Metabolism and Excretion of the Novel Bioreductive Prodrug PR-104 in Mice, Rats, Dogs, and Humans

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ABSTRACT:

PR-104 is the phosphate ester of a 3,5-dinitrobenzamide nitrogen mustard (PR-104A) that is reduced to active hydroxylamine and amine metabolites by reductases in tumors. In this study, we evaluate the excretion of [3H]PR-104 in mice and determine its metabolite profile in mice, rats, dogs, and humans after a single intravenous dose. Total radioactivity was rapidly and quantitatively excreted in mouse, with cumulative excretion of 46% in urine and 50% in feces. The major urinary metabolites in mice were products of oxidative N-dealkylation and/or glutathione conjugation of the nitrogen mustard moiety, including subsequent mercapturic acid pathway metabolites. A similar metabolite profile was seen in mouse bile, mouse plasma, and rat urine and plasma. Dogs and humans also showed extensive thiol conjugation but little evidence of N-dealkylation. Humans, like rodents, showed appreciable reduced metabolites in plasma, but concentrations of the cytotoxic amine metabolite (PR-104M) were higher in mice than in humans. The most conspicuous difference in metabolite profile was the much more extensive O-β-glucuronidation of PR-104A in dogs and humans than in rodents. The structure of the O-β-glucuronide (PR-104G) was confirmed by independent synthesis. Its urinary excretion was responsible for 13 ± 2% of total dose in humans but only 0.8 ± 0.1% in mice. Based on these metabolite profiles, biotransformation of PR-104 in rodents is markedly different from that in humans, suggesting that rodents may not be appropriate for modeling human biotransformation and toxicity of PR-104.

Tumor hypoxia is a potentially important therapeutic target because hypoxia is more severe in tumors than normal tissues, and hypoxic cells are refractory to radiotherapy and many chemotherapy drugs (Brown and Giaccia, 1998; Brown and Wilson, 2004). The importance of hypoxia as a therapeutic target has led to the development of bioreductive prodrugs that are metabolized to active cytotoxins by pathways that are inhibited by oxygen (Rauth et al., 1998; Stratford and Workman, 1998; McKeown et al., 2007; Chen and Hu, 2009). PR-104 is the first hypoxia-activated nitrogen mustard prodrug to have entered clinical development and is currently in Phase II clinical trial. It is a water-soluble phosphate ester that is rapidly converted to the corresponding alcohol PR-104A in mice (Patterson et al., 2007), rats (Patel et al., 2007), and humans (Jameson et al., 2009). PR-104A is a 3,5-dinitrobenzamide-2-mustard that is metabolized selectively under hypoxia to hydroxylamine and amine metabolites by human tumor cell lines (Patterson et al., 2007; Singleton et al., 2009). This biotransformation acts as an electronic switch to activate the nitrogen mustard moiety in nitroaromatic mustards (Denny and Wilson, 1986; Helsby et al., 2003). The 5-hydroxylamine (PR-104H) and 5-amine (PR-104M) metabolites of PR-104A are responsible for its hypoxia-selective cytotoxicity via DNA cross-linking (Patterson et al., 2007; Gu et al., 2009; Singleton et al., 2009). Reduction of the 5-nitro group of PR-104A is also catalyzed, under aerobic conditions, by human aldo-keto reductase 1C3 (Guise et al., 2010), an enzyme that is highly expressed in some human tumors (Penning and Byrns, 2009). Recent studies identifying PR-104H and PR-104M in plasma of humans (Gu and Wilson, 2009) and mice (Y. Gu, C. P. Guise, K. Patel, S. D. Holford, M. R. Abbattista, J. Lie, X. Sun, G. J. Atwell, M. Boyd, A. V. Patterson et al., manuscript submitted for publication) suggest that reductive activation of PR-104A may occur in normal tissues and in tumors.

Beyond this partial evaluation of PR-104 hydrolysis and PR-104A reduction, no systematic investigation of pathways of biotransformation of PR-104 has been reported. Expected routes of metabolism include oxidative N-dealkylation of the nitrogen mustard moiety of PR-104A, as reported for other nitrogen mustards (Kestell et al., 2000;...
Metabolism of PR-104 Across Species

Zhang et al., 2005a), and phase II conjugation of the nitrogen mustard moiety by glutathione S-transferases, as for other mustards (Dirven et al., 1996; Zhang et al., 2005b) and the primary alcohol side chain by UDP glucuronosyltransferase. Indeed, preliminary evidence for an N-dealkylated half-mustard, a cysteine conjugate, and an O-glucuronide of PR-104A has been reported in mice (Patei et al., 2007).

Here, we undertake a comparative study of the pathways of biotransformation of PR-104 in mice, rats, dogs, and humans after intravenous administration. In addition, the mass balance for excretion of radiolabeled PR-104 in mice is reported, including comparison with urinary excretion in rats and humans. The primary objectives of the study were to identify metabolic pathways of potential toxicological significance, and to assess the suitability of nonhuman species as models for biotransformation of PR-104 in humans.

Materials and Methods

Chemicals. PR-104, PR-104A (Denny et al., 2005), PR-104H (Patterson et al., 2007), PR-104M, PR-104S1 (previously called PR-104S) (Gu et al., 2009), PR-104A-d4 (Aldrich, St. Louis, MO) and silver triflate (1.03 g, 4.01 mmol) (Sigma-Aldrich, St. Louis, MO) were synthesized as described previously. Tetradeuterated internal standards of PR-104H (PR-104H-d4) and PR-104M (PR-104M-d4) were prepared using the same methods as the nonlabeled compounds. All the compounds had a purity of at least 95% by high-performance liquid chromatography (HPLC).

