In vitro and In vivo Clinical Pharmacology of Dimethyl Benzoylphenylurea, a Novel Oral Tubulin-Interactive Agent

Michelle A. Rudek, Ming Zhao, Nicola F. Smith, Robert W. Robey, Ping He, Gurulingappa Hallur, Saeed Khan, Manuel Hidalgo, Antonio Jimeno, Dimitrios Colevas, Wells A. Messersmith, Antonio C. Wolff, and Sharyn D. Baker

Abstract

Dimethyl benzoylphenylurea (BPU) is a novel tubulin-interactive agent with poor and highly variable oral bioavailability. In a phase I clinical trial of BPU, higher plasma exposure to BPU and metabolites was observed in patients who experienced dose-limiting toxicity. The elucidation of the clinical pharmacology of BPU was sought. BPU, monomethylBPU, and aminoBPU were metabolized by human liver microsomes. Studies with cDNA-expressed human cytochrome P450 enzymes revealed that BPU was metabolized predominantly by CYP3A4 and CYP1A1 but was also a substrate for CYP2C8, CYP2D6, CYP3A5, and CYP3A7. BPU was not a substrate for the efflux transporter ABCG2. Using simultaneous high-performance liquid chromatography/diode array and tandem mass spectrometry detection, we identified six metabolites in human liver microsomes, plasma, or urine: monomethylBPU, aminoBPU, G280, G308, G322, and G373. In patient urine, aminoBPU, G280, G308, and G322 collectively represented <2% of the given BPU dose. G280, G308, G322, and G373 showed minimal cytotoxicity. When BPU was given p.o. to mice in the presence and absence of the CYP3A and ABCG2 inhibitor, ritonavir, there was an increase in BPU plasma exposure and decrease in metabolite exposure but no overall change in cumulative exposure to BPU and the cytotoxic metabolites. Thus, we conclude that (a) CYP3A4 and CYP1A1 are the predominant cytochrome P450 enzymes that catalyze BPU metabolism, (b) BPU is metabolized to two cytotoxic and four noncytotoxic metabolites, and (c) ritonavir inhibits BPU metabolism to improve the systemic exposure to BPU without altering cumulative exposure to BPU and the cytotoxic metabolites.

Benzoylphenyureas were initially developed as insecticides but were found to possess in vitro antitumor activity (1, 2). However, the physicochemical properties of N-[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl]-N-(2-nitrobenzoyl)urea limited its potential clinical utility as an antitumor agent (2, 3). After multiple structure-activity relationship manipulations, a benzoylphenylurea derivative with comparable cytotoxic activity and improved physiochemical properties was developed (4, 5). The derivative was dimethyl benzoylphenylurea (BPU; NSC 639829; Fig. 1). The mechanism of action for benzoylphenyurea derivatives includes tubulin polymerization inhibition and microtubule depolymerization in vitro (4, 6).

Both BPU and the didesmethylBPU metabolite (aminoBPU, NSC 647884) showed in vitro cytotoxic activity (4, 6, 7). In mouse, rat, and dog studies characterizing both efficacy and pharmacology, BPU was observed in concentrations that were efficacious in vitro (7). BPU possessed greater in vivo activity, whereas aminoBPU was more potent in vitro (6, 7). Oral bioavailability of BPU in dogs was poor and variable ranging from 12% to 29% following administration of a 5-mg capsule and 4.4% to 26% for a 25-mg capsule (8). The relative systemic exposure to the BPU metabolites, monomethylBPU (mmBPU) and aminoBPU, was also variable (110-160% for a 5-mg capsule and 43-132% for a 25-mg capsule). Preliminary data suggested that BPU was metabolized only by CYP2D6 and CYP3A4 and was a substrate for ABCG2 (9, 10).

BPU was evaluated in a phase I clinical trial in 19 patients with refractory metastatic cancers with the drug being given p.o. once weekly on a continuous schedule (11). Plasma pharmacokinetic studies in humans revealed that BPU has a long half-life (78 ± 42, 104 ± 18, and 166 ± 24 hours for weeks 1, 4, and 8 respectively) and was extensively metabolized to mmBPU and aminoBPU; plasma exposure to mmBPU and aminoBPU represented ~200% to 740% and 40% to 240%, respectively.

Note: M.A. Rudek and M. Zhao contributed equally to this article.

