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 hydroxyamine 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 mice, with cumulative excretion of 46% in urine and 50% in feces. The major urinary metabolites in mice were products of hypoxia-activated metabolism of PR-104A to hydroxylamine and amine metabolites by human reductases in tumors. In this study, we evaluated the excretion of [3H]PR-104 in mice and determined its metabolite profile in mice, rats, dogs, and humans after a single intravenous dose. Total radioactivity was rapidly and quantitatively excreted in mice, with cumulative excretion of 46% in urine and 50% in feces. The major urinary metabolites in mice were products from 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 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 toxicology 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; Kestell et al., 2007; Kestell et al., 2008). PR-104A is a 3,5-dinitrobenzamide nitrogen mustard (PR-104A) that is reduced to active hydroxyamine 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 mice, with cumulative excretion of 46% in urine and 50% in feces. The major urinary metabolites in mice were products from 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 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 toxicology of PR-104.

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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 glucuronosyltransferases. Indeed, preliminary evidence for an N-dealkylated half-mustard, a cysteine conjugate, and an O-glucuronide of PR-104A has been reported in mice (Patel 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), and PR-104 tritiated in the carboxamide side chain ([3H]PR-104; specific activity, 28.5 GBq/mmol) (Atwell and Denny, 1996; Zhang et al., 2005a), and Phase II conjugation of the nitrogen mustard of radiolabeled PR-104 in mice is reported, including comparison with the primary alcohol side chain (M1) (Fig. 1A) (1.27 g, 3.20 mmol) (Sigma-Aldrich). The mixture was stirred at room temperature for 16 h and then washed with water, dried, and then concentrated under reduced pressure, and the residue was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:2) 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-Pr2O) (2×) to give (2,3,5,45,55,65)-6-[2-[(2-bromomethyl)[2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-ethoxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (M1) (131 mg, 79%); mp 92°C; 1H NMR [(CD3)2SO] δ 8.20 (t, J 5.6 Hz, 1H), 8.13 (d, J 5.8 Hz, 2H), 2.40 (s, 4H). Elemental analysis calculated for C11H12N4O6: C, 44.60; H, 4.08; N, 18.91; found: C, 44.58; H, 4.17; N, 18.75.

Synthesis and Characterization of Glucuronide PR-104G (M1), Intermediate 2. A solution of 2-bromo-1-bromo-1-deoxy-α-D-glucuronic acid (I; 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-Pr2O) (2×) to give (2R,3S,5H,55,65)-6-[2-[(2-bromomethyl)[2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-ethoxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (M1) (131 mg, 79%); mp 92°C; 1H NMR [(CD3)2SO] δ 8.20 (t, J 5.6 Hz, 1H), 8.13 (d, J 5.8 Hz, 2H), 2.40 (s, 4H). Elemental analysis calculated for C11H12N4O6: C, 44.60; H, 4.08; N, 18.91; found: C, 44.58; H, 4.17; N, 18.75.

