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

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Running title: Metabolism of PR-104 across species

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ABBREVIATIONS: PR-104A, 2-((2-Bromoethyl)-2-[(2-hydroxyethyl) amino] carbonyl)-4,6-dinitroanilino)ethyl methanesulfonate; CYP, Cytochrome P450s; AKR, aldo-keto reductase; PK, pharmacokinetic; AUC, area under the concentration-time curve; SPF, specific pathogen-free; IV, intravenous; CPM, counts per minute; PBS, phosphate buffered saline; i-Pr₂O, isopropyl ether; THF, Tetrahydrofuran; HRMS, High Resolution Mass Spectrometry; NMR, Nuclear Magnetic Resonance spectroscopy; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; GSH, Glutathione; CySH, Cysteine; NAC, N-acetylcysteine; Gluc, Glucuronide; OMs, Mesylate; S9, 9000g postmitochondrial supernatant.
ABSTRACT

PR-104 is the phosphate ester of a 3,5-dinitrobenzamide nitrogen mustard (PR-104A) which is reduced to active hydroxylamine and amine metabolites by reductases in tumors. Here we evaluate the excretion of [\(^{3}\)H]PR-104 in mice, and determine its metabolite profile in mice, rats, dogs and humans, following 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 modelling human biotransformation and toxicology of PR-104.
Introduction

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 which 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 which 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 crosslinking (Patterson et al., 2007; Singleton et al., 2009; Gu 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., 2009), 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 suggest that reductive activation of PR-104A may occur in normal tissues as well as 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; 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 sidechain by UDP glucuronosyltransferases. Indeed, preliminary evidence for an N-dealkylated half-mustard, a cysteine conjugate and an O-glucuronide of PR-104A have 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 following intravenous administration. In addition, the mass balance for excretion of radiolabelled 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 non-human 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), the tetra-deuterated stable isotope internal standards of PR-104 (PR-104-d4) and PR-104A (PR-104A-d4), and PR-104 tritiated in the carboxamide sidechain ([3H]PR-104; specific activity, 28.5 GBq/mmol) (Atwell and Denny, 2007) were synthesized as previously described. Tetradeca
terated internal standards of PR-104H (PR-104H-d4), and PR-104M (PR-104M-d4) were prepared using the same methods as the non-labelled compounds. All compounds had a purity of at least 95% by HPLC. PR-104H and PR-104M (including d4 internal standards) were stored in acetonitrile as stock solutions at -80°C. [3H]PR-104 had a chemical purity of 99% and radiochemical purity of 94.4%.

Synthesis and Characterization of glucuronide PR-104G (M1)

Intermediate 2. A solution of PR-104A (1.00g, 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-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate (1; Fig. 1A) (1.27 g, 3.20 mmol) (Sigma-Aldrich) and silver triflate (1.03 g, 4.01 mmol) (Sigma-Aldrich). The mixture was stirred at room temperature for 16 h, then filtered through a celite pad that was washed with CH2Cl2. The filtrate was washed with water and dried, then concentrated under reduced pressure and the residue was chromatographed on silica gel. Elution with EtOAc/ pet. ether (3:2) gave the O-acetyl derivative of PR-104A (21%, identified by MS and NMR). Further elution with EtOAc/ pet. ether (2:1) gave a product that was precipitated from a CH2Cl2 solution with i-Pr2O (2×) to give (2R,3S,4S,5S,6S)-2-[2-[2-[(2-bromoethyl)]2-(methylsulfonyloxy)ethyl]amino]-3,5-dinitrobenzamido]-3,5-dinitrobenzamido]ethoxy]-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triy triacetate (2) (670 mg, 41%): mp 72-75°C; 1H NMR [(CD3)2SO] δ 8.80-8.72 (m, 2H), 8.31 (d, J= 2.8 Hz, 1H), 5.35 (t, J= 9.6 Hz, 1H),
4.99 (t, J = 9.8 Hz, 1H), 4.95 (d, J = 8.0 Hz, 1H), 4.85 (dd, J = 9.5, 8.0 Hz, 1H), 4.48 (d, J = 9.9 Hz, 1H), 4.28 (t, J = 5.4 Hz, 2H), 3.92-3.85 (m, 1H), 3.74-3.67 (m, 1H), 3.64-3.54 (m, 5H), 3.50-3.40 (m, 6H), 3.13 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H). 13C NMR [(CD3)2SO] δ 169.3, 169.1, 168.9, 167.2, 165.4, 145.8, 145.2, 140.9, 135.8, 127.5, 122.3, 99.4, 71.2, 70.9, 70.6, 69.1, 67.5, 67.4, 54.2, 52.4, 51.1, 39.3, 36.4, 29.7, 20.2, 20.1 (2). HRMS (FAB+) calcd for C27H36BrN4O18S (MH+) m/z 815.0929, found 815.0932; calculated for C27H36BrN4O18S (MH+) m/z 817.0909, found 817.0910.

