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Demonstration of the Innate Electrophilicity of 4-(3-(Benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine (BETP), a Small-Molecule Positive Allosteric Modulator of the Glucagon-Like Peptide-1 Receptor

Heather Eng, Raman Sharma, Thomas S. McDonald, David J. Edmonds, Jean-Philippe Fortin, Xianping Li, Benjamin D. Stevens, David A. Griffith, Chris Limberakis, Whitney M. Nolte, David A. Price, Margaret Jackson, and Amit S. Kalgutkar

Pharmacokinetics, Dynamics and Metabolism–New Chemical Entities, Groton, Connecticut (H.E., R.S., T.S.M.) and Cambridge, Massachusetts (A.S.K.); Cardiovascular, Metabolic and Endocrine Diseases Medicinal Chemistry, Cambridge (D.J.E., B.D.S., D.A.G., W.M.N., D.A.P.) and Groton (C.L.); and Cardiovascular, Metabolic and Endocrine Diseases Research Unit, Cambridge (J.-P.F., X.L., M.J.) Pfizer, Inc.

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ABSTRACT

4-(3-(Benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine (BETP) represents a novel small-molecule activator of the glucagon-like peptide-1 receptor (GLP-1R), and exhibits glucose-dependent insulin secretion in rats following i.v. (but not oral) administration. To explore the quantitative pharmacology associated with GLP-1R agonism in preclinical species, the in vivo pharmacokinetics of BETP were examined in rats after i.v. and oral dosing. Failure to detect BETP in circulation after oral administration of a 10-mg/kg dose in rats was consistent with the lack of an insulinotropic effect of orally administered BETP in this species. Likewise, systemic concentrations of BETP in the rat upon i.v. administration (1 mg/kg) were minimal (and sporadic). In vitro incubations in bovine serum albumin, plasma, and liver microsomes from rodents and humans indicated a facile degradation of BETP. Failure to detect metabolites in plasma and liver microsomal incubations in the absence of NADP was suggestive of a covalent interaction between BETP and a protein amino acid residue(s) in these matrices. Incubations of BETP with glutathione (GSH) in buffer revealed a rapid nucleophilic displacement of the ethylsulfoxide functionality by GSH to yield adduct M1, which indicated that BETP was intrinsically electrophilic. The structure of M1 was unambiguously identified by comparison of its chromatographic and mass spectral properties with an authentic standard. The GSH conjugate of BETP was also characterized in NADPH- and GSH-supplemented liver microsomes and in plasma samples from the pharmacokinetic studies. Unlike BETP, M1 was inactive as an allosteric modulator of the GLP-1R.

Introduction

The incretin hormone glucagon-like peptide-1 (GLP-1) is synthesized from proglucagon-derived peptides in intestinal L-cells in response to oral nutrient ingestion (Holst, 2007). The majority of circulating GLP-1 levels comprise the 30-amino-acid peptide GLP-1(7-36)NH_2, which acts through a seven-transmembrane-spanning, heterotrimeric, class B G-protein-coupled receptor on pancreatic β cells to exert glucoregulatory and insulinotropic actions (Thorens, 1992). Binding of GLP-1 to the GLP-1 receptor (GLP-1R) activates the Gα_s subunit, leading to stimulation of membrane-associated adenylyl cyclases and increased production of cAMP, which enhances glucose-dependent insulin secretion (Thorens et al., 1993; Runge et al., 2008). Therapeutic benefits in the treatment of type 2 diabetes mellitus via agonism of the GLP-1R have been demonstrated with the s.c. administered agents exenatide in a twice-daily formulation (marketed as Byetta; Bristol-Myers Squibb Company, New York, NY and AstraZeneca Pharmaceuticals LP, Wilmington, DE) or once-weekly formulation (marketed as Bydureon; Bristol-Myers Squibb Company and AstraZeneca Pharmaceuticals LP) and liraglutide in a once-daily formulation (marketed as Victoza; Novo Nordisk A/S, Denmark) (Bode, 2011; Murphy, 2012; Jespersen et al., 2013). The efficacies of these agents have been demonstrated in multiple studies, which consistently reported clinically relevant improvements in glycemic control (i.e., reductions in hemoglobin$_{A1c}$, fasting plasma glucose, and postprandial plasma glucose excursions) (Madsbad et al., 2011; Scott et al., 2013). Additional injectable GLP-1R agonists (e.g., lixisenatide, dulaglutide, and albiglutide) are currently in late stages of clinical development (Madsbad et al., 2011; Meier, 2012).

The success of peptide-based GLP-1R agonists for the treatment of type 2 diabetes mellitus has also led to discovery efforts aimed at the

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ABBREVIATIONS: BSA, bovine serum albumin; $CDCl_3$, deuterated chloroform; CD_3OD, deuterated methanol; CHO, Chinese hamster ovary; CID, collision-induced dissociation; compound B or BETP, 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine; δ, chemical shifts expressed in ppm; DMSO-d_6, deuterated dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GSH, glutathione; HPLC, high-performance liquid chromatography; IVGTT, intravenous glucose tolerance test; *J*, NMR coupling constant in Hz; LC-MS/MS, liquid chromatography–tandem mass spectrometry; *m*CPBA, *meta*-chloroperoxybenzoic acid; MS, mass spectrometer; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; $t_{1/2}$, half-life; THF, tetrahydrofuran.

identification of orally active small-molecule agonists of the GLP-1R, which has historically proven to be a difficult task. To a large degree, this difficulty has been attributed to the biochemical mechanisms of class B G-protein-coupled receptors, which require large receptor-ligand binding sites to induce signaling. Despite this dilemma, a diverse array of low-molecular-weight nonpeptidic ligands have been recently reported as antagonists, agonists, and positive allosteric modulators of GLP-1R with intrinsic efficacy (Knudsen et al., 2007; Teng et al., 2007; Sloop et al., 2010; Willard et al., 2012a). 4-(3-(Benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine (compound B or BETP) (Fig. 1) is one such analog that has been identified as a positive allosteric modulator of the naturally occurring inactive GLP-1 metabolite, GLP-1(9-36)NH_2, while showing little modulation of the active, circulating form, i.e., GLP-1(7-36)NH_2 (Sloop et al., 2010; Wootten et al., 2012). Likewise, the insulinotropic effect of oxyntomodulin [glucagon(1-37)], a low-affinity full agonist of the GLP-1R, was also markedly enhanced in the rat i.v. glucose tolerance test (IVGTT) upon i.v. coadministration with BETP, which is consistent with the in vitro biochemical observation of the increased GLP-1R affinity of oxyntomodulin in the presence of BETP (Willard et al., 2012b). In vivo, BETP demonstrated glucose-dependent insulin secretion in the IVGTT in rats after i.v. administration. Interestingly, oral administration of BETP failed to show insulinotropic effects similar to those achieved via i.v. administration (Sloop et al., 2010). One possible reason for this discrepancy is that BETP suffers from poor oral absorption due to low aqueous solubility, low absorptive permeability, and/or extensive first-pass metabolism in the gut and liver.

As part of our general interest in examining quantitative pharmacology for the glucose-dependent insulin secretagogue properties of GLP-1R agonists in preclinical species, the in vivo pharmacokinetics of BETP were examined in rats after i.v. and oral dosing. To our surprise, little to no systemic exposure of BETP could be measured in plasma samples from both the i.v. and oral arms of the pharmacokinetic study. In vitro incubations in bovine serum albumin (BSA), plasma, and liver microsomes from rodents and humans indicated a rapid turnover of BETP. Failure to detect metabolites in BSA, plasma, and liver microsomes (in the absence of NADPH) was suggestive of a covalent interaction between BETP and a protein amino acid residue(s) in the various matrices. Consistent with this hypothesis, incubations of BETP with the endogenous antioxidant glutathione (GSH) in buffer revealed a rapid nucleophilic displacement of the ethylsulfoxide functionality by GSH. The GSH conjugate of BETP was also characterized in liver microsomes supplemented with NADPH and GSH and in plasma samples from the pharmacokinetic studies. The GSH conjugate of BETP was inactive as a positive allosteric modulator of the GLP-1R.