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Authors' Affiliations: 1Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland; 2Cancer Therapeutics Branch, National Cancer Institute, Bethesda, Maryland; and 3Investigational Drug Branch/Cancer Therapy Evaluation Program/National Cancer Institute, Rockville, Maryland

Requests for reprints: Michelle A. Rudek, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Bunting-Blaustein Cancer Research Building, Room 1M90, 1650 Orleans Street, Baltimore, MD 21231-1000. Phone: 410-502-7149; Fax: 410-614-9006; E-mail: mrudek2@jhmi.edu.

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of that for BPU. Both metabolites seemed to have longer half-lives than parent compound, which was also observed in dogs (8). Higher plasma exposure to BPU and metabolites, expressed by area under the curve (AUC), maximal plasma concentration \( (C_{\text{max}}) \), or minimal steady-state concentrations \( (C_{\text{ss,min}}) \), was observed in patients who experienced dose-limiting toxicity. The dose-limiting toxicities observed at the 320-mg dose level included a grade 5 neutropenic infection and a treatment delay due to neutropenia. Because of the observed association between drug and metabolite exposure and toxicity, we sought to determine the mechanisms of drug elimination by (a) characterizing the in vitro metabolism of BPU, (b) identifying the cytochrome P450 (CYP450) enzymes involved in drug metabolism, (c) determining whether BPU is a substrate for the efflux transporter ABCG2, (d) comprehensively characterizing the in vivo drug metabolism of BPU in the plasma and urine of patients receiving BPU, (e) characterizing the relative cytotoxicity of metabolites, and (f) modulating the pharmacokinetics of BPU with the coadministration of ritonavir.

Materials and Methods

Chemical and reagents

BPU and aminoBPU were supplied by the Developmental Therapeutics Program, Cancer Therapy Evaluation Program, NIH (Bethesda, MD). SNU-308 cells (gallbladder carcinoma) were obtained from the Korean Cell Line Bank (Seoul, Korea); HuCCT-1 cells (intrahepatic cholangiocarcinoma) were from the Health Science Research Resources Bank (Osaka, Japan); BxPC3 (pancreatic cancer), Panc-1 (pancreatic cancer), MiaPaCa (pancreatic cancer), A431 (vulvar epithelial carcinoma), and Hep2 (squamous cell carcinoma of the head and neck) cells were from the American Tissue Culture Collection (Manassas, VA); HN006 cells (squamous cell carcinoma of the head and neck) were from Dr. David Sidransky’s laboratory (Johns Hopkins, Baltimore, MD); and MCF-7 (breast cancer) and MCF-7 FLV1000 cells were from Dr. Susan E. Bates’ laboratory (National Cancer Institute). Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma, Inc. (Pittsburgh, PA). Human urine was obtained from healthy volunteers. All other chemicals and reagents were commercially available and of the highest grade.

Synthesis of unknown metabolites

The five metabolites (mmBPU, 4-(5-bromo-pyrimidin-2-yloxy)-3-methyl-phenylamine (G280), N-[4-(5-bromo-pyrimidin-2-yloxy)-3-methyl-phenyl]-formamide (G308), [4-(5-bromo-pyrimidin-2-yloxy)-3-methyl-phenyl]-urea (G322), and 1-[4-(5-bromo-pyrimidin-2-yloxy)-3-methyl-phenyl]-1H-quinazoline-2,4-dione (G373)) that were not available from the Developmental Therapeutics Program/Cancer Therapy Evaluation Program were synthesized by the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Medicinal Chemistry Core. The synthesis of mmBPU and G280 was done as described previously (12). Details of the synthesis of G308, G322, and G373 will be published elsewhere. All compounds were characterized by \(^1\)H and \(^{13}\)C nuclear magnetic resonance and liquid chromatography (LC)/mass spectrometry (MS) studies. The proposed metabolite structures were confirmed using the LC/tandem MS method and by comparing the retention times of the proposed metabolites in patient samples with that of the reference standards (13).