PR-104G (M1). An ice-cold solution of 0.05 M LiOH in MeOH/water/THF (3:1:1) (29.4 ml, 1.47 mmol) was added to 2 (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 below 30°C. Addition of a limited amount of i-Pr2O precipitated impurities which were removed by filtration, and then further addition of i-Pr2O gave (2S,3S,4S,5S,6R)-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 88-92°C; 1H NMR [(CD3)2SO] δ 12.68 (br, s, 1H), 8.74 (d, J = 2.8 Hz, 1H), 8.71 (t, J = 5.6 Hz, 1H), 8.34 (d, J = 2.8 Hz, 1H), 5.20 (br s, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.03 (d, J = 4.4 Hz, 1H), 4.32 (d, J = 7.80 Hz, 1H), 4.28 (t, J = 5.4 Hz, 2H), 3.92-3.84 (m, 1H), 3.70-3.55 (m, 4H), 3.54-3.42 (m, 6H), 3.36-3.29 (m, 1H), 3.23-3.16 (m, 1H), 3.12 (s, 3H), 3.08-3.01 (m, 1H). 13C NMR [(CD3)2SO] δ 170.2, 165.3, 145.8, 145.2, 140.9, 136.0, 127.5, 122.2, 103.0, 75.8, 75.5, 73.0, 71.3, 67.4, 67.1, 54.2, 51.1, 39.4, 36.5, 29.7. HRMS (FAB+) calculated for C20H28BrN4O15S (MH+) m/z 675.0435, found 675.0449; calculated C20H28BrN4O15S (MH+) m/z 677.0435, found 677.0434. HPLC purity, 96% at 330 nm.

Synthesis and Characterization of semi-mustards 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) and stirred at room temperature for 1.5 h. The mixture was
evaporated and the residue purified by chromatography on silica gel, and elution with EtOAc,
followed by recrystallization from EtOAc/i-Pr2O gave 2-(aziridin-1-yl)-N-(2-hydroxyethyl)3,5-
dinitrobenzamide (4) (559 mg, 55%); mp 181-182°C; 1H NMR [(CD3)2SO] δ 8.74 (d, J = 2.7
Hz, 1H), 8.61 (t, J = 5.4 Hz, 1H), 8.35 (d, J = 2.7 Hz, 1H), 4.77 (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
C11H12N4O6: C, 44.60; H, 4.08; N, 18.91. 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 diluted with saturated NaCl (100 ml) then extracted with
EtOAc, and 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)amino]carbonyl]-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 (t, 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
C12H16N4O9S: 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 (2×20 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-[(2-
bromoethyl)amino]-N-(2-hydroxyethyl)-3,5-dinitrobenzamide (M10) (177 mg, 93%): mp 122-123°C; 1H 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 = 5.9 Hz, 1H), 3.75 (t, J = 5.9 Hz, 2H), 3.63 (q, 5.8
Hz, 2H), 3.56 (q, 5.8 Hz, 2H), 3.34 (q, J = 5.8 Hz, 2H). Elemental analysis. Calculated for
C11H13BrN4O6: 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 (CD1-Foxn1<sup>nu</sup>) mice and Sprague Dawley rats (Charles River Laboratories, Margate, Kent, UK) were bred in the University of Auckland. Mice were housed in Tecniplast microisolator cages in groups of 4-6 in a temperature-controlled room (20 ± 2°C) with a 12-hour light/dark cycle and were fed <i>ad libitum</i> UV-treated Milli-RO water and a sterilized rodent diet (Harlan Teklad diet 2018s). Rats were housed in groups of 4-6 under the same conditions but received filtered tap water and diet 2018. At the time of experiments animals weighed 25-30 g (mice) and 200-220 g (rats). The rodent studies were approved by the University of Auckland Animal Ethics Committee. Beagle dogs (7 - 8 month, 10-13kg) were from Kangda Laboratory Animals S & T Co., Ltd. (Gaoyao, China). The dog study was conducted at LAB Pre-Clinical Research International Inc. (Laval, Quebec, Canada) and approved by the Institutional Animal Care and Use Committee. The dogs were individually housed in a room maintained at 21 ± 3°C and fed Teklad Certified Canine Diet (#8727C) with water <i>ad libitum</i>. A 12-hour light/dark cycle was maintained. Human subjects, from two phase I trials, were patients with a pathologically confirmed solid malignancy not amenable to standard therapy, age ≥ 18 yrs, Karnovsky performance status ≥ 70%, adequate renal and liver function, and >4 weeks since prior surgery, radiotherapy or chemotherapy.