Synthesis and Characterization of Glucuronide PR-104G (M1). Intermediate 2. A solution of PR-104A (1.00 g, 2.00 mmol) in CH2Cl2 (45 ml) containing powdered 4-Å molecular sieves was stirred at room temperature for 30 min, then cooled to 0°C, and treated sequentially with 2,3,4-tri-0-acetyl-1-bromo-1-deoxy-a-D-glucuronic (1; Fig. 1A) (1.27 g, 3.20 mmol) (Sigma-Aldrich, St. Louis, MO) and silver triflate (1.03 g, 4.01 mmol) (Sigma-Aldrich). The mixture was stirred at room temperature for 16 h and then filtered through a celite pad that was washed with CH2Cl2. The filtrate was washed with water, dried, and then concentrated under reduced pressure, and the residue was chromatographed on silica gel. Elution with EtOAc/petroleum ether (3:2) gave the O-acetyl derivative of PR-104A [21%, identified by mass spectrometry (MS) and NMR]. Further elution with EtOAc/petroleum ether (2:1) gave a product that was precipitated from a CH2Cl2 solution with isopropyl ether (i-PrOEt) (2×) to give (2R,3S,4S,5S,6S)-2-[2-[[2-bromoethyl][2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-ethoxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (M1) (131 mg, 79%); mp 88 to 92°C. [13C NMR [(CD3)2SO] δ 126.8, 126.3, 126.2, 125.1, 124.6, 122.6, 113.1, 113.0, 110.3, 106.9, 106.7, 80.3, 80.0, 77.6, 77.5, 73.8, 73.7, 72.9, 72.7, 71.9, 66.9, 67.5, 65.4, 52.4, 51.1, 39.3, 36.4, 29.7, 20.2 (2). High-resolution mass spectrometry (HRMS) (fast atom bombardment-positive) calculated for C32H26BrN10O19S (MH+) m/z 815.0929, found 815.0932; calculated for C32H26O11BrN10O19S (MH+) m/z 817.0909, found 817.0910.

PR-104G (M1). An ice-cold solution of 0.05 M LiOH in MeOH/water/tetrahydrofuran (3:1:1) (29.4 ml, 1.47 mmol) was added to (200 mg, 0.25 mmol), and the mixture was stirred at 0°C for 2.5 h. The resulting solution was diluted with water (30 ml), adjusted to pH 3 with Amberlite-120 (H+), filtered, and then concentrated to a small volume under reduced pressure at less than 30°C. Addition of a limited amount of i-PrO2 gave (25,35,45,55,66)-6-[2-[2-(2-bromoethyl)[2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-ethoxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (M1) (131 mg, 79%); mp 88 to 92°C. [13C NMR [(CD3)2SO] δ 126.8, 126.3, 126.2, 125.1, 124.6, 122.6, 113.4, 113.1, 113.0, 110.3, 106.9, 106.7, 80.3, 80.0, 77.6, 77.5, 73.8, 73.7, 72.9, 72.7, 71.9, 66.9, 67.5, 65.4, 52.4, 51.1, 39.3, 36.4, 29.7, 20.2 (2). High-resolution mass spectrometry (HRMS) (fast atom bombardment-positive) calculated for C32H26BrN10O19S (MH+) m/z 79BrN4O18S 675.0435, found 765.0434; calculated for C32H26O11BrN10O19S (MH+) m/z 79BrN4O18S 667.0435, found 677.0434. HPLC purity was 96% at 330 nm.

Synthesis and Characterization of Semimustards PR-104S1 (M8) and PR-104S2 (M10). Intermediate 4. A suspension of 2-chloro-N-(2-hydroxyethyl)-3,5-dinitrobenzamide (3) (1.00 g, 3.45 mmol) (Denny et al., 2005) in MeCN (30 ml) was treated with aziridine (0.54 ml, 10.4 mmol) (Trylead Chemicals, Hangzhou, China) and stirred at room temperature for 1.5 h. The mixture was evaporated; the residue was purified by chromatography on silica gel; and elution with EtOAc, followed by recrystallization from EtOAc/i-Pr2O, gave 2-(azirin-1-yl)-N-(2-hydroxyethyl)3,5-dinitrobenzamide (4) (559 mg, 55%); mp 181 to 182°C. [13C NMR [(CD3)2SO] δ 8.74 (d, J = 2.7 Hz, 1H); 8.61 (t, J = 5.4 Hz, 1H), 8.37 (d, J = 2.7 Hz, 1H), 8.77 (t, J = 5.5 Hz, 1H), 3.58 (q, J = 5.8 Hz, 2H), 3.38 (q, J = 5.8 Hz, 2H), 2.40 (s, 4H). Elemental analysis calculated for C12H9N2O4C: 44.60%; H: 4.08%; N: 23.81%; found: C, 44.58; H, 4.14; N, 18.92.

PR-104S1 (M8). 4 (250 mg, 0.84 mmol) was added to a solution of methanesulfonic acid (3 ml) and water (3 ml) at −5°C, and the mixture was stirred at 0°C until homogeneous and then for a further 5 min. The mixture was

Fig. 1. Synthetic schemes for reference standards. A, the β-glucuronide of PR-104A, PR-104G (M1). B, the semimustard metabolites of PR-104A (M2 and M3).
diluted with saturated NaCl (100 ml) and then extracted with EtOAc; the organic extract was washed with saturated NaCl, dried, and evaporated. The residue was purified by chromatography on silica gel, eluting with 5% MeOH/ EtOAc, and combined early fractions were concentrated and diluted with i-Pr2O to give 2-[2-[(2-hydroxyethyl)aminocarbonyl]-4,6-dinitroanilino]-ethyl methanesulfonate (M8) (89 mg, 27%): mp 122°C; 1H NMR (CD3)2SO δ 9.11 (t, J = 5.3 Hz, 1H), 8.98 (t, J = 5.4 Hz, 1H), 8.84 (d, J = 2.7 Hz, 1H), 8.41 (d, J = 2.7 Hz, 1H), 4.79 (t, J = 5.5 Hz, 1H), 4.43 (q, J = 5.0 Hz, 2H), 3.59–3.51 (m, 4H), 3.34 (q, J = 5.7 Hz, 2H), 3.19 (s, 3H). Elemental analysis calculated for C17H17BrN3O3S: C, 36.74; H, 4.41; N, 14.28; S, 8.17; found: C, 37.11; H, 4.29; N, 14.17; S, 8.20.