Materials and Methods

Materials. Unless specified otherwise, starting materials used in the synthesis of BETP and its GSH conjugate are generally available from commercial sources such as Aldrich Chemicals Co. (Milwaukee, WI) and Acros Organics (Fair Lawn, NJ). ^{1}H NMR spectra were recorded in deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD), or deuterated dimethylsulfoxide (DMSO-d_6) on a Varian Unity 400-MHz spectrometer (DG400-5 probe; available from Varian Inc., Palo Alto, CA) at room temperature. DMSO-d_6 "100%" was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Chemical shifts (δ) are expressed in ppm relative to residual solvent as an internal reference. The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants (J) are expressed as Hz. GSH, BSA, and NADPH were purchased from Sigma-Aldrich (St Louis, MO). Frozen plasma in K_3EDTA from Wistar Han rat (pooled males), CD-1 mouse (pooled males), and human (pooled males and females) was purchased from Bioreclamation, Inc. (Westbury, NY). Pooled liver microsomes from humans (pool of 50 livers from male/female), male Wistar Hannover rats, and male CD-1 mice were purchased from BD Biosciences (Woburn, MA). Jugular vein–cannulated/carotid artery–cannulated male Wistar Hannover rats were purchased from Charles River (Raleigh, NC). Solvents used for analysis were of analytical or high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Pittsburgh, PA).

Synthesis of BETP. A solution of 4-chloro-2-(methylthio)-6-(trifluoromethyl) pyrimidine (**1**, 348 mg, 1.52 mmol), 3-(benzyloxy)phenylboronic acid (**2**, 200 mg, 0.88 mmol), and cesium carbonate (252 mg, 3.88 mmol) in ethylene glycol dimethyl ether (16 ml) and water (4 ml) was degassed with N_2 gas. Tetrakis (triphenylphosphine)palladium(0) (89 mg, 0.08 mmol) was added and the reaction mixture was heated at 85°C for 18 hours under an N_2 atmosphere. The mixture was then diluted with ethyl acetate (50 ml), dried (sodium sulfate), filtered, and concentrated in vacuo. The crude product was purified by silica gel chromatography (CombiFlash, Teledyne Isco, Lincoln, NE; 0%–3% ethyl acetate in petroleum ether) to give 4-(3-(benzyloxy)phenyl)-2-(methylthio)-6-(trifluoromethyl)pyrimidine (**3**, 320 mg, 0.85 mmol, 96%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81–7.77 (m, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.54–7.33 (m, 6H), 7.18 (dd, J = 2.0, 8.5 Hz, 1H), 5.18 (s, 2H), 2.67 (s, 3H).

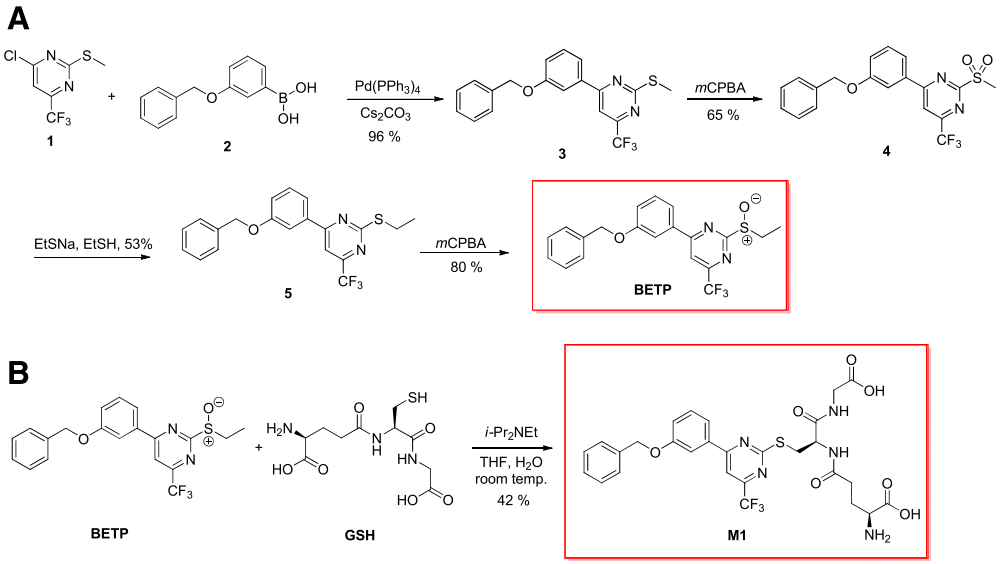

Fig. 1. Preparation of BETP and its GSH conjugate M1.

To a solution of **3** (340 mg, 0.90 mmol) in dichloromethane (10 ml) was added *meta*-chloroperoxybenzoic acid (*m*CPBA) (468 mg, 2.71 mmol) at room temperature. The reaction mixture was stirred at room temperature for 4 hours and then concentrated in vacuo. The crude product purified by silica gel chromatography (CombiFlash, 0%–15% ethyl acetate in petroleum ether) to give 4-(3-(benzyloxy)phenyl)-2-(methylsulfonyl)-6-(trifluoromethyl)pyrimidine (**4**, 240 mg, 0.59 mmol, 65%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.16 (s, 1H), 7.88 (s, 1H), 7.79 (d, J = 7.5 Hz, 1H), 7.54–7.34 (m, 6H), 7.25 (br. second., 1H), 5.20 (s, 2H), 3.47 (s, 3H).

Compound **4** (1.50 g, 3.67 mmol) was dissolved in tetrahydrofuran (THF) (6 ml) and the mixture divided between six microwave reaction tubes. To each tube were added sodium ethanethiolate (154 mg, 1.84 mmol) and ethanethiol (1 ml). The vials were sealed and heated at 100°C for 20 minutes under microwave irradiation. The six portions were recombined and concentrated, and the crude product was purified by silica gel chromatography (CombiFlash, 0%–2% ethyl acetate in petroleum ether) to give 4-(3-(benzyloxy)phenyl)-2-(ethylthio)-6-(trifluoromethyl)pyrimidine (**5**, 759 mg, 1.95 mmol, 53%) as a yellow oil. m/z = 391.0 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75–7.81 (m, 1H), 7.69 (d, J = 7.53 Hz, 1H), 7.61 (s, 1H), 7.32–7.51 (m, 6H), 7.18 (dd, J = 2.26, 7.78 Hz, 1H), 5.17 (s, 2H), 3.26 (q, J = 7.36 Hz, 2H), 1.47 (t, J = 7.28 Hz, 3H).

To a solution of **5** (700 mg, 1.79 mmol) in dichloromethane (10 ml) was added *m*CPBA (310 mg, 1.79 mmol) portionwise at 0°C. The reaction mixture was stirred at 0°C for 30 minutes and quenched by addition of sodium sulfite. The layers were separated and the aqueous portion was extracted with dichloromethane. The combined organic layers were dried over sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel chromatography (CombiFlash, 2%–24% ethyl acetate in petroleum ether) to give BETP (582 mg, 1.43 mmol, 80%) as a white solid. m/z = 407 $[M+H]^+$; ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 8.08 (s, 1H), 7.97 (d, J = 7.53 Hz, 1H), 7.48–7.58 (m, 3H), 7.37–7.44 (m, 2H), 7.28–7.37 (m, 2H), 5.25 (s, 2H), 3.35–3.47 (m, 1H), 3.19–3.30 (m, 1H), 1.33 (t, J = 7.53 Hz, 3H).