Dosing and Sample Collection

PR-104 free acid was dissolved in PBS + 1 equivalent NaHCO<sub>3</sub> and diluted in PBS (rodents and dogs) or the clinical formulation (PR-104 sodium salt lyophilized with mannitol) was reconstituted in 2 ml water and diluted in 5% dextrose (humans). Dosing was by the intravenous route in all cases. The [<sup>3</sup>H]PR-104 (free acid) was similarly titrated with NaHCO<sub>3</sub> and diluted to a specific activity of 2.85 GBq/mmol (or 77.0 mCi/mmol) with unlabelled PR-104. Mice were dosed at 326 mg/kg (975 mg/m<sup>2</sup>) via a lateral tail vein and sampled by cardiac puncture after cervical dislocation. Gallbladders were collected from 4 mice 30 min after dosing at 326 mg/kg. Rats were dosed at 244 mg/kg (1450 mg/m<sup>2</sup>) via a tail vein and
bleed serially from the saphenous veins for up to 3 hr. Urine was collected at intervals of 1 hr for up to 3 hr from 4 rats. Dogs were dosed into the cephalic or saphenous vein as a slow bolus injection at 150 mg/kg (3000 mg/m²) and blood samples were collected from the jugular vein for up to 2 hr. Patients were dosed as a 1 hr infusion, and plasma and urine samples collected following the first dose of a once every three week schedule. Urine samples were collected over 24 hr after a range of doses (135 to 1400 mg/m²) at a single clinical site (Hamilton, NZ), and blood samples were collected over 5 hr (from start of infusion) from 6 patients at the maximum tolerated dose (1100 mg/m²) (Jameson et al., 2009). In addition, blood was collected from 4 patients at the same dose level in a second phase I study following the first dose of a weekly dosing schedule. All blood samples were collected into tubes containing K₂EDTA. These samples were placed on ice immediately after collection and centrifuged within 10 min to harvest plasma.

**Excretion of Radiolabelled PR-104 in Mice**

Mice were transferred to glass metabolic cages (Minor Metabowl, Jencons UK; one animal per cage) 2 hr before treatment and had continuous access to food and water before and during experiments. [³H]PR-104 was administered i.v. via a lateral tail vein at 326 mg/kg. Urine was collected at 4, 8, 24 and 48 hrs. Metabolic cages were rinsed with 2 ml water into the urine sample to minimize carry-over between timepoints. Feces were collected at 24 and 48 hr.

**Determination of Total Radioactivity and Radioactivity Profiles**

Radioactivity was determined using a liquid scintillation analyzer (Tri-Carb® 1500, Packard), 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 cocktail (Emulsifier-Safe™, PerkinElmer, USA). Feces were dried, powdered and rehydrated by addition of deionized water. 1 ml of Soluene-350 (PerkinElmer, USA) was added and
incubated at 37°C overnight, 0.5 ml of isopropyl alcohol was added and samples were incubated for 12 hr at 37°C. 0.2 ml of 30% H₂O₂ was added dropwise 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. 10-15 ml of scintillation fluid (Hionic-Fluor, PerkinElmer, USA) 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, USA), 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 (PDA) absorbance and MS detection (Agilent 1100 LC/MSD model A, Agilent Technologies, USA). Mouse bile samples (gallbladder) were precipitated with approximately 10 vol of methanol, centrifuged and diluted into formate buffer. The chromatographic separation was performed on an Alltima C8 analytical column (150 x 4.6 mm, 5 µ; Alltech Associates Inc., Chicago, 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 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/MS/MS (Model 6410) equipped with ESI-APCI multi-mode source was used for further identification of some products in excretion samples.

Mouse and rat plasma samples were prepared by precipitating proteins with 3 vol methanol, and for dog and human plasma with 9 vol acidified methanol (methanol: ammonium acetate: acetic acid, 1000:3.5:0.2, v/w/v) as described (Patel et al., 2007). Precipitated samples were diluted into 2 vol of purified water and analyzed by the LC/MS method reported previously.
Briefly, a 150 x 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-SL ion trap mass spectrometer equipped with an Agilent capillary HPLC system was used for the further identification of some metabolites, using a Zorbax SB C18 capillary column (150 x 0.5 mm, 5 µ, Agilent Technologies) at a flow rate of 15 µl/ml (Patel et al., 2007). The electrospray ionization source was set at positive ionization mode with auto MS(n).