In vitro human metabolism by human liver microsomes

Human liver microsomes used to characterize BPU, mmBPU, and aminoBPU oxidative metabolism were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Incubations of human microsomal suspensions were done in glass vials maintained at 37°C in a shaker bath. Initial studies were conducted in a 100 mmol/L sodium
potassium phosphate buffer (pH 7.2) containing 8 mmol/L MgCl\(_2\), 0.5 or 2 mg/mL human liver microsomes, NADPH-generating system, in a final volume of 200 mL. The NADPH-generating system consisted of 0.2 mmol/L NADP\(^+\), 4 mmol/L glucose-6-phosphate, and 1.3 units/mL glucose-6-phosphate dehydrogenase (14). The final concentrations of BPU, mmBPU, and aminoBPU were 10.6, 11.0, and 11.3 mmol/L, respectively. Incubations done without NADPH-generating system were used as negative controls to control for native enzyme activities. The incubation mixtures were preincubated for 5 minutes before the initiation of the reaction by the addition of the NADPH-generating system. At the end of the incubation period, reactions were terminated by the addition of 2.5 mL of a mixture of 0.2 mmol/L paclitaxel in acetonitrile-n-butyl chloride (1:4, v/v) and 0.5 mL water. The samples were then processed and analyzed by LC/tandem MS (13). Incubations were done in triplicate.

**Metabolite formation by cDNA-expressed human cytochrome P450 isoforms**

Microsomes prepared from cDNA-transfected baculovirus insect cells containing human P450 reductase and control (wild-type baculovirus), CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C18, CYP2C19, CYP2D6*1, CYP2E1, CYP3A4, CYP3A5, CYP3A7, CYP4A11, or CYP19 (aromatase) were purchased from BD Gentest Corp. (Woburn, MA). Initial incubations were done using 50 pmol/mL recombinant CYP450, 1 mmol/L BPU, NADPH-generating system, 5 mmol/L MgCl\(_2\), and 100 mmol/L potassium phosphate buffer (pH 7.4) in a final volume of 450 mL. Control CYP450 and incubations done without NADPH-generating system were used as negative controls to control for native enzyme activities. The incubation mixtures were preincubated for 5 minutes before initiation of the reaction by the addition of the NADPH-generating system. At the end of the incubation period, reactions were terminated by transferring 20 mL of the reaction mixture to 2.5 mL of a mixture of 1 ng/mL temazepam in n-butyl chloride and 0.5 mL water. The samples were then processed and analyzed by LC/tandem MS. Incubations were done in duplicate and on at least two occasions if no activity was observed. After confirmation of CYP450 isoform activity, studies were done to determine linearity with respect to protein concentration and time.

**Relative resistance to elucidate ABCG2 substrate specificity**

Cytotoxicity assays were done as previously described using cells that lack ABCG2 (MCF-7) and ABCG2-overexpressing cells (MCF-7 FLV1000 which are MCF-7 cells maintained in 1,000 nmol/L flavopiridol) to determine the relative resistance to BPU, mmBPU, aminoBPU, or mixtoxantrone (control; refs. 15, 16). Each concentration was tested in quadruplicate and controls were tested in replicates of eight. Relative resistance values were obtained by dividing the IC\(_{50}\) value of each drug for the resistant cell line by the IC\(_{50}\) value for the parental line. Attempts were made to generate cells resistant to BPU, aminoBPU, and mmBPU by incubating MCF-7 breast cancer cells in progressively increasing concentrations of the drugs as described previously for flavopiridol (15). The resulting cell lines MCF-7 BPU150, aminoBPU110, and mmBPU125 cells were maintained in 50, 10, and 25 mmol/L BPU, aminoBPU, and mmBPU, respectively. To determine if ABCG2 was up-regulated in the resistant sublines, the 5D3 antibody was used to recognize the epitope of ABCG2 (17).

**Characterization of metabolites in human plasma and urine**

Human subjects participated in a phase 1 clinical study of BPU and received a dose of 150 or 320 mg given p.o. once weekly. The drug was formulated as a 5- or 25-mg capsule containing polyglycolized glycercides and polyethylene glycol and stored under refrigeration. The protocol was approved by the Institutional Review Board of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, and the patients provided written informed consent. Serial blood samples were collected in heparin-containing tubes before drug administration and at 0.25, 0.5, 1.0, 1.5, 2, 4, 6, 8, 24, 48, and 72 hours after administration of BPU during week 1. In addition, pretreatment trough samples were collected weekly before BPU administration. Samples were processed within 30 minutes by centrifugation for 10 minutes at 3,000 x g at ambient temperature. Plasma supernatant was split into two aliquots and stored at −70°C until subsequent analysis. Urine was collected continually during five collection intervals: 0 to 4, 4 to 8, 8 to 24, 24 to 48, and 48 to 72 hours after administration of the BPU dose during week 1. Urine was collected in plastic containers and was refrigerated during collection. For each collection period, the total volume of urine was recorded and two 15-mL aliquots were frozen at −70°C until subsequent analysis. A catheter was not used during the collection of urine.

