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

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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)NH2, which acts through a seven-transmembrane-spanning, heterotrimeric, class B G-protein-coupled receptor on pancreatic β cells to exert glucoregulatory and insulino tropic actions (Thorens, 1992). Binding of GLP-1 to the GLP-1 receptor (GLP-1R) activates the Goα subunit, leading to stimulation of membrane-associated adenyl 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 hemoglobinA1c, 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; CDC18, deuterated chloroform; CD2, OD, 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-d6, 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; mCPBA, meta-chloroperoxybenzoic acid; MS, mass spectrometer; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; t1/2, half-life; THF, tetrahydrofuran.

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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)NH₂, while showing little modulation of the active, circulating form, i.e., GLP-1(7-36)NH₂. Consistent with this, the insulino içer effect 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 insulinoic 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.

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

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). 1H NMR spectra were recorded in deuterated chloroform (CDCl3), deuterated methanol (CD3OD), or deuterated dimethyl-sulfoxide (DMSO-d6) on a Varian Unity 400-MHz spectrometer (DG400-5 probe; available from Varian Inc., Palo Alto, CA) at room temperature. DMSO-d6 “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 K3EDTA 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 Hanover rats, and male CD-1 mice were purchased from BD Biosciences (Woburn, MA). Jugal vein–cannulated/carotid artery–cannulated male Wistar Hanover 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-(benzoylphosphine)boronic 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 N2 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 N2 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-(benzoxylphosphine)phenyl)-2-(methylthio)-6-(trifluoromethyl)pyrimidine (3, 320 mg, 0.85 mmol, 90%) as a yellow oil. 1H NMR (400 MHz, CDCl3) δ 7.81–7.77 (m, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.54–7.33 (m, 4H), 7.18 (dd, J = 2.0, 8.5 Hz, 1H), 5.18 (s, 2H), 2.67 (s, 3H), 2.14 (s, 3H).
To a solution of 3 (340 mg, 0.90 mmol) in dichloromethane (10 ml) was added meta-chloroperoxybenzoic acid (mCPBA) (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 was purified by silica gel chromatography (CombiFlash, 0–15% ethyl acetate in petroleum ether) to give 4-(3-benzyloxy)phényl)-2-(ethylthio)-6-(trifluoromethyl)pyrimidine (1, 759 mg, 1.95 mmol, 65%) as a yellow oil. 1H NMR (400 MHz, CDCl3) 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 tetrhydrofuran (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)phényl)-2-(ethylthio)-6-(trifluoromethyl)pyrimidine (5, 759 mg, 1.95 mmol, 53%) as a yellow oil. m/z 391.0 [M+H]+; 1H NMR (400 MHz, CDCl3) δ = 7.77–7.81 (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 mCPBA (310 mg, 1.84 mmol) and acetonitrile (1 ml). 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, 0–2% ethyl acetate in petroleum ether) to give BETP (m/z 582, 1.43 mmol), as a white solid. The preparative HPLC conditions were as follows: HPLC Column: DMSO and acetonitrile in the incubation media were 0.01% and 0.99% (v/v), respectively. Microsomal stability assays 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 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 metabolite 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% acetoneitrile. The final concentrations of DMSO and acetoneitrile 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.
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,
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 Turbolonspray 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. 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 was 150°C. The capillary voltage was set to 27 kV, and the cone voltage to 7 V. The SYNAPT G2 was operated in V modes: 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.

**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 mCPBA 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 mCPBA. An authentic sample of M1 was prepared by reacting BETP with GSH in aqueous THF in the presence of disopropylethyl amine (Fig. 1B).

### Data Analysis

Substrate disappearance half-lives ($t_{1/2}$) 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.

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**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 mCPBA 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 mCPBA. An authentic sample of M1 was prepared by reacting BETP with GSH in aqueous THF in the presence of disopropylethyl amine (Fig. 1B).

**Table 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>NADPH</th>
<th>GSH</th>
<th>$t_{1/2}$ ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>–</td>
<td>–</td>
<td>47 ± 5.0</td>
</tr>
<tr>
<td>Mouse</td>
<td>–</td>
<td>+</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Rat</td>
<td>–</td>
<td>+</td>
<td>8.4 ± 0.3</td>
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</tbody>
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$a_{1/2}$ could not be determined; samples were below the limit of analytical quantitation for BETP at all time points.
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
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 thiouether 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 analyzed 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 $t_{1/2}$ 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).
Intrinsic Electrophilicity of BETP

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 et al., 2007; Obach et al., 2008). 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 addition 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
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 Laperesse, 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,ω-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 insulitotoxic 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 EC₅₀ 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)NH₂ at the GLP-1R.

**Authorship Contributions**

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

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

**References**


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