Hepatic S9 metabolism of PR-104A

In vitro hepatic metabolism of PR-104A was studied using liver S9 fraction prepared from pooled CD-1 nu/nu mice in-house and pooled human liver S9 purchased from CellzDirect Inc. Reactions (final volume 0.1 ml/well in 96 well plates) comprised hepatic S9 (2 mg of protein/ml), PR-104A (150 µM), 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 were incubated for 30 min at 37°C under air or in an anaerobic chamber (Sheldon Manufacturing Inc.). Incubations with boiled S9 preparations were used as controls. All solutions for the anoxic experiment were equilibrated (along with the 96-well plates) in the chamber for at least 3 d to remove oxygen, and S9 was pre-gassed 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 12,000g 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). Briefly, 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 UHPLC–MS/MS using a Zorbax Eclipse XDB-C18 Rapid Resolution HT (50×2.1mm, 1.8 µ) 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 i.v. dose of [3H]PR104, radioactivity was rapidly excreted in urine with 90% of the urinary excretion occurring within 4 hr. Total radioactivity in excreta (urine plus feces) by 24 hr after dosing accounted for 94.0 ± 1.6% of the administered dose, rising to 96 ± 0.8% by 48 hr (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) which failed to separate M2 from PR-104. This made it possible to quantify 8 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-PDA at 370 nm to estimate the contributions of PR-104 and M1 to the radioactivity peak. The major urinary metabolites were the “semi-mustard” product PR-104S1 (M8) resulting from oxidative N-dealkylation of the bromoethyl moiety of PR-104A, and its corresponding cysteiny (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 metabolites in urine include glutathionyl (M16; 1.2% of total dose), cysteiny1 (M17; 7.0%) and N-acetylcysteiny1 (M18; 1.3%) conjugates related to M14, an intra-molecular 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-acetylcysteiny1 adduct (M5) derived from M2 and semi mustard 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 1-2 h window, and shown for other times in supplement Fig. S2. The profile was broadly similar to mice although the cysteiny1 adducts M12 and M3 were not detected. The N-acetylcysteiny1 adduct (M5), a downstream product of M3, was present in the earlier (0-1 h) urine (supplement Fig. S2A). In addition, a metabolite not observed in mice, M21, became more prominent between 2-3 h post-dose (supplement Fig. S2B).

Humans. After administration of PR-104 (135 mg/m^2) 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 cysteiny1 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 demonstrated 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. Surprisingly, PR-104 itself was not detected in urine. A more sensitive LC/MS/MS method for PR-104 (Patel et al., 2007) was therefore used to evaluate PR-104 in the same samples. PR-104 was below the limit of quantitation of 10 nM in 2 of 4 patients dosed at 135 mg/m^2, and in the
patient 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 and dog and human) at early times after i.v. administration of PR-104 are shown in Fig. 4. A full catalogue of plasma metabolites in the four species is provided in Table 1. The hydrolysis product, PR-104A, was a major metabolite in all species. Glucuronide PR-104G (M1) was the main metabolite in both dogs and humans. The cysteinyI conjugate M8 was observed in all species along with its presumed precursor glutathionyl conjugate (M2) in mice, rats and dogs and its downstream N-acetylcysteinyI conjugate in mice and rats only. Both semi mustards 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 while hydroxylamine PR-104H was the major reduced metabolite in humans (Gu and Wilson, 2009). Rats also showed PR-104M >> PR-104H, while 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 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 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 (λ_max) at 370 nm, as for PR-104 itself,
and had a strictly symmetric spectral shape. The semi-mustard metabolites resulting from N-dealkylation of one arm of the mustard all showed a spectral shift to $\lambda_{\text{max}} \sim 360$ nm and a broad shoulder at $\sim 420$ nm. Products of intramolecular alkylation showed similar spectra to the semi-mustards, but with a less pronounced shoulder, while the reduction products showed a major spectral changes consistent with electronic perturbation of the chromophore. Spectral features are summarized in Table 1 with supporting spectra in supplemental data. 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, 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 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 (supplement data). To confirm the identity of M1, the $\text{O-}\beta$-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, $^{13}$C NMR and high resolution mass spectroscopy (see 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 not given) showed a doublet for the anomic proton at $\delta = 5.94$ ppm with $J = 4.2$ Hz. In addition, *E. 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-β-glucuronide on the hydroxyethyl side chain of PR-104A.