**In vitro cytotoxicity of synthesized metabolites**

In vitro drug sensitivity of G280, G308, G322, and G373 was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye conversion assay (18). Each experiment was done in six replicates for each drug concentration and was carried out independently at least thrice. Response to drug treatment was assessed by standardizing treatment groups to untreated controls.

**Dimethyl benzoylphenylurea pharmacokinetics in combination with ritonavir**

Female C57BL/6 mice (6 weeks old) were kept in a controlled environment with food and sterilized water available ad libitum. Animals weighed ~ 20 g at the time of experiment. BPU was dissolved in 0.05% Tween 80 in 0.9% NaCl adjusted to pH 3 with 0.1 N HCl. Ritonavir was supplied as 80 mg/mL in ethanol/Cremophor EL/propylene glycol/water (43.9:8.25:22.2) and further diluted in the same vehicle. BPU was given p.o. by gavage at a dose of 10 mg/kg in the presence and absence of ritonavir, which was given 30 minutes before BPU at a dose of 12.5 mg/kg (19, 20). The dose and schedule for the concomitant administration of BPU and ritonavir was selected on results from previous studies (7, 19). Mice were humanely killed, and plasma and brain tissues were harvested as a function of time after administration of BPU. Each time point represents three samples from three different mice. Blood was collected by cardiac puncture under anesthesia into heparinized syringes and centrifuged to obtain plasma. Brain tissues were rapidly dissected and snap frozen in liquid nitrogen. Samples were stored frozen at −70°C until analysis. Animal experimentation complied with local and national requirements.

Mean plasma and brain tissue concentrations at each sampling point were calculated for BPU and metabolites. Pharmacokinetic variables were calculated from mean BPU and metabolite concentration-time data using noncompartmental methods as implemented in WinNonlin version 3.1 (Pharsight Corp., Mountain View, CA). C\(_{max}\) and t\(_{max}\) were the observed values from the mean data. The AUC\(_{last}\) was calculated using the linear trapezoidal method. The method of Baier (21) was used to estimate the variance of AUC\(_{last}\) based on the variance of the mean concentration at each time point. To determine whether there was a significant difference between exposure as expressed by AUC\(_{last}\), a pairwise comparison was done using a Z test (22). The a priori level of significance was P < 0.05. Relative systemic exposure to BPU was calculated using the AUC\(_{last}\)~BPU~/AUC\(_{last}\)~BPU~:

\[
\text{Analyte AUC}_{\text{last}}/\text{BPU AUC}_{\text{last}}
\]

Relative systemic exposure in brain compared with plasma was calculated using the AUC\(_{last}\)~Brain~/AUC\(_{last}\)~Plasma~:

\[
\text{Brain Analyte AUC}_{\text{last}}/\text{Plasma Analyte AUC}_{\text{last}}
\]
Sample preparation and analysis by liquid chromatography/tandem mass spectrometry

In vitro metabolism using microsomes. BPU and the metabolites were extracted by acetonitrile-n-butyl chloride (1:4, v/v) and separated on a Waters (Milford, MA) X-Terra MS C18 (50 × 2.1 mm, 3.5 μm) column with acetonitrile/water mobile phase (80:20, v/v) at 0.1% formic acid using isocratic flow at 0.15 mL/min for 5 minutes. The published method was modified to detect mmBPU, aminoBPU, and G280 in addition to BPU by Micromass Quattro LC triple-quadrupole MS detector (Beverly, MA) with electrospray-positive ionization.5 Results were assessed qualitatively comparing the area ratio of BPU or metabolite with the internal standard, paclitaxel.