Synthesis and Characterization of Seminustards PR-104S1 (M8) and PR-104S2 (M10). A suspension of 2-chloro-1-bromo-1-deoxy-α-D-glucuronic acid (2; 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-Pr2O) (2×) to give (2R,3S,5H,55,65)-6-[2-[(2-bromomethyl)[2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-ethoxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (M1) (131 mg, 79%); mp 92°C; 1H NMR [(CD3)2SO] δ 8.20 (t, J 5.6 Hz, 1H), 8.13 (d, J 5.8 Hz, 2H), 2.40 (s, 4H). Elemental analysis calculated for C11H12N4O6: C, 44.60; H, 4.08; N, 18.91; found: C, 44.58; H, 4.17; N, 18.75.
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 dried with i-Pr2O to give 2-[2-[(2-hydroxyethyl)amino]carbononyl]-4,6-dinitroanilino]-ethyl methanesulfonate (M8) (89 mg, 27%): mp 122 C; H 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 (d, J = 5.5 Hz, 1H), 4.43 (d, J = 5.0 Hz, 1H), 3.59–3.51 (m, 4H), 3.34 (q, J = 5.7 Hz, 2H), 3.19 (s, 3H). Elemental analysis calculated for C15H12N4O4S: C, 36.74; H, 4.11; 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 (150 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 x 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-bromo-2-methylpropyl)-N-(2-hydroxyethyl)-N,N-dimethylbenzamide (M10) (177 mg, 93%): mp 122 to 123°C; H NMR [CD3]2SO δ 9.09 (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), 2.36 (q, J = 5.8 Hz, 2H), 2.34 (q, J = 5.8 Hz, 2H). Elemental analysis calculated for C15H19BNO4S: 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 nahu(CD1-Foxn1nu) mice and Sprague-Dawley rats (Charles River, Margate, Kent, UK) were bred in the University of Auckland. Mice were housed in Tecniplast (Burgugiate, 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 (diet 2018s; Harlan Teklad, Madison, WI). Dogs were dosed into the cephalic or saphenous vein as a slow bolus veins for up to 3 h. Urine was collected at intervals of 1 h for up to 3 h from the dogs. Rats were dosed into the tail vein at 326 mg/kg. Urine was collected at 4, 8, 24, and 48 h. Metabolic carryover between time points. Feces were collected at 24 and 48 h.

Determination of Total Radioactivity and Radioactivity Profiles. Radioactivity was determined using a liquid scintillation analyzer (Tri-Carb 1500; PerkinElmer Life and Analytical Sciences, Waltham, MA), with samples counted for 10 min unless 2σ of 0.4% was reached. An external standard quench curve was used to correct for differences in counting efficiency. Urine samples for total radioactivity measurement were diluted into water-accepting scintillation mixture (Emulsifier-Safe; PerkinElmer Life and Analytical Sciences, Waltham, MA). Feces were dried, powdered, and rehydrated by addition of deionized water. One millilitre of Soluene-350 (PerkinElmer Life and Analytical Sciences) was added and incubated at 37°C overnight; 0.5 ml of isopropanol alcohol was added; and samples were incubated for 12 h at 37°C. Next, 0.2 ml of 30% H2O2 was added drop-wise with swirling to bleach. After standing for 10 min at ambient temperature, samples were warmed to 37°C for 15 min to decompose peroxides and thus minimize chemiluminescence. Ten to 15 ml of scintillation fluid (Hionic-Flour; PerkinElmer Life and Analytical Sciences) was added. For quantification of radioactivity in HPLC eluents, fractions were collected at 0.1-min intervals using an Agilent 1100 auto-fraction collector (Agilent Technologies, Santa Clara, CA), transferred to scintillation vials, mixed with Emulsifier-Safe, and counted for radioactivity.

