium (DMEM with NEAA, 1% v/v) and were used for conducting studies. To determine the rates of permeation, BZA was added to either the apical or basolateral compartment at concentrations of 0.7, 7, and 70 µM. The substrate concentrations were selected based on
the analytical sensitivity and solubility of the compound. To evaluate the involvement of P-gp, BZA (0.7 and 7 μM) was added to the apical (A) or basolateral (B) compartment in the absence or presence of verapamil (100 μM), a P-gp inhibitor. The inhibitor (verapamil) was added into both apical and basolateral compartments. All stock solutions were prepared at 20 mM in 100% DMSO and diluted in incubation medium to achieve final concentrations for studies. The maximum concentration of DMSO in the incubation medium was less than 2%. Incubations were performed in triplicates at 37°C and 5% CO₂ for 2 hours in a CO₂ incubator with 40 rpm shaking. Media from the apical and basolateral compartments were collected and the radioactivity was determined using a liquid scintillation counter.

**HPLC Analysis**

Metabolite profiles were determined by HPLC with radioactivity detection. A Waters model 2695 Alliance system (Waters Corp., Milford, MA) with a built-in autosampler was used for analysis. A Flo-One β Model A625 radioactivity flow detector (PerkinElmer Life and Analytical Sciences) with a 250 μL LQTR flow cell and a Waters model 996 photodiode array UV detector set to monitor at 300 nm were used for data acquisition. Separations were accomplished on a Discovery C18, 250 x 4.6 mm column (Supelco Analytical, Bellefonte, PA) using a mobile phase flow rate of 1 mL/min. The flow rate of Ultima Flo M scintillation fluid was 3 mL/min, providing a mixing ratio of scintillation cocktail to mobile phase of about 3:1. The sample chamber in the autosampler was maintained at 4°C, while the column was at an ambient temperature of about 20°C. The mobile phase consisted of 10 mM ammonium acetate, pH 4.5 (A) and 90% methanol in water (B). A linear gradient was used: B was set at 20% for 5 min,
increased to 40% in 25 min, 50% in 30 min, 60% in 10 min, and to 80% in 5 min, and isocratic at 80% for another 5 min.

**Liquid Chromatography/Mass Spectrometry Analysis (LC/MS)**

The HPLC system used for mass spectrometric analysis was an Agilent Model 1100 HPLC system equipped with a binary pump and diode array UV detector (Agilent Technologies, Palo Alto, CA). The HPLC separation conditions were as described above for metabolite profiling except that the UV detector was set to monitor 200-600 nm. During LC/MS sample analysis, up to 10 min of the initial flow was diverted away from the mass spectrometer prior to evaluation of metabolites. The mass spectrometer used for metabolite characterization was a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA). It was equipped with an electrospray ionization (ESI) interface and operated in the positive ionization mode. The capillary temperature was 300°C, and the spray voltage was set at 4.5 kV. The nebulizer gas pressure was 90 psi and the auxiliary gas was set to 40. MS\(^2\) analysis was performed with a relative collision energy of 30%. Synthetic BZA-4'-glucuronide and BZA-5-glucuronide standards were used to aid the structural characterization.

**Data Analysis**

Flo-One analytical software (version 3.60) was utilized to integrate the radioactive peaks for the quantitative calculation of metabolite formation. Xcalibur software (version 1.3, Thermo Electron Corp.) was used to control LC/MS equipment and for analysis of LC/MS data. The apparent permeability coefficient (P\text{app}) was calculated by

\[
P_{\text{app}} = \frac{(dQ/dt)}{(A \times C_0)},
\]

where dQ/dt is amount of drug transported within a given time.
period. \( C_0 \) is the initial drug concentration in the donor compartment at \( t = 0 \), and \( A \) is the surface area of the monolayer. Results were expressed as a mean \( \text{P}_{\text{app}} \) (cm/sec) \( \pm \) standard deviation (SD) from three separate monolayers, unless otherwise noted. The efflux ratio (\( B \rightarrow A/A \rightarrow B \) ratio) was obtained from a ratio of \( \text{P}_{\text{app}}(B \rightarrow A)/\text{P}_{\text{app}}(A \rightarrow B) \).
**Results**

**Metabolite Profiles in Microsomes and Hepatocytes**

HPLC-radiochromatograms of $[^{14}\text{C}]$BZA (50 µM) incubated in microsomes of human liver, duodenum, jejunum and ileum and hepatocytes are depicted in Figure 2.