In vitro metabolism using cDNA-expressed human cytochrome P450 enzymes. BPU, mmBPU, aminoBPU, G280, G308, and G322 were extracted by n-butyl chloride extraction and separated on a Waters X-Terra MS C18 (50 × 2.1 mm, 3.5 μm) column with acetonitrile/water mobile phase (70:30, v/v) containing 0.1% formic acid using isocratic flow at 0.15 mL/min for 5 minutes. Sample preparation was modified to use only 20-μL sample with half of the n-butyl chloride volume with the addition of 0.5 mL water to decrease the salt content in the reconstitute. The analytes of interest were monitored by API 3000 triple-quadrupole MS detector (Applied Biosystems, Foster City, CA) with electrospray-positive ionization.5 The spectrometer was programmed to allow the [MH+] ion and product ion of BPU (470.1, 134.0), aminoBPU (442.1, 105.9), mmBPU (322.8, 105.9), and the internal standard (301.0–254.8). The retention times were 2.85 minutes for BPU, 2.65 minutes for mmBPU, 1.90 minutes for aminoBPU, 1.20 minutes for G280, 1.40 minutes for G308, 1.25 minutes for G322, and 3.88 minutes for the internal standard. Results were assessed qualitatively comparing the area ratio of BPU or metabolite with the internal standard, temazepam.

Mouse pharmacokinetics. BPU and metabolites were extracted from mouse plasma using acetonitrile-n-butyl chloride (1:4, v/v). Brain tissue homogenates were prepared at a concentration of 200 mg/mL in PBS and further diluted 1:10 in human plasma before extraction using acetonitrile-n-butyl chloride (1:4, v/v). The analytes of interest were monitored by API 3000 triple-quadrupole MS detector with electrospray-positive ionization.5 BPU, mmBPU, and aminoBPU were quantitatively assessed for plasma and brain tissue, whereas G208, G308, and G322 were quantitatively assessed for plasma and qualitatively assessed in brain tissues. Plasma calibration curves were prepared over the range of 0.5 to 106.4 nmol/L BPU, 0.6 to 109.7 nmol/L mmBPU, 1.1 to 226.2 nmol/L aminoBPU, 1.8 to 357.1 nmol/L G280, 1.6 to 324.7 nmol/L G308, and 1.6 to 310.6 nmol/L G322. Brain tissue calibration curves were tested over the range of 31.2 to 63.8, 829.8 nmol/g BPU, 329.0 to 65,789.5 nmol/g mmBPU, and 339.4 to 67,873 nmol/g aminoBPU.

Human pharmacokinetics. Initially, the original plasma LC/tandem MS method was modified to use simultaneous LC/diode array and tandem MS detection to identify unknown metabolites in plasma and urine (13). After the metabolites were identified and synthesized, the LC/tandem MS method was further modified and validated using the API 3000 triple-quadrupole MS detector with electrospray-positive ionization.5 BPU and metabolites were extracted from plasma by acetonitrile precipitation and from urine using n-butyl chloride extraction. BPU and metabolites were separated on a Waters X-Terra MS C18 (50 × 2.1 mm, 3.5 μm) column with acetonitrile/water mobile phase (70:30, v/v) containing 0.1% formic acid using isocratic flow at 0.15 mL/min for 5 minutes. Plasma calibration curves were generated over the range of 2.5 to 500 ng/mL for BPU, mmBPU, and aminoBPU. Urine calibration curves over the range of 0.1 to 20 ng/mL for BPU and mmBPU, 0.5 to 100 ng/mL for aminoBPU, 10 to 2,000 ng/mL for G280, 1 to 200 ng/mL for G308, and 3 to 600 ng/mL for G322. The values for precision and accuracy for both plasma and urine during method validation and in-study evaluation were within 15%, except for G308, which was outside acceptable limits and therefore not assessed quantitatively.5

Results

In vitro human metabolism

Cytochrome P450-mediated metabolism of dimethyl benzoyl-phenyurea, mmBPU, and aminoBPU. NADPH-dependent metabolism was observed when BPU, mmBPU, and aminoBPU were incubated with human liver microsomes. BPU (~75%) was converted to metabolites after a 60-minute incubation using 0.5 mg/mL microsomes. Two major metabolites have been identified thus far as mmBPU and aminoBPU by LC/tandem MS. Incubations using a human liver microsome concentration of 0.5 mg/mL were insufficient to detect a decrease in mmBPU and aminoBPU or the appearance of metabolites. Only 15% of mmBPU and 10% of aminoBPU was converted to metabolites following a 60-minute incubation with 2 mg/mL microsomes. G280, G308, G322, and G373 were not identified in metabolism experiments as NADPH-dependent metabolites of BPU, mmBPU, or aminoBPU.