PR-104S2 (M10). A suspension of 4 (185 mg, 0.51 mmol) in 24 wt.% aqueous HBr (6 ml) was stirred at room temperature for 3 h, then diluted with water (25 ml) and extracted with EtOAc (2 × 20 ml). The combined extract was dried, filtered through a plug of silica gel, concentrated to a small volume under reduced pressure, and then diluted with i-Pr2O to give 2-(2-bromoethyl)-amin-o-(2-hydroxyethyl)-3.5-dinitrobenzamide (M10) (177 mg, 93%): mp 122 to 123°C; 1H NMR (CD3)2SO δ 8.09 (t, J = 5.4 Hz, 1H), 8.97 (t, J = 5.5 Hz, 1H), 8.85 (d, J = 2.8 Hz, 1H), 8.38 (d, J = 2.7 Hz, 1H), 4.80 (t, J = 5.5 Hz, 1H), 3.75 (t, J = 5.9 Hz, 2H), 3.63 (q, J = 5.8 Hz, 2H), 3.56 (q, J = 5.8 Hz, 2H), 3.34 (q, J = 5.8 Hz, 2H). Elemental analysis calculated for C17H14BrN5O5: C, 35.03; H, 3.47; N, 14.86; Br, 21.19; found: C, 35.20; H, 3.54; N, 14.95; Br, 21.04.

Subjects. Specific-pathogen-free homozygous nu/nu (CD-1-Foxn1nu) mice and Sprague-Dawley rats (Charles River, Margate, Kent, UK) were bred in the University of Auckland. Mice were housed in Tecniplast (Buggagiate, Italy) microisolator cages in groups of four to six in a temperature-controlled room (20 ± 2°C) with a 12-h light/dark cycle and were fed ad libitum UV-treated Milli-RO water (Millipore Corporation, Billerica, MA) and a sterilized rodent diet (diet 2018s; Harlan Teklad, Madison, WI). Rats were housed in groups of four to six under the same conditions but received filtered tap water and diet 2018s (Harlan Teklad). At the time of experiments, animals weighed 25 to 30 g before the next injection.

Absorbance detection was at 370 nm (bandwidth, 4 nm). An Agilent LC/tandem MS (MS/MS; model 6410) equipped with an electrospray ionization/atmospheric pressure chemical ionization multimode source was used for further identification of some products in excretion samples.

Mouse and rat plasma samples were prepared by precipitating proteins with 3 volumes of methanol, and for dog and human plasma with 9 volumes of acetonitrile/methanol (methanol/ammonium acetate-acetic acid, 1000:5:0.2, v/v, 20 min, returned to the initial composition over 2 min, and maintained for 5 min before the next injection. Absorbance detection was at 370 nm (bandwidth, 4 nm). An Agilent LC/tandem MS (MS/MS; model 6410) equipped with an electrospray ionization/atmospheric pressure chemical ionization multimode source was used for further identification of some metabolites using a Zorbax SB C18 capillary column (150 × 0.5 mm, 5 μm; Agilent Technologies) at a flow rate of 0.15 μl/min. The electrospray ionization source was set at positive ionization mode with auto MS(n).

Hepatic 9000g Postmitochondrial Supernatant Metabolism of PR-104A. In vitro hepatic metabolism of PR-104A was studied using liver 9000g postmitochondrial supernatant (S9) fraction prepared from pooled CD-1 nu/nu mice in vivo in-house and pooled human liver S9 purchased from CelzDirect (Durham, NC). Reactions (final volume, 0.1 ml/well in 96-well plates) comprised hepatic S9 (2 mg of protein/ml), PR-104A (150 μM), and cofactor (NADPH, NADH, or both, 1 mM each) in sodium/potassium phosphate buffer (67 mM, pH 7.4) with 5 mM MgCl2 and 1 mM EDTA and were incubated for 30 min at 37°C under air or in an anaerobic chamber (Sheldon Manufacturing, Cornelius, OR).

Incubations with boiled S9 preparations were used as controls. All the solutions for the anoxic experiment were equilibrated (along with the 96-well
plates) in the chamber for at least 3 days to remove oxygen, and S9 was preaged with nitrogen by flushing vigorously on ice for 3 min. The reaction was terminated, and metabolites were extracted by addition of 0.1 ml of ice-cold acidified methanol (methanol/ammonium acetate/acetic acid, 1000: 3.5:0.5, v/w/v) with centrifugation at 12,000 g for 5 min. The supernatant was diluted into an equal volume of water, and 25 μl was analyzed by LC/MS/MS as reported (Gu and Wilson, 2009).

Pharmacokinetic Studies. Mouse and human plasma samples were analyzed by LC/MS/MS (Gu and Wilson, 2009). In brief, mouse and human plasma samples were prepared by precipitating proteins with methanol or acidified methanol as above. The supernatant was diluted into water containing internal standards, and aliquots were analyzed by ultra-HPLC/MS/MS using a Zorbax Eclipse XDB-C18 Rapid Resolution HT (50 × 2.1 mm, 1.8 μm; Agilent Technologies) column and gradient of acetonitrile and 0.01% formic acid with a 6-min run time. Plasma concentration versus time data for unchanged PR-104 and metabolites PR-104A, PR-104H, PR-104M, PR-104S, and PR-104G were analyzed noncompartmentally using WinNonlin (version 5.0; Pharsight, Mountain View, CA).

Results

Excretion of Radioactivity in Mouse Urine and Feces. After a single intravenous dose of [3H]PR104, radioactivity was rapidly excreted in urine, with 90% of the urinary excretion occurring within 4 h. Total radioactivity in excreta (urine plus feces) by 24 h after dosing accounted for 94.0 ± 1.6% of the administered dose, increasing to 96 ± 0.8% by 48 h (Fig. 2A). By that time, excretion of total radioactivity was approximately equal for the two routes, with 46% in urine and 50% in feces.