Metabolite Profiling. Urine samples were diluted 1:10 with water, filtered through 0.22-μm filters, and analyzed directly by HPLC with photodiode array absorbance and MS detection (Agilent 1100 LC/MSD model A; Agilent Technologies). Mouse bile samples (gallbladder) were precipitated with approximately 10 volumes of methanol, centrifuged, and diluted into formate buffer. The chromatographic separation was performed on an Altima C8 analytical column (150 × 4.6 mm, 5 μm; Alltech Associates, Deerfield, IL) with a flow rate of 0.7 ml/min maintained at 25°C. The mobile phase was an acetonitrile gradient constructed using 80% acetonitrile/20% water v/v (A) and 45 mM ammonium formate buffer in water at pH 4.5 (B) with 20% of A for 2 min and then increasing linearly to 80% A over 13 min, held for a further 5 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 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 acidified methanol (methanol/ammonium acetate/acetic acid, 1000:3.5:0.2, v/v/v) (Patel et al., 2007). Precipitated samples were then diluted into 2 volumes of purified water and analyzed by the LC/MS method reported previously (Patel et al., 2007). In brief, a 150 × 2.1-mm column with flow rate of 0.3 ml/min was used, with positive and negative mode atmospheric pressure electrospray ionization. The mass/charge (m/z) ratio was scanned from 200 to 800 with fragmentor voltage of 100 V. An Agilent LC/MSD trap-SI ion trap mass spectrometer equipped with an Agilent capillary HPLC system 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 15 μl/min (Patel et al., 2007). 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 nahu mice in vivo and in-house prepared liver S9 purchased from CellzDirect (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 plate cover the assigned content.
plated into an equal volume of water, and 25 mg of urine and 50% in feces. The radioactivity was approximately equal for the two routes, with 46% in urine and 50% in feces. Total radioactivity in excreta (urine plus feces) by 24 h after a single intravenous dose of [3H]PR-104 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 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. 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 were the "semimustard" product PR-104S1 (M8) resulting from oxidative N-dealkylation of the bromoethyl moiety of PR-104A, and its corresponding cysteinyl (M12) and N-acetylcysteinyl (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
find that PR-104 itself was not detected in urine. Therefore, a more
metabolites were detected as shown in Fig. 3B. We were surprised to
both) (Fig. 3D). Including all 11 patients, 2% of the total dose was
hydrolysis product M19, which represented
plasma but not urine (Table 1). Another minor metabolite was the
metabolites in urine include glutathionyl (M16; 1.2% of total dose),
cysteinyl (M17; 7.0%), and N-acetylcysteinyl (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. Analy-
sis 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 ad-
ducts 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 metab-
olite in urine and showed an approximately linear increase in renal
excretion of PR-104A and PR-104G with dose level (R² = 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-104A, was a 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-acetyl-
cysteinyl 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,
M. Boyd, A. V. Patterson, et al., manuscript submitted for publica-
tion), whereas hydroxylamine PR-104H was the major reduced me-
tabolite in humans (Gu and Wilson, 2009). Rats also showed PR-
H, whereas in dog plasma concentrations of 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**

Metabolites identified in mouse, rat, dog, and human samples collected after administration of a single intravenous dose of PR-104

<table>
<thead>
<tr>
<th>Met ID</th>
<th>Name</th>
<th>λmax (nm)</th>
<th>m/z</th>
<th>Halide Isotope</th>
<th>Biotransformation</th>
<th>Dealkylation</th>
<th>Oxidization</th>
<th>Nitroreduction</th>
<th>Glucuronidation</th>
<th>Hydrolysis</th>
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<tbody>
<tr>
<td>PR-104</td>
<td></td>
<td>370</td>
<td>579</td>
<td>Br</td>
<td>Hydrolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR-104A</td>
<td></td>
<td>370</td>
<td>499</td>
<td>Br</td>
<td>O-Glucuronidation</td>
<td>+ / + +</td>
<td></td>
<td></td>
<td></td>
<td>+ / + +</td>
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<tr>
<td>M1</td>
<td></td>
<td>370</td>
<td>675</td>
<td>Br</td>
<td>Hydrolysis</td>
<td>+ / + +</td>
<td>+ / + +</td>
<td>+ / + +</td>
<td></td>
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<tr>
<td>M2</td>
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<td>376</td>
<td>726</td>
<td>GSH conjugate</td>
<td>(Br displacement)</td>
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<td>+ / +</td>
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<td>376</td>
<td>540</td>
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<td>(Br displacement)</td>
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<td>376</td>
<td>716</td>
<td>CySH conjugate</td>
<td>O-glucuronidation</td>
<td>+ / + / +</td>
<td>+ / + / +</td>
<td>+ / + / +</td>
<td></td>
<td>+ / + / +</td>
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<tr>
<td>M5</td>
<td></td>
<td>376</td>
<td>582</td>
<td>NAC conjugate</td>
<td>(Br displacement)</td>
<td>+ / + / +</td>
<td>+ / + / +</td>
<td>+ / + / +</td>
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<tr>
<td>M6</td>
<td></td>
<td>380</td>
<td>710</td>
<td>GSH conjugate</td>
<td>(OMs displacement)</td>
<td>+ / + / +</td>
<td></td>
<td></td>
<td></td>
<td>+ / + / +</td>
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<tr>
<td>M7</td>
<td></td>
<td>380</td>
<td>524</td>
<td>CS conjugate</td>
<td>(OMs displacement)</td>
<td>+ / + / +</td>
<td></td>
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<td>+ / + / +</td>
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<td>M8</td>
<td></td>
<td>355</td>
<td>393</td>
<td>Oxidative N-dealkylation</td>
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<td></td>
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<td>+ / + / +</td>
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<td>355</td>
<td>569</td>
<td>Oxidative N-dealkylation/O-glucuronidation</td>
<td>+ / + / +</td>
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<td></td>
<td></td>
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<tr>
<td>M10</td>
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<td>355</td>
<td>377</td>
<td>Oxidative N-dealkylation</td>
<td>+ / + / +</td>
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<td>Intramolecular alkylation/oxidative N-dealkylation</td>
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<td>Nucleophilic displacement</td>
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<td>373</td>
<td>Oxidation/hydrolysis</td>
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<td>240</td>
<td>485</td>
<td>Br</td>
<td>Nitroreduction</td>
<td>+ / + / +</td>
<td></td>
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<td>Nitroreduction</td>
<td>+ / + / +</td>
<td></td>
<td></td>
<td></td>
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</table>