Dimethyl benzoylphenyurea metabolism by cDNA-expressed human cytochrome P450 isomors. When BPU was incubated with cDNA-expressed human CYP450 isoform preparations, CYP1A1 and CYP3A4 had the greatest activity for BPU metabolism (Fig. 2). CYP3A4 seems to be the most potent CYP450 because within 5 minutes both mmBPU and aminoBPU are formed using 12.5 pmol/mL. CYP1A1 also has increased turnover of BPU with optimization to linearity using 12.5 pmol/mL protein for 5 minutes; aminoBPU was formed under these conditions by 10 minutes. Substantial BPU metabolism was also observed with CYP2C8, CYP2D6, CYP3A5, and CYP3A7 (Fig. 2). CYP3A5 was optimized to protein concentration and time at 25 pmol/mL at 15 minutes with the conversion to only mmBPU. The remaining CYP450 enzymes, CYP2C8, CYP2D6, and CYP3A7, were optimized to 25 pmol/mL protein and 20 minutes with conversion to only mmBPU. CYP2D6 seems to be the least potent CYP450 involved in BPU metabolism because aminoBPU was not formed until 20 minutes when it appears by 15 minutes for both CYP2C8 and CYP3A7. With sufficient incubation time (i.e., 30 minutes), mmBPU and aminoBPU were formed by all isoforms. G280, G308, G322, and G373 were not identified in metabolism experiments as NADPH-dependent metabolites.

Relative resistance to elucidate ABCG2 substrate specificity

The relative resistance to BPU, mmBPU, or aminoBPU is ~3, which suggests that these compounds are not transported by ABCG2 (Table 1). In addition, none of the BPU, mmBPU, or aminoBPU resistant MCF-7 cell lines had increased 5D3 staining compared with the parental line, suggesting that ABCG2 up-regulation was not responsible for the resistance (data not shown).

In vivo identification of metabolites in human plasma and urine

Previously, high-performance LC analysis with UV absorbance detection at 225 nm revealed accumulation of two metabolites, designated as M1 and M2 in human plasma from several patients at the 320-mg dose level (23). Fraction collection of M1 with subsequent direct injection on the mass spectrometer revealed a fragmentation pattern consistent with mmBPU (data not shown). Synthesized mmBPU verified the peak in plasma. There was an interfering peak at the same retention time as M2 using the LC/UV assay, which was identified as aminoBPU using tandem MS detection. The simultaneous LC/diode array and tandem MS detection were used to determine if the metabolites were present in both plasma and urine. In addition to mmBPU, aminoBPU was identified as a metabolite in human plasma at both 150- and 320-mg dose levels.

In fresh patient urine, G280, G308, G322, and G373 were detected and collectively represented <2% of the given BPU dose. G280, G308, G322, and G373 were synthesized to verify the peaks in urine. G373 was the only synthesized metabolite that was not confirmed during analysis of urine that had been frozen but was initially detected in fresh urine. Therefore, G373 was removed from method development and validation procedures.

In vitro cytotoxicity of synthesized metabolites

The potential clinical relevance of the metabolites that were formed either in vitro or in vivo was determined by assessing the relative cytotoxicity. G280, G308, G322, and G373 showed minimal activity against the cell lines tested at concentrations as high as 10 μmol/L (see Fig. 3).

### Table 1. BPU and metabolite lack of resistance in ABCG2-overexpressing cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>MCF-7*</th>
<th>FLV1000*</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPU</td>
<td>0.9</td>
<td>2.5 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>mmBPU</td>
<td>0.95 ± 0.07</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>aminoBPU</td>
<td>0.009</td>
<td>0.026 ± 0.006</td>
<td>3</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.008 ± 0.003</td>
<td>19.5 ± 14.8</td>
<td>2,437</td>
</tr>
</tbody>
</table>

*IC_{50}'s were reported in micromolar and were determined by the sulforhodamine B assay (16).
Dimethyl benzoylphenylurea pharmacokinetics in combination with ritonavir