Metabolite profiles of BZA were qualitatively similar in human hepatic and intestinal microsomes. BZA was extensively metabolized to two glucuronides, BZA-4'-glucuronide and BZA-5-glucuronide, in all incubations examined. However, differences were observed in the proportions of these two glucuronides formed in hepatic and intestinal microsomes. Both BZA-4'-glucuronide and BZA-5-glucuronide were major metabolites in jejunum, duodenum and ileum microsomes, while BZA-4'-glucuronide was the predominant glucuronide formed in liver microsomes and BZA-5-glucuronide was observed in trace or small amounts. Under the conditions utilized, the ratios of BZA-4'-glucuronide to BZA-5-glucuronide were 73, 2, 3 and 3 in incubations of liver, duodenum, jejunum, and ileum microsomes, respectively. BZA was extensively metabolized via glucuronidation in hepatocytes. Similar to liver microsomes, the 4'-glucuronide was the predominant metabolite formed by hepatocytes. The ratio of BZA-4'-glucuronide to 5-glucuronide in hepatocytes was 56. No oxidative metabolites were observed in any significant amounts when BZA was incubated in the presence of NADPH with microsomal proteins or hepatocytes. Under similar incubation conditions, $[^{14}\text{C}]$testosterone and $[^{14}\text{C}]$4-nitrophenol were metabolized to 6β-hydroxyt testosterone and the 4-nitrophenol glucuronide conjugate, respectively, indicating that the human liver microsomes used in this study were metabolically active. Similar results were observed in cryopreserved hepatocytes, the positive control $[^{14}\text{C}]$7-ethoxycoumarin was
metabolized to both phase I (hydroxycoumarin) and phase II (7-ethoxycoumarin glucuronide) metabolites (data not shown).

Structural Characterization of Metabolites

The identities of the two glucuronides BZA-4'-glucuronide and BZA-5-glucuronide formed in the in vitro samples were confirmed by LC/MS analysis as well as by chromatography with authentic reference standards. Both BZA-4'-glucuronide and BZA-5-glucuronide produced a \([\text{M+H}]^+\) at \(m/z\) 647. The product ions of \(m/z\) 647 mass spectra and proposed fragmentation schemes for both glucuronides are shown in Figure 3. The mass spectra were similar for both glucuronides. Neutral loss of 176 Da yielded the product ion at \(m/z\) 471, which indicated conjugation with glucuronic acid. Product ions at \(m/z\) 252 and 126 were also observed for BZA. However, the mass spectra did not indicate the site of conjugation. The HPLC retention time of these metabolites matched those of synthetic 4'-glucuronide and 5-glucuronide confirming identification of these two metabolites.