Synthesis of the GSH Conjugate of BETP (M1). To a solution of BETP (100 mg, 0.25 mmol) in a mixture of THF (2.5 ml) and water (1.0 ml) at room temperature were added GSH (154 mg, 0.50 mmol) and diisopropylethylamine (175 μl, 1.0 mmol), and the mixture was stirred for 20 hours at room temperature. The solution was concentrated in vacuo and the crude residue was purified by preparative HPLC to afford M1 (165 mg, 0.25 mmol) as a white solid. The preparative HPLC conditions were as follows: HPLC Column: DIKMA (Lake Forest, CA) Diamonsil(2) C18 5 μm, 200 × 20 mm. Gradient elution: 30% acetonitrile in water (0.1% trifluoroacetic acid) to 50% acetonitrile in water (0.1% trifluoroacetic acid). The purified product was assessed as >95% purity by analytical HPLC and ^{1}H NMR. m/z = 636.0 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (t, J = 5.52 Hz, 1H), 8.56 (d, J = 8.53 Hz, 1H), 8.30 (s, 1H), 8.01–8.04 (m, 1H), 8.00 (d, J = 8.03 Hz, 1H), 7.46–7.54 (m, 3H), 7.37–7.44 (m, 2H), 7.31–7.37 (m, 1H), 7.27 (dd, J = 2.01, 8.03 Hz, 1H), 5.24 (s, 2H), 4.73 (dt, J = 4.77, 8.91 Hz, 1H), 3.96 (dd, J = 4.52, 13.55 Hz, 1H), 3.67–3.80 (m, 2H), 3.55 (t, J = 6.78 Hz, 1H), 3.30 (dd, J = 9.54, 13.55 Hz, 1H), 2.34 (t, J = 7.28 Hz, 2H), 1.82–2.04 (m, 2H).

Incubations in Plasma and BSA. Stock solutions of BETP were prepared in DMSO. BETP (final concentration = 1 μM) was incubated in 0.1 M potassium phosphate buffer (pH 7.4) supplemented with BSA (10 mg/ml) or in plasma from rat (n = 3), mouse (n = 3), and human (n = 3) at 37°C (pH 7.4). Total incubation volume was 0.6 ml and the final DMSO concentration in the incubations was 1% (v/v). Plasmas were thawed and adjusted to pH 7.4. Incubation matrices (594 μl) were prewarmed to 37°C and maintained at that temperature for 5 minutes before adding substrate (6 μl). Periodically (0–60 minutes), aliquots (50 μl) of the incubation mixtures were added to acetonitrile (200 μl) containing terfenadine (mol. wt. = 472; 0.02 μg/ml) as an internal standard. Samples were vortexed and then centrifuged at 2300g for 10 minutes. Supernatants were analyzed for disappearance of BETP by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Incubations in Buffer. BETP (final concentration = 1 μM) was incubated in 0.1 M potassium phosphate buffer (pH 7.4) in the absence or presence of GSH (5 mM) at 37°C (n = 3). Total incubation volume was 0.6 ml and the final DMSO concentration in the incubations was 5% (v/v). Periodically (0–60 minutes), aliquots (50 μl) of the incubation mixtures were added to acetonitrile (200 μl) containing terfenadine (mol. wt. = 472; 0.02 μg/ml) as an internal standard. Samples were vortexed and then centrifuged at 2300g for 10 minutes. Supernatants were analyzed for disappearance of BETP (and concomitant appearance of M1) by LC-MS/MS. The metabolic fate of BETP (10 μM) in 0.1 M phosphate buffer (pH 7.4) in the presence of GSH (5 mM) was also examined qualitatively by LC-MS/MS after incubation at 37°C for 30 minutes.

Incubations in Liver Microsomes. Stock solutions of BETP were prepared in a solution of 1% DMSO and 99% acetonitrile. The final concentrations of DMSO and acetonitrile in the incubation media were 0.01% and 0.99% (v/v), respectively. Microsomal stability assessments were determined in triplicate after incubation of BETP (1 μM) with rat, mouse, and human liver microsomes (cytochrome P450 concentration, 0.25 μM) in 0.1 M potassium phosphate buffer (pH 7.4), containing 3.3 mM magnesium chloride, at 37°C. Incubations were conducted in the presence or absence of NADPH (1.3 mM) and GSH (5 mM). The total incubation volume was 0.6 ml. Incubations were prewarmed at 37°C for 5 minutes before the addition of BETP. Aliquots (50 μl) of the reaction mixture at 0, 2, 5, 10, 20, 40, and 60 minutes (time period associated with reaction linearity) were added to acetonitrile (200 μl) containing terfenadine (mol. wt. = 472; 0.02 μg/ml) as an internal standard. The samples were centrifuged at 2300g for 10 minutes before LC-MS/MS analysis for the disappearance of BETP and appearance of M1. For the purposes of qualitative metabolite identification studies, the concentration of BETP in the liver microsomal incubations was raised to 10 μM and that of P450 in rat, mouse, and human liver microsomes was raised to 0.5 μM. After quenching the incubation mixtures with acetonitrile (1 ml), the solutions were centrifuged (2300g, 15 minutes) and the supernatants were dried under a steady nitrogen stream. The residue was reconstituted with the mobile phase and analyzed for metabolite formation by LC-MS/MS.

Animal Pharmacokinetic Studies. Rat studies were conducted at Pfizer; all animal care and in vivo procedures conducted were in accordance with guidelines of the Pfizer Animal Care and Use Committee. Jugular vein–cannulated male

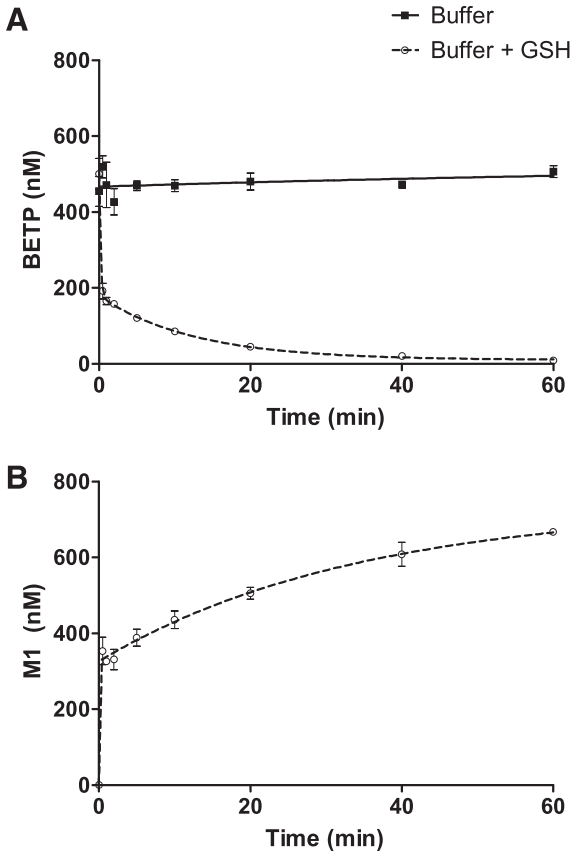

Fig. 2. (A) Depletion of BETP and (B) appearance of GSH conjugate M1 in incubations of BETP in buffer with and without GSH (n = 3). Mean values and standard deviations are shown.