BPU pharmacokinetics was given to mice in the presence and absence of ritonavir to determine whether a known CYP3A inhibitor would alter the pharmacokinetic profile of BPU. Ritonavir also inhibits ABCG2, but the experiments done in BPU-resistant cell lines did not indicate that BPU and metabolites are transported by this protein (24). Ritonavir given 30 minutes before BPU resulted in an ~5-fold increase in BPU AUC$_{last}$ from 518 nmol/L h for BPU given alone to 2,491 nmol/L h when BPU was given with ritonavir ($P < 0.05$; Fig. 4). A simultaneous decrease in mmBPU AUC$_{last}$ from 4,492 to 2,371 nmol/L h ($P < 0.05$), aminoBPU AUC$_{last}$ from 8,523 to 4,085 nmol/L h ($P < 0.05$), and G280 AUC$_{last}$ from 390 to 316 nmol/L h ($P < 0.05$) was observed when BPU was given with ritonavir. G308 and G322 were not detected in mouse plasma. The relative systemic exposure when compared with BPU decreased from 867% to 131% for mmBPU and from 1645% to 164% for aminoBPU. The time to peak concentration was not altered for BPU or mmBPU but was increased from 4 to 24 hours for aminoBPU. The combined exposure to BPU and the metabolites was affected to a lesser extent by ritonavir (13,924 versus 10,163 nmol/L h) than each of the compounds individually. Similar trends were observed in brain tissue. Ritonavir resulted in an ~5-fold increase in BPU AUC$_{last}$ from 686 to 3,343 nmol/g h when BPU was given with ritonavir ($P < 0.05$). A noticeable, but not statistically significant, decrease in mmBPU AUC$_{last}$ from 11,471 to 8,540 nmol/g h ($P > 0.05$) was observed with coadministration. However, there was a significant decrease in aminoBPU AUC$_{last}$ from 9,138 to 6,203 nmol/g h ($P < 0.05$). G280, G308, and G322 were not detected in mouse brain tissue. As with the plasma, a change in time to peak concentration was only observed for aminoBPU (2 versus 24 hours). The combined exposure to BPU and the metabolites was affected to a lesser extent by ritonavir (21,296 versus 18,085 nmol/g h) than each of the compounds individually. The relative systemic exposure in brain compared with plasma was similar regardless of ritonavir coadministration for BPU (132% versus 134%) and mmBPU (255% versus 261%) but was increased for aminoBPU (107% versus 152%).

![Fig. 3. In vitro cytotoxicity of G280, G308, G322, and G373 against a panel of eight human-derived cancer cell lines. Relative growth after exposure to increasing concentrations of G280 (A), G308 (B), G322 (C), and G373 (D). ▼ and long dashed line, A431; ○ and solid line, BxPC3; △ and dash-dot-dotted line, Hep2; ▽ and medium dashed line, HN006; ■ and solid line, HuCCT-1; ● and dash-dotted line, MiaPaCa; ◆ and long dashed line, Panc-1; ○ and dotted line, SNU-308.](image_url)
Discussion

BPU is a compound with complex pharmacologic issues, including poor and variable oral absorption. In humans, the dose-limiting toxicity of BPU seems to be related to higher plasma exposure (AUC, $C_{\text{max}}$, and $C_{\text{ss,min}}$) of BPU, mmBPU, and aminoBPU (11). This prompted our exploration of the in vitro and in vivo metabolism of BPU. Initial studies done in human liver microsomes showed increased metabolism of BPU when compared with mmBPU or aminoBPU. This was evident by 75% of BPU being converted to mmBPU, aminoBPU, and unknown metabolites by only one quarter of the microsomes that metabolized 15% of mmBPU and 10% of aminoBPU. The minimal turnover of mmBPU and aminoBPU in vitro could be one possible explanation for the longer half-life of these metabolites compared with BPU in vivo. In addition, in vitro experiments suggested that BPU is sequentially N-demethylated to mmBPU and aminoBPU, because aminoBPU is only formed once mmBPU is present.

Preliminary data suggested that only CYP2D6 and CYP3A4 were responsible for the conversion of BPU to mmBPU and aminoBPU (9). Data presented in this article confirmed that these two CYP450 enzymes and four additional CYP450 enzymes (CYP1A1, CYP2C8, CYP3A5, and CYP3A7) were involved in BPU metabolism (see Fig. 2). CYP3A4 and CYP1A1 seem to be the most potent metabolizing enzymes involved in the conversion of BPU to mmBPU and aminoBPU. The initial exploration by Garimella only included a screen of seven CYP450 isoenzymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) and showed the need for full exploration of all CYP450 enzymes. Experiments were done with the CYP3A subfamily to distinguish the differences in activity, with CYP3A4 showing the highest activity followed by CYP3A5 and finally by CYP3A7. These results could be important in select populations because CYP2C8, CYP2D6, CYP3A5, and CYP3A7 have genetic alterations or variations that might result in functional differences in in vivo drug metabolism (25–29). CYP1A1 is an extrahepatic CYP450, which has been observed in tumor specimens, is inducible and polymorphic, may play a role in drug resistance, and is known to have low activity toward flutamide, imatinib, tamoxifen, and toremifene (30). For BPU, the conversion by CYP1A1 was significant to both mmBPU and aminoBPU and therefore may provide therapeutic benefit in tumors that have CYP1A1 expressed.