Incubations with cDNA Expressed Human UGT Isozymes

To identify the UGT enzymes responsible for the formation of the two BZA glucuronides, \([^{14}\text{C}])BZA was incubated with recombinant human UGT enzymes UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17, which represented the most abundant UGT isoforms. Among the 12 isozymes examined, UGT 1A1, 1A8 and 1A10 were the most active isoforms involved in the glucuronidation of BZA in humans. UGT 1A3, 1A7, 1A9, 2B7, and 2B15 were less active, while no activity was observed for UGT 1A4, 1A6, 2B4, and 2B17. Under the similar conditions, trifluoperazine, naphthol, and dihydrotestosterone were converted to their glucuronide...
conjugates by UGT1A4, 1A6, and 2B17 with 81%, 100%, and 100% turnovers, respectively, indicating those UGT isozymes used in this study were metabolically active. Glucuronidation activities of BZA for UGT isozymes are shown in Figure 4. It should be noted that in this experiment, incubations were performed using same amount of supersomes (0.25 mg/mL protein) for each isozyme. However, the relative content of enzyme for each UGT preparation was unknown. Representative radiochromatograms of $^{[14}C\]BZA following incubation with UGT 1A1, 1A8 and 1A10, the three major isozymes involved in the glucuronidation of BZA, are shown in Figure 5. BZA-4'-glucuronide formation was catalyzed most efficiently by UGT 1A1, 1A8, and 1A10, while UGT 1A3, 1A7, 1A9, 2B7 and 2B15 showed weak to moderate activity. In the formation of BZA-5-glucuronide, UGT1A1, 1A3, 1A7, 1A8, 1A9, and 1A10 displayed some activity, while UGT1A10 showed the greatest catalytic activity. UGT 1A1, 1A3, 1A7, 1A8, 1A9, and 1A10 generated both the 4'-glucuronide and 5-glucuronide, but the ratios of 4'-glucuronide to 5-glucuronide were variable and were 20, 1.6, 0.4, 8.6, 0.3, and 3.9, respectively. UGT 2B7 and 2B15 formed only the 4'-glucuronide metabolite under the experimental conditions used in this study. In these experiments, all commercially available (12) UGT isozymes were tested. However, they do not include the full complement of known UGTs. Therefore, the possible contribution of other UGT enzymes that were not tested cannot be ruled out.

In Vitro Kinetic Analysis

The kinetic parameters for the formation of 4'-glucuronide and 5-glucuronide were investigated in pooled female human liver, duodenum and jejunum microsomes and in recombinant UGT 1A1, 1A8, and 1A10; the results are summarized in Table 1. For the
formation of BZA-4'-glucuronide, $K_m$ and $V_{max}$ values of UGT 1A1, 1A8, 1A10, liver, duodenum, and jejunum microsomes were 11.3, 33.1, 6.6, 5.4, 8.5, and 5.1 µM and 0.8, 2.9, 1.1, 1.9, 2.4, and 1.2 nmol/min/mg, respectively. For the formation of BZA-5-glucuronide, $K_m$ and $V_{max}$ values of UGT 1A8, 1A10, duodenum, and jejunum microsomes were 2.5, 7.4, 11.1, and 5.1 µM, and 0.1, 0.1, 1.2, 0.5 nmol/min/mg, respectively. $K_m$ and $V_{max}$ values for the formation of BZA-5-glucuronide in liver microsomes and UGT 1A1 could not be determined due to low turnover. The kinetic parameters ($K_m$ or $V_{max}$) for the formation of BZA glucuronides were in a similar range in hepatic and intestinal microsomes. These values were also in a similar range in the individual UGT 1A1, 1A8, and 1A10 isozymes. In addition, the intrinsic clearance values ($V_{max}/K_m$) for the formation of BZA-4'-glucuronide by UGT 1A8, 1A10, duodenum, and jejunum microsomes were 2.2, 11.8, 2.6, and 2.4-fold higher, respectively, than those for BZA-5-glucuronide, indicating that the 4'-glucuronide was formed more preferentially than the 5-glucuronide. Attempts were made to determine the kinetic parameters for the formation of bazedoxifen-5-glucuronide by UGT1A7 and UGT1A9 enzymes, but the activities appeared to be too low to calculate these parameters for these two enzymes.

**Permeability of BZA and Effect of BZA Concentration and a P-gp Inhibitor on the Transport of BZA**

BZA was found to have moderate absorptive permeability ($P_{app}$ (A$\rightarrow$B) = 0.18 - 0.73 $\times$ 10$^{-6}$ cm/sec) at the drug concentrations studied, ranging from 0.7 to 70 µM (Table 2). A B$\rightarrow$A/A$\rightarrow$B ratio efflux ratio of 8.5 was observed at 0.7 µM, that decreased to 1.0 as the concentration of BZA increased to 70 µM, consistent with saturation of an efflux
transporter at higher concentrations. Efflux ratios of BZA at both 0.7 and 7 μM were decreased to approximately 1.0 in the presence of verapamil (100 μM), consistent with the efflux of BZA being due to P-gp.
DMD # 30999