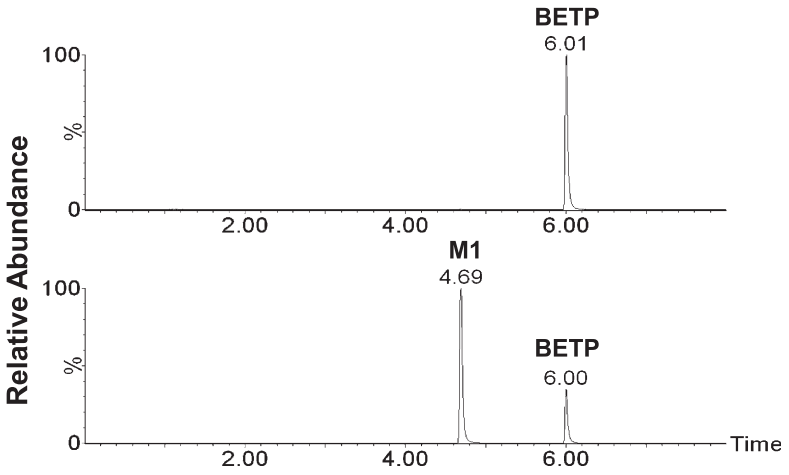

Fig. 3. Extracted ion chromatogram of an incubation mixture of BETP (10 μM) and GSH (5 mM) in 0.1 M potassium phosphate buffer at 37°C at t = 0 minutes (top) and t = 30 minutes (bottom).

Wistar Hannover rats (0.25–0.27 kg) were used for pharmacokinetic analysis. For oral pharmacokinetic studies, animals were fasted overnight before dosing, whereas access to water was provided ad libitum. BETP was administered i.v. via the jugular vein of rats (n = 2). For oral studies, BETP was administered by oral gavage to rats (n = 2). BETP was administered at 1.0 mg/kg i.v. and 10 mg/kg orally. Orally dosed rats were fed after collection of the 4-hour blood samples. BETP was formulated as a solution in DMSO–polyethylene glycol 400–water [10:50:40 (v/v/v)] and 0.5% (w/v) methylcellulose with 2% (v/v) DMSO for the i.v. and oral studies, respectively. After dosing, serial plasma samples were collected at appropriate times via the jugular vein cannula and kept frozen at −20°C until LC-MS/MS analysis for presence of BETP and M1.

LC-MS/MS Methodology for Quantification of BETP and M1. Concentrations of BETP and its GSH adduct (M1) in various matrices (buffer, plasma, and/or liver microsomes) were determined by LC-MS/MS. Briefly,

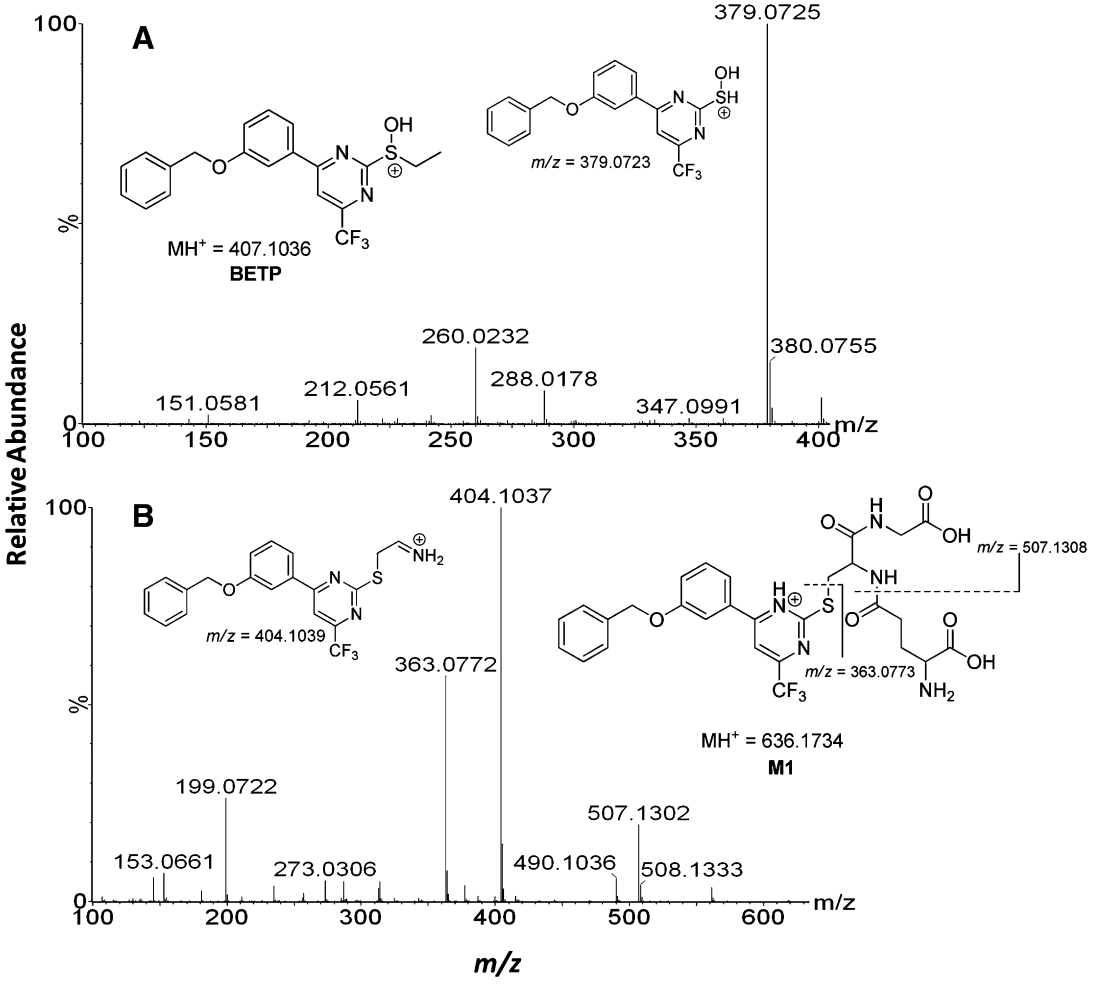

Fig. 4. CID spectrum of BETP (A) and GSH conjugate M1 (B).

samples/sample extracts were injected by a fixed-loop CTC PAL Auto-sampler onto a Shimadzu LC-20AD HPLC system coupled to an AB Sciex API4000 triple quadrupole mass spectrometer (MS) fitted with a TurboIonspray source operating in positive ion mode (AB Sciex, Framingham, MA). Chromatographic separation was performed by gradient elution on a Waters HSS T3 XP (30 × 2.1 mm, 2.5 μm) reverse-phase column (Waters Corporation, Milford, MA), using a binary solvent mixture consisting of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 600 μl/min. Quantitation was performed by multiple reaction monitoring mode with transitions of 407.2 → 379.2 (BETP), 636.2 → 507.1 (M1), and 472.2 → 436.2 (internal standard: terfenadine). Standards of BETP and M1 in each matrix were fit by least-squares regression, and unknown concentrations were determined from the resultant best-fit equation.