Profiles of Excreted Metabolites. Mice and rats. We developed a chromatographic method to optimize separation of urinary metabolites from mice as illustrated in Fig. 2B. Improved resolution of PR-104 and its metabolites in mouse urine samples was achieved, relative to an earlier method (Patel et al., 2007) that failed to separate M2 from PR-104. This made it possible to quantify eight major radioactive peaks (Fig. 2C), all of which were subsequently identified as detailed below and summarized in Table 1. Metabolites were identified based on comparison of retention times, UV spectra, and mass spectra with authentic standards, or structures were inferred from UV and mass spectra as described below (see Fig. 5 for proposed structures). Unchanged PR-104 and its dephosphorylated alcohol metabolite, PR-104A, in urine accounted for 6.3 and 4.6% of the injected dose, respectively (Fig. 2C). The PR-104 radioactivity peak was incompletely resolved from metabolite M1 (identified as the O-β-glucuronide of PR-104A, PR-104G). The cumulative urinary excretion of M1 (PR-104G) was calculated as 0.8% of injected dose by using HPLC/photodiode array at 370 nm to estimate the contributions of PR-104 and M1 to the radioactivity peak. The major urinary metabolites were the “semimustard” product PR-104S1 (M8) resulting from oxidative N-dealkylation of the bromoethyl moiety of PR-104A, and its corresponding cysteiny1 (M12) and N-acetylcysteiny1 (M13) conjugates, which accounted for 5.3, 6.6, and 2.7% of injected dose, respectively. A cysteine adduct of PR-104A (M3), resulting from displacement of the Br-leaving group, was also a major urinary metabolite, accounting for 6.1% of injected dose. Other major polar
metabolites in urine include glutathionyl (M16; 1.2% of total dose), cysteinyl (M17; 7.0%), and N-acetylcytistinyl (M18; 1.3%) conjugates related to M14, an intramolecular alkylation product detected in plasma but not urine (Table 1). Another minor metabolite was the hydrolysis product M19, which represented <1% of total dose. Analysis of bile collected 30 min after dosing showed a different pattern from urine (Fig. 2D), with both glutathione conjugates of PR-104A (M2 and M6) along with the N-acetylcysteinyl adduct (M5) derived from M2 and semimustard M8, all at high concentrations, as well as its glutathionyl adduct (M11).

The metabolite profile in rat urine is illustrated in Fig. 3A for the 1- to 2-h window and shown for other times in Supplemental Fig. S2. The profile was broadly similar to mice, although the cysteinyl adducts M12 and M3 were not detected. The N-acetylcysteinyl adduct (M5), a downstream product of M3, was present in the earlier (0–1 h) urine (Supplemental Fig. S2A). In addition, a metabolite not observed in mice, M21, became more prominent between 2 and 3 h postdose (Supplemental Fig. S2B).

**Humans.** After administration of PR-104 (135 mg/m²) to patients, metabolite M1 (PR-104G) was identified as the main metabolite in urine (Fig. 3B) and accounted for ~12% of the injected dose (Fig. 3C). A cysteinyl adduct of PR-104A (M3) was the second most prominent metabolite and accounted for 3% of total dose. PR-104A accounted for ~1%. Evaluation of a limited number of patients at higher doses confirmed the predominance of the glucuronide metabolite in urine and showed an approximately linear increase in renal excretion of PR-104A and PR-104G with dose level ($R^2 = 0.9$ for both) (Fig. 3D). Including all 11 patients, 2% of the total dose was excreted as PR-104A and 13% at PR-104G. A number of minor metabolites were detected as shown in Fig. 3B. We were surprised to find that PR-104 itself was not detected in urine. Therefore, a more sensitive LC/MS/MS method for PR-104 (Patel et al., 2007) was used to evaluate PR-104 in the same samples. PR-104 was below the limit of quantitation of 10 nM in two of four patients dosed at 135 mg/m² and in the patient dosed at 346 mg/m², with very low levels in the other patients (<0.005% of total dose in all 11 patients; data not shown).

**Metabolite Profiles in Plasma.** Representative chromatograms for plasma from all four species (mouse, rat, dog, and human) at early times after intravenous administration of PR-104 are shown in Fig. 4. A full catalog of plasma metabolites in the four species is provided in Table 1. The hydrolysis product, PR-104M (data not shown). PR-104H, whereas in dog plasma concentrations of PR-104H were much lower. As reported elsewhere, the amine PR-104M was the major metabolite in all the species. Glucuronide PR-104G (M1) was the main metabolite in both dogs and humans. The cysteinyl conjugate M8 was observed in all the species along with its presumed precursor glutathionyl conjugate (M2) in mice, rats, and dogs and its downstream N-acetylcysteinyl conjugate in mice and rats only. Both semimustards of PR-104A (M8, M10) were observed in mice and rats, with M8 the more prominent of the two. The corresponding thiol conjugates M11 and M12 were also found in plasma of mice and rats. Some minor nucleophilic displacement products (M19 and M20) were also present in mice and rats. As reported elsewhere, the amine PR-104M was the predominant reduced metabolite in mouse plasma (Y. Gu, C. P. Guise, K. Patel, S. D. Holford, M. R. Abbattista, J. Lie, X. Sun, G. J. Atwell, M. Boyd, A. V. Patterson, et al., manuscript submitted for publication), whereas hydroxylamine PR-104H was the major reduced metabolite in humans (Gu and Wilson, 2009). Rats also showed PR-104M ≫ PR-104H, whereas in dog plasma concentrations of PR-104H > PR-104M (data not shown).

**Metabolite Identification.** Metabolites were characterized by LC/MS (including photodiode array detection) and LC/MS/MS in plasma and urine samples of all the species, where possible including comparison of retention times and spectra with synthetic standards (PR-104A, PR-104G, PR-104S1, PR-104S2, PR-104H, and PR-104M). In general, the [M + H]$^+$ ion was observed for all the metabolites, occasionally with appearance of [M + Na]$^+$ and [M + K]$^+$ ions, and the presence of

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**Table 1. The hydrolysis product, PR-104A, was a major metabolite in all the species along with its presumed precursor glutathionyl conjugate in mice and rats only. Both semimustards of**

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<th>Name</th>
<th>$\lambda_{max}^*$</th>
<th>$m/z$</th>
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<th>Isotope</th>
<th>Biotransformation</th>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>360</td>
<td>460</td>
<td>Oxidative N-dealkylation/NAC conjugation</td>
<td>+/-</td>
<td>+/-</td>
<td>-/-</td>
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<td></td>
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<tr>
<td>M14</td>
<td></td>
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<td>403</td>
<td>Intramolecular alkylation</td>
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<td>+/-</td>
<td>-/-</td>
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<td></td>
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<td></td>
<td>365</td>
<td>297</td>
<td>Intramolecular alkylation/oxidative N-dealkylation</td>
<td>-/+-</td>
<td>-/+-</td>
<td>-/+-</td>
<td>-/+-</td>
<td></td>
<td></td>
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<tr>
<td>M16</td>
<td></td>
<td>345</td>
<td>630</td>
<td>Intramolecular alkylation/GSH conjugate</td>
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<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
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<tr>
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<td>444</td>
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<td>-/+-</td>
<td>-/+-</td>
<td>-/+-</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>486</td>
<td>Intramolecular alkylation/NAC conjugation</td>
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<td>-/+-</td>
<td>-/+-</td>
<td>-/+-</td>
<td></td>
<td></td>
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<tr>
<td>M19</td>
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<td>421</td>
<td>Br</td>
<td>H</td>
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<td>+/+/+</td>
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<td>-/+-</td>
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<tr>
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<td>Oxidation/hydrolysis</td>
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<td>-/+</td>
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<td>240</td>
<td>485</td>
<td>Br</td>
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<td>Nitroreduction</td>
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<td>+/-</td>
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<td>Nitroreduction</td>
<td>+/-</td>
<td>+/+</td>
<td>+/+</td>
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</table>