Discussion

In the present study, the in vitro metabolism of BZA was investigated in human hepatic and intestinal microsomes and cryopreserved hepatocytes. In addition, UGT isozymes involved in the glucuronidation of BZA were identified by using cDNA expressed individual enzymes. Kinetic parameters ($K_m$, $V_{max}$) for the formation of BZA glucuronides were also determined in microsomes and UGTs. The permeability and efflux of BZA and inhibition of P-gp were evaluated using Caco-2 monolayers.

After the incubation with BZA, two glucuronides were identified in hepatic and intestinal microsomes in the presence of UDPGA, and in human hepatocytes. Based on matching chromatographic retention times and mass spectra with those of reference standards, metabolites were identified as BZA-4'-glucuronide and BZA-5-glucuronide. BZA-4'-glucuronide was the major glucuronide formed in hepatic liver microsomes and hepatocytes, while both BZA-4'-glucuronide and BZA-5-glucuronide were the major metabolites generated in intestinal microsomes. No major oxidative metabolites were detected in microsomes in the presence of NADPH or in hepatocytes, suggesting BZA undergoes little or no P450 mediated metabolism.

UGT 1A1, 1A8, and 1A10 appear to be the three most active human UGT isoforms involved in the glucuronidation of BZA, although multiple isoforms showed some catalytic abilities. UGT1A1 efficiently converted BZA to BZA-4'-glucuronide predominantly, while UGT 1A8 and 1A10 showed relatively higher catalytic activities towards the formation of BZA-5-glucuronide than UGT1A1. UGT1A1 is known to be a major UGT enzyme expressed in the human liver (Court, 2010), while UGT 1A8 and 1A10 are mainly expressed in the intestines (Cheng et al., 1999 and Jeong et al., 2005).
These data suggest that both hepatic and extrahepatic metabolism play major roles in the presystemic clearance and disposition of BZA, since BZA is converted efficiently to BZA-4'-glucuronide by both hepatic and intestinal enzymes and to BZA-5-glucuronide by intestinal enzymes.

The predominant circulating metabolite in rats and monkeys following oral administration of [14C]bazedoxifene was the 5-glucuronide. Both 4'-glucuronide and 5-glucuronide were the major plasma metabolites in mice. BZA underwent rapid and extensive glucuronidation in women following a single oral dose at 20 mg (Chandrasekaran et al., 2009). Both BZA-4'- and 5-glucuronide metabolites were observed, with little or no cytochrome P450-mediated metabolites detected in plasma.

The in vitro metabolic pathways for BZA were qualitatively consistent with the in vivo results reported in humans. A major difference between in vitro and in vivo metabolism of BZA in humans is that BZA-5-glucuronide was the predominant circulating metabolite in women following oral administration of BZA, while BZA-4'-glucuronide was the major metabolite in the in vitro incubations, especially in hepatocytes and liver microsomes, where the 5-glucuronide is formed only in trace or very small amounts. A hypothesis for this in vitro/in vivo difference is that the large amounts of BZA-4'-glucuronide likely formed in the liver following oral administration of BZA in women may be excreted efficiently via the bile and undergo enterohepatic recirculation and/or elimination in feces, since almost all of the orally administered dose is known to be excreted in the feces.

Raloxifene, another SERM containing a benzothiophene core with two phenolic groups in place of the indole base of BZA has also been reported to be metabolized primarily via
glucuronidation in humans (Morello, 2003 and Kemp, 2002). As with BZA, the majority of the circulating radioactivity in post-menopausal women receiving radiolabeled raloxifene was due to a single glucuronide metabolite (raloxifene-4′-glucuronide), although it was a minor metabolite in human liver microsomes compared to the other glucuronide, raloxifene-6-glucuronide. Raloxifene-4′-glucuronide was produced in higher proportions in intestinal microsomes than raloxifene-6-glucuronide (Heringa, 2003). In addition, UGT1A1, 1A8 and 1A10 have been found to be the primary contributors to raloxifene glucuronidation, in vitro (Kemp et al., 2002).