LC-MS/MS Methodology for Metabolite Identification Studies. Separation of BETP and metabolites was achieved using an ACQUITY UPLC system (Waters Corporation) with a 2.1 × 150 mm, 1.8-μm ACQUITY UPLC HSS C18 column, maintained at a column temperature of 40°C. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). The flow rate was 0.3 ml/min and the gradient was as follows: 5% solvent B (0 minutes), 80% solvent B (5 minutes), and 5% solvent B (5.2–8.0 minutes). The injection volume was 15 μl. Detection of BETP and metabolites was performed on a SYNAPT G2 (Waters MS Technologies, Manchester, UK) orthogonal acceleration quadrupole time-of-flight MS. The MS was operated in positive ion mode using electrospray ionization. The desolvation gas was set to 700 l/h at a temperature of 350°C. The cone gas was set to 30 l/h and the source temperature to 150°C. The capillary voltage was set to 4 kV, the cone voltage to 28 V, and the extraction cone to 7 V. The SYNAPT G2 was operated in V optics mode (sensitivity mode) with resolution greater than 10,000 at full width at half maximum. The data acquisition rate was 0.10 s/scan; data were collected from 1 to 8 minutes in MS^e acquisition mode with a collision energy ramp of 25–45 V for high-energy scans. The MS was calibrated to a mass accuracy under 5 mDa. Data were collected in continuum mode from *m/z* 100 to 900. MassLynx version 4.1, SCN 803 software (Waters Corporation) was used for data processing.

Cell Culture and cAMP Assay. Chinese hamster ovary (CHO) cells stably expressing the human GLP-1R (CHO-GLP1R cells) were maintained in a Dulbecco's modified Eagle's medium–F-12 mixture (Invitrogen #11330032; Invitrogen, Carlsbad, CA) supplemented with 500 μg/ml G418 (Invitrogen #10131035) and 10% heat-inactivated fetal bovine serum. Cells were grown at 37°C in a 95% humidified atmosphere consisting of 5% CO_2. A cell-based time-resolved fluorescence resonance energy transfer (FRET) assay (Cisbio Bioassays #62 AM4PEJ; Cisbio Bioassays, Codolet, France) was used to measure receptor-mediated cAMP production. This method is based on generation of a FRET signal upon the interaction between 1) an anti-cAMP antibody coupled to a FRET donor (cryptate) and 2) a cAMP derivative coupled to a FRET acceptor (d2). Endogenous cAMP produced by cells competes with labeled cAMP for binding to the cAMP antibody, thus reducing the FRET signal. Briefly, CHO-GLP1R cells were dissociated from tissue culture plates using enzyme-free cell dissociation buffer and resuspended in an appropriate volume of assay buffer [1× Hanks' balanced salt solution (Gibco #14025-092; Invitrogen), 1 M HEPES (Gibco #15630-080)] supplemented with 500 μM 3-isobutyl-1-methylxanthine. A total of 2500 cells/well was dispensed into white 384-well plates (BD Falcon # 353988; BD Biosciences, San Jose, CA). The metabolite GLP-1(9-36)NH_2 was serially diluted in assay buffer containing BETP (10 μM), M1 (10 μM), or the corresponding vehicle DMSO. Ligands (5 μl of 2× concentration) were added to the appropriate wells, and plates were incubated 30 minutes at room temperature. Labeled cAMP (5 μl) and anti-cAMP antibody (5 μl) were then added to each well, and the plates were further incubated at 37°C for 1 hour. Time-resolved FRET signal was measured using an Envision 2103 Multilabel Plate Reader (PerkinElmer, Waltham, MA) with a laser excitation at 337 nm and dual emissions at 665 nm and 590 nm. A cAMP standard curve diluted in assay buffer was included and used to calculate the amount of cAMP produced, as specified by the manufacturer.

Data Analysis. Substrate disappearance half-lives ($t_{1/2}$s) were calculated using E-WorkBook 2011 (IDBS, Guildford, Surrey, UK). Sigmoidal curve fitting of ligand concentration-response curves was executed using GraphPad Prism software version 5.02 (GraphPad, San Diego, CA). The same software package was used for calculating the EC_{50} values, an index of ligand potency.

TABLE 1

In vitro stability of BETP in liver microsomes (n = 3) in the presence and absence of NADPH (1.3 mM) and GSH (5 mM)

Species	NADPH	GSH	$t_{1/2}$ ± S.D.
			min
Human	−	−	47 ± 5.0
	−	+	<0.5[a]
	+	−	8.4 ± 0.3
	+	+	<0.5[a]
Mouse	−	−	5.6 ± 1.3
	−	+	<0.5[a]
	+	−	0.81 ± 0.05
	+	+	<0.5[a]
Rat	−	−	8.8 ± 0.3
	−	+	<0.5[a]
	+	−	2.2 ± 0.2
	+	+	<0.5[a]

[a] $t_{1/2}$ could not be determined; samples were below the limit of analytical quantitation for BETP at all time points.

Results

Preparation of BETP and Its GSH Conjugate M1. BETP was prepared as shown in Fig. 1A. Suzuki coupling (Suzuki, 2005) of chloropyrimidine (**1**) and boronic acid (**2**) derivatives yielded sulfide **3**, which was oxidized with excess *m*CPBA to yield the corresponding sulfone **4**. Displacement with sodium ethanethiolate introduced the ethyl sulfide (compound **5**), which was oxidized to afford BETP using one equivalent of *m*CPBA. An authentic sample of M1 was prepared by reacting BETP with GSH in aqueous THF in the presence of diisopropylethyl amine (Fig. 1B).

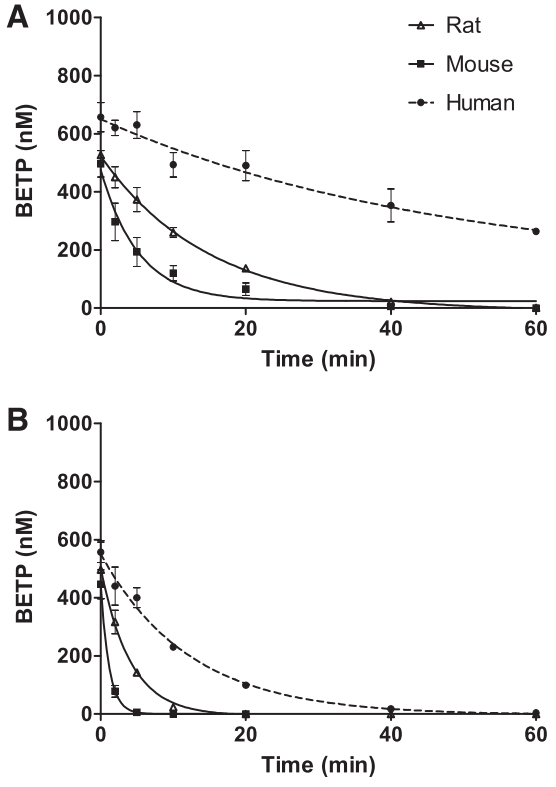

Fig. 5. Disappearance of BETP in rat, mouse, and human liver microsomes in the absence (A) and presence (B) of NADPH (1.3 mM) (n = 3). Mean values are plotted and standard deviations are indicated with error bars.

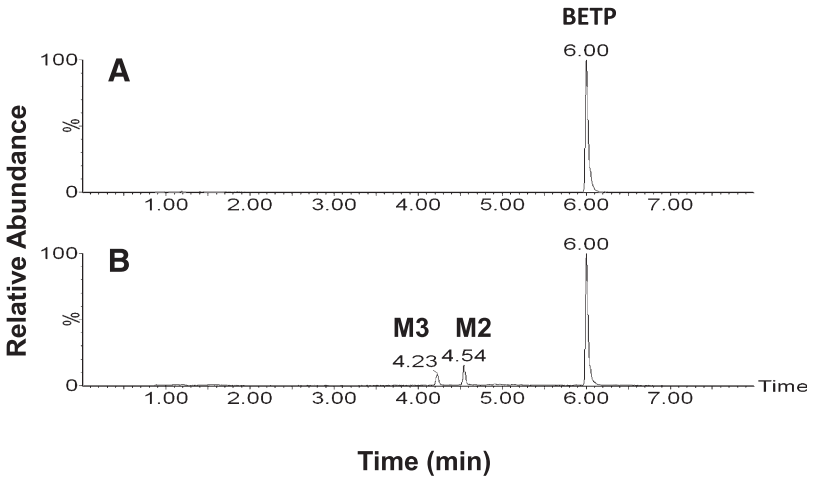

Fig. 6. Extracted ion chromatogram of an incubation mixture of BETP (10 μM) in rat liver microsomes in the absence (A) and presence (B) of NADPH conducted at 37°C for 60 minutes.