Drug efflux by outward-directed ABC transporters is another potential source of variability in oral absorption, drug disposition, and drug-drug interactions. Because BPU is a substrate for CYP3A4, CYP3A5, and CYP3A7 and there tends to be an overlap between CYP3A and ABCB1 and ABCG2
substrates, we anticipated that BPU or one of its metabolites would be a substrate for ABCG2 (10, 31–36). However, preliminary experiments refute that BPU, mmBPU, and amino-BPU are substrates for ABCG2 transport using cytotoxicity or 5D3 antibody methodology. Therefore, it is unlikely that ABCG2 is involved in resistance to BPU, mmBPU, and aminobPU or plays a role in their disposition.

In humans, BPU, mmBPU, and aminobPU were found in plasma, whereas aminobBPU, G280, G308, G322, and G373 were found in urine. mmBPU, G280, G308, and G322 were synthesized and confirmed against patient specimens. G373 was synthesized but not confirmed against patient specimens that could be due to a misinterpretation of the structure when initially detected in fresh (i.e., not frozen) patient urine or instability of G373 when frozen in urine. Previous reports showed that aminoBPU was the most cytotoxic metabolite followed by mmBPU and BPU (7, 12). We have shown that the metabolites that were primarily excreted in urine (G280, G308, G322, and G373) possessed minimal cytotoxic activity. Therefore, it is thought that the cytotoxicity of aminoBPU and mmBPU may contribute and have clinical relevance due to the increased exposure noted in the two patients with dose-limiting toxicity in the phase I clinical trial with continuous weekly dosing (11).

Noting the unique metabolic profile of BPU, we determined whether the use of ritonavir, a known CYP3A4 inhibitor, could be used to exploit the tissue-specific differences in metabolic enzyme activities to enhance efficacy and safety of BPU. It was thought that temporary and simultaneous inhibition of intestinal and hepatic activity of total Cyp3A would improve the low and variable oral absorption characteristics of BPU and decrease systemic exposure to cytotoxic metabolites, which have been linked to dose-limiting toxicity in humans. Ritonavir temporarily prevented the conversion of BPU to mmBPU and aminoBPU as was noted with the significant increase in BPU exposure in plasma and decrease in mmBPU and aminoBPU exposure when ritonavir was given 30 minutes before BPU (see Fig. 4). However, the combined exposure to BPU and the metabolites was affected to a lesser extent by ritonavir than each of the compounds individually, confirming in vitro transporter studies that metabolism is the major factor involved in the observed interaction.

In conclusion, we have characterized the in vivo and in vitro metabolism of BPU to six metabolites. mmBPU and aminoBPU were the major products following incubation with human liver microsomes and in plasma with formation by CYP1A1, CYP2C8, CYP2D, CYP3A4, CYP3A5, and CYP3A7. CYP3A4 seems to play a major role in liver and intestinal metabolism relative to the other CYP450 isozymes. G280, G308, and G322 were found in human urine but not in NADPH-dependent metabolic experiments, suggesting that these metabolites are chemical breakdown products. BPU and the cytotoxic metabolites mmBPU and aminoBPU were not substrates for ABCG2 in vivo or in vitro. Finally, we determined that intentional pharmacokinetic biomodulation of the extensive first-pass metabolism of BPU improved bioavailability and decreased exposure to the cytotoxic metabolites. Further studies are ongoing to determine the kinetics of the CYP450 reactions responsible for the metabolism of BPU, mmBPU, and aminobPU. In addition, the role of CYP1A1 in BPU cytotoxicity will be explored because CYP1A1 is typically linked to cancer incidence and tumor drug sensitivity (30). Finally, the use of ritonavir to boost the systemic exposure of BPU provides a promising combination for further testing in patients.

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