In Caco-2 cell monolayer flux experiments, BZA appeared to act as a P-gp substrate, as suggested by a significant efflux ratio (>2), apparent saturation of efflux as concentration of BZA increased, and sensitivity to verapamil. On the basis of a review of absorption of drugs in human and permeability in Caco-2 monolayers, it was proposed that $P_{\text{app}}$ values $> 1 \times 10^{-6}$ cm/sec, were consistent with complete absorption (Artursson et al 2001). BZA displayed moderate $A \rightarrow B$ fluxes (0.18-0.73x10^{-6} cm/s) over the concentration range 0.7 – 70 $\mu$M. Under conditions where efflux of BZA was saturated (70 $\mu$M BZA) or inhibited (in the presence of 100 $\mu$M verapamil), $A \rightarrow B$ and $B \rightarrow A$ fluxes were similar, with $P_{\text{app}}$ values ranging between 0.69 and 1.33 x 10^{-6} cm/s. Overall, this suggests moderate to high passive permeability of BZA that is likely to support good absorption. Raloxifene was also reported to be a substrate for P-gp and other transporter enzymes in the intestine (Jeong et al., 2004). The interplay of P-gp, other transporters such as MRP, and intestinal metabolism were suggested to be important in limiting absorption of raloxifene in the intestine and in supporting enteric recycling (Jeong et al., 2004). Taken together, the in vitro metabolism and transcellular flux data suggest that
similar enzymes and pathways may be involved in the metabolism and disposition of the
two SERM drugs, BZA and raloxifene in vivo. However, oral bioavailability of BZA,
whilst low (6%), was found to be approximately three-fold higher than raloxifene (2%)
(Ermer et al., 2003; Heringa, 2003). An analysis of the factors that may contribute to the
oral bioavailability of raloxifene suggested that intestinal glucuronidation catalysed by
UGT1A8 and UGT1A10 is important in its first-pass metabolism (Mizuma, 2009).
Hence, it is possible that a difference in the balance of absorption, intestinal and hepatic
metabolism of the two drugs underlies the difference in their bioavailability.

In conclusion, glucuronidation was the primary metabolic pathway for BZA in human in
vitro systems, consistent with observations in vivo. Little P450-mediated metabolism of
BZA was evident. UGT 1A1, 1A8 and 1A10 were the most active isoforms involved in
the glucuronidation of BZA. Both hepatic and extra hepatic metabolism appear to play
major roles in the presystemic clearance and disposition of BZA, since BZA is converted
efficiently to BZA-4'–glucuronide by both hepatic and intestinal enzymes and to 5-
glucuronide by intestinal enzymes. In Caco-2 cells, BZA also appeared to be a substrate
for P-gp.
ACKNOWLEDGMENTS

The authors would like to thank Robin Moore and Zeqi Huang for their contributions in some of the initial experiments.
References


Mizuma, T. (2009) Intestinal glucuronidation metabolism may have a greater impact on oral bioavailability than hepatic glucuronidation metabolism in humans: A study with raloxifene, substrate for UGT1A1, 1A8, 1A9 and 1A10. Int. J. Pharmaceutics 378, 140-141.


DMD # 30999


Footnote

Results from this study have been previously presented as a poster at the Annual Meeting of American Association of Pharmaceutical Scientists. Atlanta, GA, November, 2008.