Plasma and BSA Stability of BETP. To examine the interspecies stability in plasma, BETP at a concentration of 1 μM was incubated in rat, mouse, and human plasma at 37°C; periodically, aliquots of the incubation mixture were examined for depletion of BETP. The $t_{1/2}$ for depletion of BETP in rat, mouse, and human plasma was 35 ± 3.0, 55 ± 19, and 60 ± 13 minutes, respectively. Incubation of BETP (1 μM) in potassium phosphate buffer supplemented with 10 mg/ml BSA at 37°C also indicated a decline of the parent compound with a $t_{1/2}$ of 54 ± 5.0 minutes.

Stability of BETP in Potassium Phosphate Buffer in the Presence and Absence of GSH. BETP (1 μM) was stable in phosphate buffer (pH 7.4) at 37°C ($t_{1/2}$ > 120 minutes). However, inclusion of GSH (5 mM) in the incubation mixture resulted in a rapid

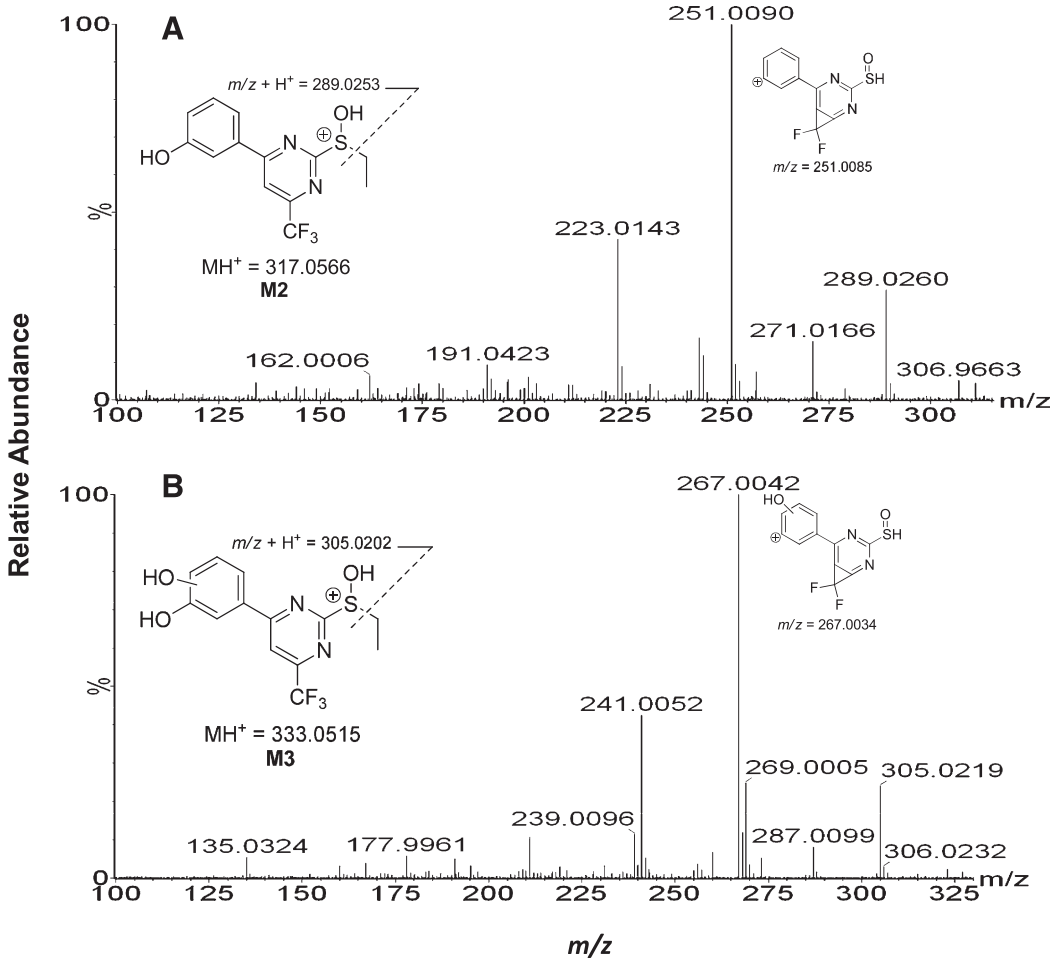

Fig. 7. CID spectra of metabolites M2 (A) and M3 (B) generated upon incubating BETP (10 μM) in rat liver microsomes in the presence of NADPH at 37°C for 60 minutes.

disappearance of BETP, with a $t_{1/2}$ of <0.5 minute (Fig. 2A). LC-MS/MS analysis of a reaction mixture comprising BETP (10 μM) in potassium phosphate buffer and GSH (5 mM), incubated at 37°C for 30 minutes, revealed the formation of a single metabolite denoted as M1 (Fig. 3). Under reversed-phase HPLC conditions, M1 eluted before BETP (BETP: retention time (t_R) = 6.00 minutes; M1: t_R = 4.69 minutes). The collision-induced dissociation (CID) spectra of BETP and M1 are depicted in Fig. 4, A and B, respectively. M1 displayed a protonated molecular ion (MH^+) at *m/z* 636.1734, an addition of 229.0698 Da to the molecular weight of BETP (MH^+ = 407.1036). The CID spectrum of M1 yielded a diagnostic fragment ion at *m/z* 507.1302, which corresponds to the neutral loss of the pyroglutamate component in GSH (i.e., 129.0426 Da), suggesting that M1 was a GSH adduct. Furthermore, the occurrence of the fragment ion at *m/z* 363.0772 is consistent with the presence of an aromatic thioether motif in M1 (Baillie and Davis, 1993). A proposed structure of M1 that is compatible with the observed fragmentation pattern is depicted in Fig. 4B. To unambiguously prove the proposed structure, an authentic standard of M1 was synthesized via an independent route. The LC-MS/MS attributes (t_R and CID spectrum) of the M1 synthetic standard were identical to the one generated in the chemical reaction between BETP and GSH in buffer (unpublished data).

Based on the qualitative metabolite identification studies, incubations of BETP in GSH-supplemented phosphate buffer were simultaneously monitored for the disappearance of BETP and the appearance of the GSH adduct, respectively (Fig. 2B). In phosphate buffer containing GSH, following the 60-minute incubation period, the amount of BETP remaining was 9.0 ± 1.0 nM, resulting in a 492 ± 40 nM consumption of BETP when compared with 0 minutes (500 ± 41 nM). At 60 minutes, the amount of GSH adduct was 667 ± 6.0 nM, resulting in similar loss of parent substrate BETP and formation of the GSH conjugate.

Liver Microsomal Stability of BETP. To examine liver microsomal stability, BETP at a concentration of 1 μM was incubated in rat, mouse, and human liver microsomes at 37°C for 60 minutes in the presence and absence of NADPH cofactor and in the presence and absence of GSH; periodically, aliquots of the incubation mixture were examined for depletion of BETP and the appearance of the GSH adduct of BETP (in liver microsomal incubations supplemented with the thiol nucleophile) (Table 1). The $t_{1/2}$ for depletion of BETP in rat, mouse, and human liver microsomes in the absence of NADPH and GSH was 8.8 ± 0.3, 5.6 ± 1.3, and 47 ± 5.0 minutes, respectively (Fig. 5A). In the presence of NADPH (but absence of GSH), the $t_{1/2}$ for depletion of BETP in rat, mouse, and human liver microsomes was 2.2 ± 0.2, 0.81 ± 0.05, and 8.4 ± 0.3 minutes, respectively (Fig. 5B). In the presence of both NADPH and GSH, the $t_{1/2}$s for depletion of BETP in rat, mouse, and human liver microsomes were <0.5 minute.