* Maximal UV absorbance.

1 Plasma/urine.

2 Plasma only.

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For proposed structures, see Fig. 5. Relative abundance (+, ≥10%; +, <10%; –, not detected) was estimated from absorbance at 370 nm, or for M22 and M23 from the MS signal.
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 $\lambda_{\text{max}}$ at 360 nm and a broad shoulder at ~420 nm. Products of intramolecular alkylation showed similar spectra to the semimustards 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 spectra in the supplemental data. Inferred structures are shown in Fig. 5.

**PR-104A.** PR-104A showed a UV spectrum with $\lambda_{\text{max}}$ at 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 $\text{OSO}_2\text{Me}$, ($\text{CH}_2$)$_2\text{OSO}_2\text{Me}$, and further $\text{Br}$ and $\text{OH}$ 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 $O$-$\text{H}$-glucuronide of PR-104A was synthesized by reaction of PR-104A with 2,3,4-tri-$O$-acetyl-1-bromo-1-deoxy-$\text{D}$-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 $O$-$\text{H}$-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 ($\text{OSO}_2\text{Me}$), 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 $m/z$ 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

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**Fig. 5.** Proposed metabolic pathways of PR-104 in mice, rats, dogs, and humans. GS, glutathione; CyS, cysteine; NAC, N-acetylcysteine; Gluc, glucuronic acid.
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 glucurononyl 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-acetylcyesteine-, 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 glucuronolytic 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, cysteiny1, 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 cysteiny1 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.

**Metabolites M22 and M23.** Identification of the reduced metabolites PR-104H (M22) (Patterson et al., 2007) and PR-104M (M23) (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) are detailed elsewhere.

**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-leaving groups of the nitrogen mustard moiety, most notably by glutathione (M2, M6), leading to the corresponding cysteinyl (M3, M7) and N-acetylcysteiny1 adducts (M5) via the mercapturic acid pathway [nucleophilic displacements of the mustard-leaving 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 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-

[Paper details placeholders are used instead of the actual URLs.]
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 (Patel et al., 2007; Patterson et al., 2007) and humans (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 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 ectophosphatases. 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 Wilson, 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 those of the quantified metabolites (PR-104A and PR-104G), the total cumulative urinary excretion was 25.4 ± 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.
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(2-Et-X)[Et-Y]] decreased the order X = Y = H > X = Y = Br > X = Br or Cl, Y = 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 α-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 mustard forms chloroacetdehyde, which has been reported to cross-link DNA (Spengler and Singer, 1988), induce DNA breaks, and inhibit DNA synthesis (Brüggemann et al., 2006). Chloroacetdehyde 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 alkylating 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). Inmate 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-104H in humans. The reduced metabolites were detected at very low concentrations in dog plasma (PR-104H > PR-104M, Cmax ~0.14 and 0.1 μM, 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-β-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
species difference 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 toxicity of PR-104.

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References