Table 1. Kinetic Parameters for BZA Glucuronidation in Human Liver, Duodenum, and Jejunum Microsomes and in cDNA Expressed UGT1A1, UGT1A8 and UGT1A10

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (µL/min/mg protein)</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (µL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver microsomes</td>
<td>5.4</td>
<td>BZA 4'-Glucuronide 1.9</td>
<td>352</td>
<td>ND</td>
<td>BZA 5-Glucuronide ND</td>
<td>ND</td>
</tr>
<tr>
<td>Duodenum microsomes</td>
<td>8.5</td>
<td>2.4</td>
<td>282</td>
<td>11.1</td>
<td>1.2</td>
<td>108</td>
</tr>
<tr>
<td>Jejunum microsomes</td>
<td>5.1</td>
<td>1.2</td>
<td>235</td>
<td>5.1</td>
<td>0.5</td>
<td>98</td>
</tr>
<tr>
<td>UGT 1A1</td>
<td>11.3</td>
<td>0.8</td>
<td>71</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UGT 1A8</td>
<td>33.1</td>
<td>2.9</td>
<td>88</td>
<td>2.5</td>
<td>0.1</td>
<td>40</td>
</tr>
<tr>
<td>UGT 1A10</td>
<td>6.6</td>
<td>1.1</td>
<td>167</td>
<td>7.4</td>
<td>0.1</td>
<td>14</td>
</tr>
</tbody>
</table>

ND: not determined
Table 2. Permeability of Bazedoxifene in Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>BZA (μM)</th>
<th>Treatment</th>
<th>( P_{\text{app}} ) (AB) ( \times 10^{-6} ) cm/sec (Mean ± SD)</th>
<th>( P_{\text{app}} ) (BA) ( \times 10^{-6} ) cm/sec (Mean ± SD)</th>
<th>( P_{\text{app}} ) (BA)/( P_{\text{app}} ) (AB) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>No Inhibitor</td>
<td>0.18 ± 0.02</td>
<td>1.52 ± 0.14</td>
<td>8.5</td>
</tr>
<tr>
<td>7</td>
<td>No Inhibitor</td>
<td>0.73 ± 0.04</td>
<td>2.05 ± 0.22</td>
<td>2.8</td>
</tr>
<tr>
<td>70</td>
<td>No Inhibitor</td>
<td>0.69 ± 0.06</td>
<td>0.71 ± 0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>0.7</td>
<td>(+) Verapamil (100 μM)</td>
<td>0.73 ± 0.18</td>
<td>0.71 ± 0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>(+) Verapamil (100 μM)</td>
<td>1.33 (n=2)</td>
<td>1.12 ± 0.09</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Note: Incubations were performed for 2 hours at 37°C with Bazedoxifene administered in the apical (AB) or basolateral (BA) compartment.

Note: Results are mean ± SD of n = 3 inserts.
Legends for Figures

Figure 1. Proposed Metabolic Pathways for BZA in Human Liver and Intestinal Microsomes and in Cryopreserved Human Hepatocytes

Figure 2: Radiochromatograms of $[^{14}\text{C}]$BZA in Human Liver and Intestinal Microsomes and in Cryopreserved Human Hepatocytes

Figure 3: Product Ions of [M+H]$^+$ Mass Spectra for BZA-4’-Glucuronide and BZA-5-Glucuronide

Figure 4: Formation of BZA-glucuronides by UGT Isozymes

Figure 5: Radiochromatograms of $[^{14}\text{C}]$BZA Following Incubation with cDNA Expressed Human Isozymes
Figure 1.

Bazedoxifene (BZA)

BZA-5-glucuronide

BZA-4'-glucuronide
Figure 2.

$^{14}$C CPM

Control

Liver microsomes

Duodenum microsomes

Jejunum microsomes

Ileum microsomes

Hepatocytes

Time (min)

$4'$-glu

$5'$-glu

BZA
Figure 3.

![Chemical structures of BZA-4'-glucuronide and BZA-5-glucuronide with m/z values](image-url)
Figure 4.
Figure 5.

![Graph showing time (min) vs. CPM for A1, A8, and A10 samples with peaks labeled 4'-glu and 5-glu](image-url)

**Figure 5.**

![Graph showing time (min) vs. CPM for A1, A8, and A10 samples with peaks labeled 4'-glu and 5-glu](image-url)

**Figure 5.**

![Graph showing time (min) vs. CPM for A1, A8, and A10 samples with peaks labeled 4'-glu and 5-glu](image-url)