Metabolite Identification Studies. No metabolites were detected upon qualitative LC-MS/MS examination of incubation mixtures of plasma (rat, mouse, and human) and BSA with BETP (10 μM) conducted at 37°C for 60 minutes. Likewise, no metabolite formation was discerned upon incubation of BETP (10 μM) with rat, mouse, and human liver microsomes in the *absence* of NADPH at 37°C for 60 minutes. LC-MS/MS analysis of incubation mixtures of BETP (10 μM) with rat, mouse, and human liver microsomes in the *presence* of NADPH at 37°C for 60 minutes revealed the formation of two metabolites (M2 and M3) in each species. A representative chromatogram of a rat liver microsomal incubation with BETP (± NADPH) is shown in Fig. 6. The CID spectra of M2 and M3 are depicted in Fig. 7, A and B, respectively. M2 (t_R = 4.54 minutes) displayed a MH^+ at *m/z* 317.0566, which is consistent with *O*-dealkylation in BETP (Fig. 7A). M3 (t_R = 4.23 minutes) displayed a MH^+ at *m/z* 333.0515, which is consistent with a monohydroxylation of M2. A proposed structure for M3 that is compatible with the fragmentation pattern is shown in Fig. 7A. Incubation mixtures of BETP (10 μM) in NADPH- and GSH-supplemented rat, mouse, and human liver microsomes revealed the exclusive (and quantitative) conversion to M1 following a 60-minute incubation at 37°C (data not shown).

Pharmacokinetic Studies in Rats. The in vivo formation of M1 was examined in rats after administration of single i.v. (1 mg/kg) and oral (10 mg/kg) doses of BETP. Figure 8 illustrates the mean observed plasma concentration– versus–time profiles of BETP and M1 after i.v. (panel A) or oral dosing (panel B). A small quantity (≤114 ng/ml, 0.28 μM) of BETP was observed after i.v. dosing, while none was detected after oral dosing. The formation of M1 was observed in both dose groups. The rapid appearance of M1 in rat plasma (as early as 1.0 minute with peak total plasma concentrations of ~3.0 μM) after i.v. dosing indicates the efficiency of the reaction between BETP and GSH in rats. As such, we were unable to estimate pharmacokinetic parameters for BETP due to small and sporadic amounts measurable in the plasma samples.

Activity of BETP and its GSH Conjugate at the Human GLP-1R. Recent work demonstrated that coincubation with BETP markedly enhances the activity of truncated metabolite of GLP-1(7-36)NH_2, i.e., GLP-1(9-36)NH_2, at the GLP-1R (Wootten et al., 2012). Here, we took advantage of the latter procedure to test whether the GSH conjugate of BETP (i.e., M1) retains positive allosteric modulator properties of BETP. GLP-1(9-36)NH_2 failed to activate the GLP-1R in the presence of M1 (10 μM) or corresponding DMSO vehicle (Fig. 9). In contrast, consistent with previous observations, a dose-dependent

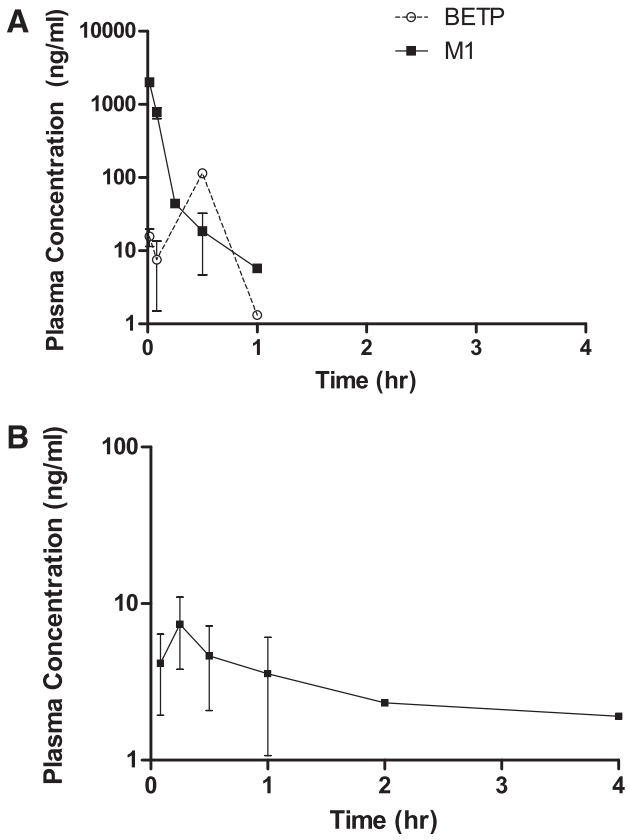

Fig. 8. Mean concentration-versus-time profiles of BETP and its GSH conjugate in rat plasma (n = 2) after i.v. (A) or oral (B) dosing at 1 mg/kg and 10 mg/kg, respectively. The error bars represent concentration range.

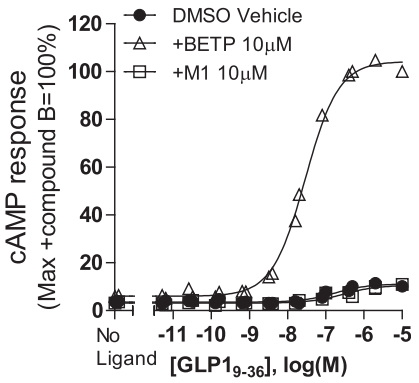

Fig. 9. Allosteric modulation of the human GLP-1R by BETP and the GSH conjugate M1. Ligand-stimulated cAMP production was measured in CHO-GLP1R cells. Concentration-response curves are shown for the weak partial agonist GLP-1(9-36)NH_2 in the presence or BETP, the metabolite M1, or the vehicle DMSO. In contrast to BETP, M1 failed to positively modulate the activity of GLP-1(9-36)NH_2. All data represent the mean ± S.E.M. of three independent experiments conducted in quadruplicate.

increase in cAMP production was observed when CHO-GLP1R cells were stimulated with GLP-1(9-36)NH_2 in the presence of BETP (10 μM).