* Maximal UV absorbance.
1 Plasma/urine.
2 Plasma only.
FIG. 3. Excretion of PR-104 and metabolites in rat and human urine. A, representative chromatogram of rat urine, 1 to 2 h after an intravenous dose of 244 mg/kg. B, representative chromatogram of human urine (90 min postinfusion, dose 135 mg/m²). C, metabolite profiles in human urine (135 mg/m², four subjects). “Others” includes the minor metabolites for which HPLC peaks could be integrated, assuming the same extinction coefficient at PR-104A at 370 nm. D, dose dependence of urinary excretion of PR-104A ($R^2 = 0.90$) and PR-104G ($R^2 = 0.91$) in humans (11 subjects).

FIG. 4. Representative HPLC of PR-104 metabolites in mouse, rat, dog, and human plasma at early times (15 min for mouse and dog, 20 min for rat, and end of infusion for human) after dosing.
characteristic Br and Cl isotope peaks provided guidance for the number of halides. The UV/visible absorbance spectra were also diagnostic. Thus, all the metabolites retaining a full nitrogen mustard moiety showed absorption maxima ($\lambda_{\text{max}}$) at 370 nm, as for PR-104 itself, and had a strictly symmetric spectral shape. The semimustard metabolites resulting from N-dealkylation of one arm of the mustard all showed a spectral shift to $\lambda_{\text{max}}$ 360 nm and a broad shoulder at ~420 nm. Products of intramolecular alkylation showed similar spectra but with a less pronounced shoulder, whereas the reduction products showed major spectral changes consistent with electronic perturbation of the chromophore. Spectral features are summarized in Table 1 with supporting data in the supplemental information. Inferred structures are shown in Fig. 5.

**PR-104A.** PR-104A showed a UV spectrum with $\lambda_{\text{max}}$ 370 nm and molecular ion [M + H]$^+$ at m/z 499 with single bromine doublet pattern (m/z 501 at 98% abundance) and major fragment ions at m/z 403, 375, 297, and 279 in LC/MS and MS/MS analysis corresponding to loss of $-$OSO$_2$Me, (CH$_2$)$_2$OSO$_2$Me, and further $-$Br and $-$OH loss. These characteristic ions of the well characterized primary metabolite were used as marker fragments to interpret the mass spectra of other metabolites.

**Metabolite M1.** M1 exhibited an essentially identical UV spectrum to PR-104A and a molecular ion at m/z 675. Subsequent neutral loss of 176 atomic mass units (amu) from the ion yielded a fragment ion at m/z 499, which is a specific signature for glucuronide conjugates. In addition, a loss of glucuronic acid (GlcA) resulting in m/z 481 was also observed. Further loss of the mesylate group yielded another fragment ion at m/z 403 (supplemental data). To confirm the identity of M1, the O-$\beta$-glucuronide of PR-104A was synthesized by reaction of PR-104A with 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-$\alpha$-d-glucuronate and subsequent deprotection to form PR-104G (Fig. 1), which was fully characterized by $^1$H NMR, $^1$C NMR, and HRMS (see under Materials and Methods). In particular, $^1$H NMR of intermediate 2 showed a doublet for the anomic proton at $\delta = 4.95$ ppm with J = 8.0 Hz, clearly indicating a $\beta$ configuration. For comparison, the corresponding $\alpha$-glucuronide (prepared by inclusion of 2,4,6-collidine in the glycosylation reaction; experimental data not given) showed a doublet for the anomic proton at $\delta = 5.94$ ppm with J = 4.2 Hz. In addition, *Escherichia coli* $\beta$-glucuronidase was shown to hydrolyze the PR-104G reference standard (data not shown). Further comparison of the MS fragmentation patterns and retention time of M1 with the PR-104G reference standard confirmed M1 as the O-$\beta$-glucuronide on the hydroxyethyl side chain of PR-104A.

**Metabolites M2 through M7.** M2 and M3 showed similar UV spectra to PR-104A and [M + H]$^+$ at m/z 726 and 540, respectively. Both showed MS fragment ion loss of 96 amu ($-$OSO$_2$Me), giving prominent MS$^2$ m/z values of 630 and 444 in the ion trap. Particularly characteristic for protonated glutathione conjugates (Levsen et al., 2005), loss of glycine (75 amu) and anhydroglutamic acid (129 amu) was observed, resulting in m/z 651 and 597. Two other fragment ions from M2, at m/z 334 and 274, were consistent with the ethyl-glutathione moiety in the structure with cleavage at the amino- and mercapto-positions, respectively (supplemental data). Cleavage of the same amino-position in the M3 molecular ion resulted in fragments at m/z 148 and 393, corresponding to ethyl-cysteine and the residual semimustard, respectively. M4 showed a similar UV spectrum to PR-104A and a molecular ion of 716. The characteristic fragments of 120 and 148, corresponding to cysteine and ethyl-cysteine, indicated a conjugate of cysteine; a fragment ion at m/z 620 (corresponding to loss of a mesylate group) showed this to be the result of displacement...
of the Br-leaving group of PR-104A. In addition, neutral loss of 176 resulting in fragment ion of 540 showed the presence of a glucuronoyl moiety, showing M4 to be the product of dual phase II conjugation of PR-104A. Metabolite M5, with a UV spectrum similar to M2 and M3 and a molecular ion at m/z 582, was identified as the N-acetylcysteine adduct, the downstream product of M3 through the mercapturic acid route. Fragment ions of 130, 162, and 190, consistent with thiol residues resulting from cleavage at S-CH₂, N-acetylcysteine-, and amino-positions, respectively, supported this assignment. Metabolites M6 and M7 showed UV spectra similar to PR-104A and molecular ions [M + H]+ at m/z 710 and 524. The presence of a bromine signature in the mass spectra of both identified them as the glutathione and cysteine conjugates from displacement of the mesylate group of PR-104A.