Discussion

Our present studies establish the electrophilic nature of BETP by virtue of its facile chemical reaction with the endogenous nucleophile GSH, which affords the corresponding sulfydryl conjugate M1. A likely mechanism (Fig. 10) for the formation of M1 involves nucleophilic attack of GSH on the C2 pyrimidine carbon in BETP to yield the negatively charged σ-complex or Meisenheimer complex followed by elimination of the alkylsulfoxide group as the corresponding sulfenic acid species. The electron-withdrawing substituents (pyrimidine nitrogen atoms in positions 1 and 3 and the trifluoromethyl substituent at position 6) serve to increase the electrophilicity of the C2 carbon via resonance and/or inductive stabilization of the transition state, and favor reaction with the nucleophilic thiol. Certainly, the role of the trifluoromethyl group in accelerating the nucleophilic displacement of 2-halopyridines has been studied (Schlosser and Rausis, 2005). In hindsight, our finding on the innate electrophilicity of BETP is not surprising when one examines the plethora of publications dealing with seemingly "chemically inert" compounds, which are prone to nucleophilic displacement by GSH under non-enzymatic (pH 7.4, phosphate buffer, 37°C) and/or enzymatic conditions (mediated by glutathione transferases in liver microsomes and/or liver cytosol). From a structure-activity relationship perspective, a recurring structural theme in these examples is the presence of the methylsulfone/sulfonamide and/or halide leaving group, which is attached to an electron-deficient heteroaromatic ring system (e.g., pyridine, pyridone, benzothiazole, thiadiazole, benzofuran, indole, etc.) (Clapp, 1956; Colucci and Buyske, 1965; Conroy et al., 1984; Graham et al., 1989; Woltersdorf et al., 1989; Graham et al., 1990; Kishida et al., 1990; Huwe et al., 1991; Zhao et al., 1999; Teffera et al., 2008; Inoue et al., 2009; Litchfield et al., 2010). More recently, Yang et al. (2012) have also demonstrated the susceptibility of 2-(alkylthio)-1,3,4-thiadiazoles and 2-(alkylthio)-1,3-benzothiazoles to undergo nucleophilic displacement with GSH in human liver microsomes. The requirement of NADPH cofactor in the GSH displacement reactions suggested that the rate-limiting step involved oxidation of the alkylthio functionality to the corresponding electrophilic sulfoxide and sulfone metabolites, followed by nucleophilic displacement of the formed sulfoxide and/or sulfone by GSH. In the present work, we did not observe further oxidation of the *S*-oxide motif in BETP to the corresponding sulfone metabolite in liver microsomal incubations supplemented with the cytochrome P450 cofactor NADPH.

Our studies also revealed that BETP was unstable in BSA and plasma from rat, mouse, and human, which is contrary to a previous speculation that BETP is stable in plasma (Willard et al., 2012a). Similar to the experience with BSA/plasma, incubations of BETP in rat, mouse, and human liver microsomes in the absence of NADPH led to a steady decline in BETP concentrations. Failure to detect products/metabolites in these incubations suggests that the mechanism of BETP depletion proceeds via a covalent displacement reaction between BETP and a nucleophilic amino acid residue(s) in plasma and liver microsomal proteins, similar to the pathway depicted with GSH in Fig. 10. Inclusion of NADPH and GSH in liver microsomal incubations led to an even more rapid decline of BETP and the quantitative conversion to GSH adduct M1, which is indicative of a detoxifying metabolic pathway that competes with protein covalent binding. The propensity of GSH to reduce microsomal covalent binding has been noted with several drugs that are bioactivated to electrophilic species (Zhao et al., 2007; Obach et al., 2008).

Although the protein (plasma/liver microsome) covalent adduction theory has not been proven with a radiolabeled version of BETP, our hypothesis is reasonably supported by literature reports. There are numerous published accounts of covalent interactions between plasma and/or liver microsomal proteins and electrophilic xenobiotics, including drugs. Covalent binding of electrophilic acyl glucuronide metabolites has been demonstrated in plasma, notably to albumin, and has been detected in vivo in humans for a number of acyl glucuronide–forming drugs (Smith and Wang, 1992; Ding et al., 1993, 1995; Sallustio et al., 1997). Likewise, covalent modification of lysine residues in human serum albumin has been noted with the

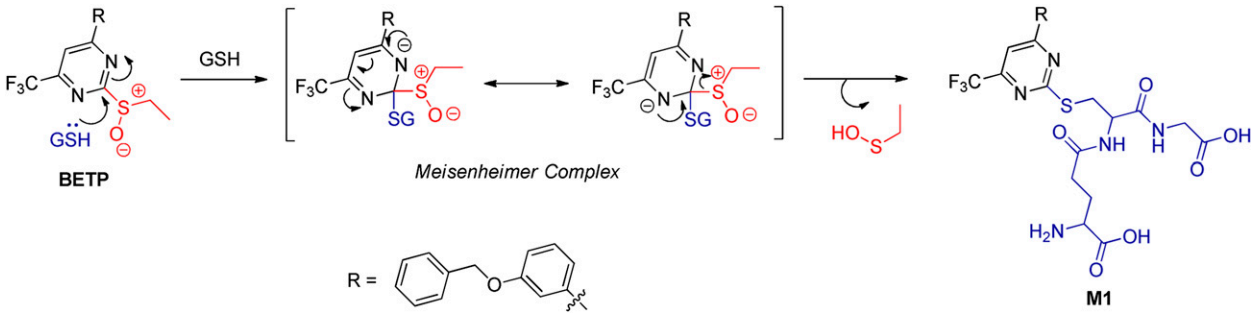

Fig. 10. Nucleophilic displacement of the ethylsulfoxide moiety in BETP by GSH.

electrophilic β-lactam antibiotics such as penicillin G (Levine and Ovary, 1961; Yvon and Wal, 1988; Yvon et al., 1990; Bertucci et al., 2001). Such covalent reactions have also been reported to occur in patients treated with high dosages of β-lactam antibiotics, and are thought to be responsible for the adverse effects associated with this class of compounds (Batchelor et al., 1965; Ahlstedt and Kristofferson, 1982; Lafaye and Lapresle, 1988). Finally, the plasma instability observed with the loop diuretic ethacrynic acid and HKI-272 (an irreversible, covalent inhibitor of tyrosine kinase) have also been attributed to a covalent interaction of their respective α,β-unsaturated carbonyl moieties with amino acid residues in plasma proteins (Bertucci et al., 1998; Bertucci and Domenici, 2002; Chandrasekaran et al., 2010; Wang et al., 2010). With respect to covalent binding to liver, both NADPH-dependent and -independent covalent interactions have been demonstrated between liver microsomal proteins and xenobiotics (Evans et al., 2004; Shin et al., 2007).

The failure to detect BETP in circulation following oral administration to rats comes as no surprise considering the in vitro chemical/biochemical instability of this electrophilic molecule, and the corresponding impact this attribute can have on oral absorption. As such, the lack of an insulinotropic effect of orally administered BETP in the rat IVGTT (Sloop et al., 2010) parallels our inability to detect systemic concentrations of BETP upon administration by the oral route. With reference to the glucose-dependent insulin secretion noted over a course of ~20 minutes after a single i.v. bolus dose of BETP at 10 mg/kg (Sloop et al., 2010), it is possible that enough BETP systemic exposure was achieved at the i.v. dose of 10 mg/kg to cover the in vitro EC_{50} of 0.75 μM of BETP against the rat GLP-1R (Sloop et al., 2010). Based on our present work, systemic concentrations of BETP in the rat i.v. pharmacokinetic study at the 1-mg/kg dose were minimal (and sporadic), but did yield total plasma concentrations of ~0.28 μM. The detection of the GSH conjugate M1 at total circulating concentrations significantly higher than BETP in the i.v. pharmacokinetic study (~3.0 μM) also led us to examine its role in the positive allosteric modulation of GLP-1R. However, unlike BETP, M1 failed to enhance the activity of GLP-1(9-36)NH_2 at the GLP-1R.

Authorship Contributions

Participated in research design: Kalgutkar, Eng, Sharma, McDonald, Griffith, Stevens, Fortin, Jackson.

Conducted in vitro experiments: Eng, Sharma, McDonald, Li, Fortin, Nolte.

Contributed new reagents or analytic tools: Edmonds, Stevens, Griffith, Limberakis, Price.

Performed data analysis: Kalgutkar, Eng, Sharma, McDonald. Fortin.

Wrote or contributed to the writing of the manuscript: Kalgutkar, Eng, Sharma, McDonald, Fortin, Griffith.

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Address correspondence to: Amit S. Kalgutkar, Pharmacokinetics, Dynamics, and Metabolism–New Chemical Entities, Pfizer Worldwide Research and Development, Cambridge, MA 02139. E-mail: amit.kalgutkar@pfizer.com