Metabolites M8 through M13. M8 and M10 exhibited distinctive UV spectra (λmax 355 nm) and molecular ions of m/z 393 and 377, respectively. M10 gave a doublet peak + 2 amu indicative of a single bromine. M8 had an MS product ion of m/z 297, similar to that of PR-104A. Further comparison of the UV spectra, MS fragmentation patterns, and retention time of M8 and M10 with those of the reference standard confirmed that they were the mesylate and bromo semimustards, respectively. M9 was identified as the O-glucuronide of PR-104S1 (M8) based on its similar UV spectrum to the semimustards and molecular ion of 569, further supported by characteristic glucuronide neutral loss of 176 and subsequent loss of the mesylate group resulting in fragment ions of 393 and 297, respectively. In addition, loss of glucuronic acid (GlcA) resulting in m/z 375 was also observed. M11, M12, and M13 showed UV spectra (λmax 360 nm) similar to M8 and M10, and had molecular ions of m/z 604, 418, and 460, consistent with the glutathione, cysteinyl, and N-acetylcysteiny1 adducts, respectively, arising from the metabolism of M8 or M10 via the mercapturic acid pathway. These assignments were confirmed by residual thiol fragment ions (120 and 148 for M12; 130, 162, and 190 for M13) resulting from cleavage at amino- and mercapto-positions.

Metabolite M14 through M18. M14 gave a molecular ion of m/z 403 with a single bromine doublet peak. It was provisionally identified as a cyclized product arising via intramolecular alkylation of the side chain amide nitrogen by the mesylate moiety. M15 showed a molecular ion of 297 and a distinct UV spectrum (λmax = 365 nm), with a fragmentation pattern in the MS² spectrum consistent with the proposed structure (Supplemental Fig. S1). M16, M17, and M18 all showed a distinctive UV spectrum with λmax = 345 nm. M16 gave a molecular ion of 630 and fragment ion of 274 and 145, corresponding to cleavage at the S-CH₂ bond and further loss of anhydroglutamic acid resulting in a glycine residue, suggesting it to be the glutathionyl conjugate of M15. M17 showed a molecular ion of m/z 444 with a major fragment ion at 357, which corresponded to loss of 87 resulting from cleavage at the S-CH₂ bond in the cysteinyl adduct of M15. M18 showed a higher mass by 42 amu, consistent with the corresponding N-acetylcysteine adduct.

Metabolites M19 and M20. M19 and M20 showed similar UV spectra to PR-104A and molecular ions of m/z 421 and 455. Because of the presence of single bromine and chloride doublet peaks (base peaks m/z 423 and 457), these two metabolites were assigned as the products of nucleophilic displacement of the mesylate moiety of PR-104A by the hydroxyl ion (M19) and bromo group by a chloride ion (M20), resulting in less lipophilic products with shorter retention times.

Metabolite M21. Metabolite M21 was identified as a carboxylic acid derivative resulting from oxidation of the hydroxyethyl side chain of PR-104A and hydrolysis of both mustard moieties. This assignment is based on its similar UV spectrum to PR-104A, molecular ion of m/z 373, and lack of halide isotopes. Negative-mode ionization gave a base peak at m/z 371, corresponding to [M-H]-, supporting this assignment. This was further confirmed by the fragment ions of 355 and 377, corresponding loss of one or two hydroxyl groups, with further loss of a carboxyl group resulting in fragment ions of 297 and 279, respectively.


Metabolic Pathways for PR-104. The biotransformation pathways inferred from the above structural assignments are shown in Fig. 5. PR-104 is extensively and rapidly hydrolyzed to the alcohol PR-104A in all the species; no metabolites retaining the phosphate ester moiety were detected. The identified downstream metabolites of PR-104A can be classified as arising from five types of biotransformation: 1) reduction of the nitro group para to the mustard (PR-104H and PR-104M); 2) O-glucuronidation, predominantly on the hydroxethyl side chain (PR-104G, M1) [A glucuronide of the semimustard PR-104S1 was also identified (M9), and a minor metabolite (M4) identified as a glucuronide of the cysteine conjugate M3 was also detected.]; 3) nucleophilic displacement of the bromo- or mesylate-eliminating groups of the nitrogen mustard moiety, most notably by glutathione (M2, M6), leading to the corresponding cysteinyl (M3, M7) and N-acetylcysteinyl adducts (M5) via the mercapturic acid pathway [nucleophilic displacements of the mustard-eliminating groups by OH− (hydrolysis products M19 and M21) and Cl− (M20) were also seen as minor pathways.]; 4) oxidative N-dealkylation of the mustard moiety to the semimustards M8 and M10, which in turn give rise to the corresponding thiol adducts (M11, M12, M13) and O-glucuronide (M9); and 5) intramolecular cyclization of mustard moiety and carboxamide side chain, represented by M14 with further N-dealkylation (M15) and thiol conjugation (M16–M18).

Comparison of Metabolite Concentration-Time Profiles in Mice and Humans. Concentration-time profiles of PR-104 and its metabolites in mice and humans dosed at similar body surface area-scaled doses (975 and 1100 mg/m², respectively) are shown in Fig. 6. Estimation of noncompartmental pharmacokinetic parameters showed terminal half-lives of the all the metabolites of ~20 min in mice, similar to PR-104A itself (Table 2). The plasma area under the concentration-time curve (AUC) of PR-104M was ~20% of that for PR-104A, with lower concentrations of PR-104H. The metabolites in humans showed longer half-lives (~40 min), again similar to that of PR-104A itself. This quantitative comparison of mice and humans confirmed that the two species have distinctly different PR-104A metabolite profiles; in humans, PR-104H rather than PR-104M was the dominant reduced metabolite; PR-104G was much more prominent; and the semimustard PR-104S1 was present at much lower levels. The plasma AUC of PR-104H in humans was ~5% of that for PR-104A, whereas PR-104G showed equal or higher AUC than PR-104A.

Metabolism of PR-104A in Mouse and Human Liver S9. The differing profiles of the cytotoxic reduced metabolites of PR-104A (with PR-104H predominant in mice and PR-104M in humans) led us to evaluate NAD(P)H-supported metabolism of PR-104A in liver S9 preparations (Table 3). No reaction products were observed in boiled S9 controls (data not shown). Under aerobic conditions, PR-104S1 was the dominant metabolite, with intermediate concentrations of PR-104H and low concentrations of PR-104M, in both species. When the reaction was performed in an anaerobic chamber, oxidative metabolism to PR-104S1 was strongly inhibited, consistent with a re-
requirement for O$_2$ as a cosubstrate for the mixed function oxidase reaction. In turn, these results suggest that the oxygen concentration in the anaerobic chamber reactions was well below the $K_m$ for O$_2$ for cytochromes P450 ($\sim$5 $\mu$M) (Jones and Mason, 1978). Under these anoxic conditions, the reductive pathway was markedly increased relative to 21% O$_2$. Notably, in anoxic human liver S9 concentrations of PR-104H were 5- to 6-fold higher than PR-104M, but in murine S9 concentrations were higher than PR-104H, broadly consistent with the metabolite profiles seen in plasma. The cofactor dependence for the in vitro metabolism showed a strong preference for NADPH for both reductive (PR-104H, PR-104M) and oxidative (PR-104S1) pathways, under both aerobic and anoxic conditions, consistent with a major role of NADPH:cytochrome P450 oxidoreductase in both.

**Discussion**

This study identifies the major biotransformations of PR-104 in humans and in the three nonclinical species (mice, rats, and dogs) used during preclinical development of this bioreductive prodrug. It confirms the previously reported extensive and rapid hydrolysis of the phosphate “preprodrug” to PR-104A in rodents (Patterson et al., 2007; Patterson et al., manuscript submitted for publication) and extends this to dogs. The absence of other phosphorylated metabolites identifies this as the only significant biotransformation of PR-104 itself, which presumably reflects its exclusion from cells and therefore limited access to enzymes other than ectophosphatas. The reduced metabolites PR-104H (Patterson et al., 2007) and PR-104M have also been previously identified, fully structurally characterized, and shown in plasma of mice (Y. Gu, C. P. Guise, K. Patel, S. D. Holford, M. R. Abbattista, J. Lie, X. Sun, G. J. Atwell, M. Boyd, A. V. Patterson, et al., manuscript submitted for publication) and humans (Gu and Wil-son, 2009).

None of these previously characterized metabolites (PR-104A, PR-104H, PR-104M) represent major end-products in mouse urine or bile. These two routes of excretion each account for approximately half of the elimination of [3H]PR-104 in mice (Fig. 2A), with the dominant end metabolites arising through oxidative N-dealkylation of the mustard moiety of PR-104A (M8, M12, M13), intramolecular alkylation (M17), and the subsequent formation of thiol conjugates of these metabolites (as well as of PR-104A itself). The three most prominent urinary metabolites were all cysteine conjugates (M3, M12, and M17; Fig. 2C).

The cysteine conjugate M3 was also a major urinary metabolite in humans, but the O-glucuronide PR-104G was more prominent. Although not all the human metabolites could be quantified with reference to authentic standards, assuming that extinction coefficients were similar to the quantified metabolites (PR-104A and PR-104G), the total cumulative urinary excretion is $25.4 \pm 2.5\%$ (Fig. 3C). This is suggestive of substantial biliary excretion in humans, which would be consistent with the obvious prominence of the glucuronidation pathway.

The plasma metabolite profiles were broadly consistent with those in urine. Combining the information from both, marked species differences in PR-104A biotransformation can be discerned. The oxidative N-dealkylation of the nitrogen mustard of PR-104A is conspicuously lacking in dogs, but the resulting semimustard metabolites are prominent in rodents. This route appears to be a minor one in humans, given that it is represented only by low concentrations of PR-104S1, although this difference could also reflect faster clearance of the N-dealkylation products in humans. Of the two semimustard metabolites, PR-104S1 is much more prominent than PR-104S2 in all three species where N-dealkylation is observed. Again, this could reflect either faster N-dealkylation of the bromoethyl than ethyl mesylate arm.

**TABLE 2**

* Pharmacokinetic parameters of PR-104 metabolites in mouse and human plasma after intravenous administration of 975 mg/m$^2$ (bolas) and 1100 mg/m$^2$ (60-min infusion), respectively.  

<table>
<thead>
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<th>Metabolite</th>
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<th>Human*</th>
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<tr>
<td></td>
<td>$\text{AUC}_{\text{tot}}$</td>
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<tr>
<td></td>
<td>$\mu\text{mol-l}^{-1} \text{min}$</td>
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<td>PR-104G (M1)</td>
<td>2.7</td>
<td>13.1</td>
</tr>
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* The PK for PR-104 and PR-104A are in good agreement with the values reported elsewhere (Jameson et al., 2009), for 6 of the same 10 subjects, using a different analytical method.

FIG. 6. Plasma concentration-time profiles of PR-104 metabolites. A, mice dosed at 975 mg/m$^2$ (326 mg/kg), mean $\pm$ S.E.M. for three mice. B, humans dosed at 1100 mg/m$^2$, mean $\pm$ S.E.M. for 10 subjects.
of the mustard or faster clearance of the PR-104S2 metabolite. An in vitro metabolism study of PR-104A analogs showed that kinetics of dealkylation of the diethylamino group \( [N(\text{Et-X})(\text{Et-Y})] \) decreased the order \( X = Y = H \geq X = Y = \text{Br} > X = \text{Br} \) or Cl, \( Y = \text{mesylates} \) (Helsby et al., 2008), which suggests that the mesylate-leaving group suppresses dealkylation. In the latter study, neither of the semimustard metabolites was detected from PR-104A, but it suggests that the bromoethyl moiety may be selectively dealkylated. The enzymology and mechanism of this dealkylation are undetermined. In addition to the classic cytochrome P450-mediated \( \alpha \)-hydroxylation of tertiary amines, oxidation to the \( N \)-oxide of aromatic nitrogen mustards can lead to dealkylation (Tercel et al., 1995). Although the semimustard products of dealkylation are not themselves able to cross-link DNA and thus have low cytotoxic potency (Gu et al., 2009), the other dealkylation products are reactive aldehydes. Thus, dealkylation of oxazaphosphorine mustards forms chloroacetaldehyde, which has been reported to cross-link DNA (Spengler and Singer, 1988). Both dealkylation products are reactive aldehydes. Thus, dealkylation of oxazaphosphorine mustards forms chloroacetaldehyde, which has been reported to cross-link DNA (Spengler and Singer, 1988), induce DNA breaks, and inhibit DNA synthesis (Brugemann et al., 2006). Chloroacetaldehyde is a potent cytotoxin in vitro (Brüggemann et al., 1997), shows antitumor activity in vivo (Börner et al., 2000), is neurotoxic (Goren et al., 1986; Lewis and Meanwell, 1990), is nephrotoxic (Skinner et al., 1993), and is considered to contribute to the clinical toxicity of oxazaphosphorine mustards (Zhang et al., 2005a).

The relative absence of dealkylated metabolites of PR-104 in humans suggests that formation of analogous reactive aldehydes is unlikely, and that the toxicity profile of PR-104 therefore may differ from the oxazaphosphorine TH-302, which is also a hypoxia-activated nitrogen mustard prodrug (Duan et al., 2008).

In addition to enzymatic \( N \)-dealkylation, a set of metabolites (M14–M18) arise via intramolecular alkylation of the carboxamide nitrogen. The presumed initial metabolite in this series (M14) is a semimustard, and the subsequent dealkylation (M15) and thiol conjugation products (M16–M18) lack alkylation potential and thus are not expected to be of toxicologic significance. Likewise, hydrolysis of the mustard-leaving groups results in monoalkylating (M19) or non-alkylating (M21) metabolites that are not expected to contribute significant toxicity.

Thiol conjugates were prominent in all the species, including humans, especially a conjugate of PR-104A, in which cysteine has displaced the bromine. The cysteinyl and \( N \)-acetylcysteinyl adducts presumably arise from the processing of glutathionyl adducts (which were observed as M2 and M6 in rodents) via the mercapturic acid pathway. This was also a major pathway for detoxification of the semimustards and intermolecular cyclization products in rodents as shown by excretion studies. It is well known that glutathione conjugation, catalyzed by glutathione S-transferases, contributes to the detoxification of nitrogen mustards (Arrick and Nathan, 1984). Innate or acquired overexpression of this enzyme family is often observed in tumor cell lines, which in turn causes drug resistance (Colvin et al., 1993; Dirven et al., 1996). The abundant metabolites resulting from glutathione conjugation in all the species suggest that PR-104 will not be immune from this resistance mechanism, although local release of the active metabolites in tumors has the potential to overwhelm local detoxification pathways.

The biodistribution of the nitroreduction products of PR-104A in mice has been described recently (Y. Gu, C. P. Guise, K. Patel, S. D. Holford, M. R. Abbattista, J. Lie, X. Sun, G. J. Atwell, M. Boyd, A. V. Patterson, et al., manuscript submitted for publication), with amine PR-104M (M23) at higher concentrations than hydroxylamine PR-104H (M22) in plasma and all the normal tissues. Here we confirmed this pattern, with a 3-fold higher AUC of PR-104M than PR-104H in mouse plasma (Table 2). The same pattern was seen in rats, but the converse (AUC of PR-104H 6-fold higher than PR-104M) in human plasma (Table 2) was reported recently for a single patient (Gu and Wilson, 2009). In vitro metabolism of PR-104A also showed higher concentrations of PR-104H than PR-104M in human liver S9, but in anoxic mouse S9 concentrations of PR-104M were slightly higher than PR-104H (Table 3). Although this is broadly in agreement with the metabolite profile in plasma, the mouse S9 preparations did not support the extensive conversion of PR-104H to PR-104M inferred from the plasma metabolite profile and recently confirmed in mouse liver itself (Y. Gu, C. P. Guise, K. Patel, S. D. Holford, M. R. Abbattista, J. Lie, X. Sun, G. J. Atwell, M. Boyd, A. V. Patterson, et al., manuscript submitted for publication). The total of circulating reduced metabolites, normalized for PR-104A, is approximately 4 times higher in mice than humans. This could reflect a greater capacity for reductive activation of PR-104A in murine than human normal tissues, although the similarity of the total reductive metabolism of PR-104A in murine and human liver S9 suggests the difference in vivo is more likely to reflect faster clearance of PR-104M in humans. The reduced metabolites were detected at very low concentrations in dog plasma (PR-104H > PR-104M, \( C_{\max} \approx 0.14 \) and 0.1 \( \mu \text{M} \), respectively), although these samples were stored for longer periods than human and rodent samples before analysis; therefore, we cannot exclude the possibility of loss during storage.

The \( O \)-\( \beta \)-glucuronide of PR-104A (PR-104G, M1) was found to be the major urinary metabolite in humans (10% of total dose), but this route was responsible for <1% of urinary excretion in mice. This
sider differences was also seen in plasma, with PR-104G the dominant metabolite in humans and dogs, and much lower levels in rodents. A similar species difference in PR-104A glucuronidation was seen using liver microsomes in a preliminary in vitro study (Helsby et al., 2008). In addition, glucuronides of a semimustard (M9) and cysteine conjugate (M4) were found in human urine, emphasizing the importance of the glucuronidation pathway. The extensive glucuronidation of PR-104A in humans raises the possibility that this may be a significant determinant of clearance of PR-104A. In addition, biliary excretion of PR-104G could result in regeneration of PR-104A by gut microflora and gastrointestinal toxicity analogous to that reported for the irinotecan metabolite 7-ethyl-10-hydroxycamptothecin (Takasuna et al., 1996). Although gastrointestinal toxicity has not been reported to date, further evaluation of PR-104A glucuronidation in humans is clearly warranted.

In conclusion, PR-104 is uniformly hydrolyzed to PR-104A as the obligatory first metabolic step in all four species studies. However, the subsequent metabolism of PR-104A shows marked species differences; although glutathione conjugation is prominent in all the species, glucuronidation is essentially restricted to humans and dogs, whereas N-dealkylation of the mustard moiety is more prominent in rodents. The results suggest that rodents may be less suitable than dogs as models for the human toxicology of PR-104.

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References


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