Metabolites M2 - 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 (-OSO2Me), giving prominent MS² 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) were 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 semi mustard 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 due to displacement of the Br leaving group of PR-104A. In addition, neutral loss of 176 resulting in fragment ion of 540 demonstrated the presence of a glucuronol moiety, demonstrating 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, NAC- 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 - M13. M8 and M10 exhibited distinctive UV spectra (λ_max 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 a 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 semi mustards respectively. M9 was identified as the O-glucuronide of PR-104S1 (M8) based on its similar UV spectrum to the semi-mustards 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 ($\lambda_{\text{max}}$ 360 nm) similar to M8 and M10, and had molecular ions of m/z 604, 418 and 460, consistent with the glutathionyl, cysteinyl and N-acetyl-cysteinyl 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 mecapto- positions.

**Metabolite M14 - 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 ($\lambda_{\text{max}}$ 365 nm), with a fragmentation pattern in the MS$^2$ spectrum consistent with the proposed structure (Fig. S1P). M16, M17 and M18 all showed a distinctive UV spectrum with $\lambda_{\text{max}}$=345 nm. M16 gave a molecular ion of 630 and fragment ion of 274 and 145, corresponding to cleavage at the S-CH$_2$ 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-CH$_2$ 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 was identified as a carboxylic acid derivative resulting from oxidation of the hydroxyethyl sidechain 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) 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 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 hydroxyethyl side chain (PR-104G, M1). A glucuronide of the semi-mustard 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 cysteiny1 (M3, M7) and N-acetylcysteiny1 adducts (M5) via the mercapturic acid pathway. Nucleophilic displacement 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 semi-mustards M8 and M10, which in turn give rise to the corresponding thiol
adducts (M11, M12, M13) and O-glucuronide (M9). (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 mg/m² and 1100 mg/m² respectively) are shown in Fig. 6. Estimation of non-compartmental pharmacokinetic parameters showed terminal half lives of the all metabolites of ~ 20 min in mice, similar to PR-104A itself (Table 2). The plasma 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 semi-mustard PR-104S1 was present at much lower levels. The plasma AUC of PR-104H in humans was ~ 5% of that for PR-104A while 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 requirement for O₂ as a co-substrate for the mixed function oxidase reaction. In turn, these results suggest that the oxygen concentration in the anaerobic chamber reactions
were well below the $K_m$ for $O_2$ for CYPs ($\sim 5 \mu M$; 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 5-6-fold higher than PR-104M but in murine S9 PR-104M concentrations were higher than PR-104H, broadly consistent with the metabolite profiles seen in plasma. The cofactor dependence for the \textit{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 non-clinical species (mice, rats and dogs) utilized during pre-clinical development of this bioreductive prodrug. It confirms the previously reported extensive and rapid hydrolysis of the phosphate “pre-prodrug” to PR-104A in rodents (Patterson et al., 2007; Patel et al., 2007) and humans 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 demonstrated in plasma of mice 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 $[^3]$H]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 semi-mustard metabolites are prominent in rodents. This route appears to be a minor in humans given that it is represented only by low concentrations of PR-104S1, although this differences could also reflect faster clearance of the N-dealkylation products in humans. Of the two semi-mustard 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 ethylmesylate arm of the mustard or faster clearance of the PR-104S2 metabolite. An in vitro metabolism study of PR-104A analogues showed that kinetics of dealkylation of the diethylamino group \( N(Et-X)(Et-Y) \) decreased the order \( X=Y=H > X=Y=Br > X= Br \) or Cl, \( Y = OMs \) (Helsby et al., 2008) which suggests that the mesylate leaving group suppresses dealkylation. In the latter study neither of the semi-mustard metabolites were detected from PR-104A, but it suggests that the bromoethyl moiety may be selectively dealkylated. The enzymology and mechanism of this dealkylation is undetermined. In addition to the classical CYP-mediated \( \alpha \)-hydroxylation of tertiary amines, oxidation to the N-oxide of aromatic nitrogen mustards can lead to dealkylation (Tercel et al., 1995). While the semi-mustard products of dealkylation are not themselves able to crosslink 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 crosslink DNA (Spengler and Singer, 1988), induce DNA breaks and inhibit DNA synthesis (Bruggemann et al., 2006). Chloroacetaldehyde is a potent cytotoxin in vitro (Bruggemann et al., 1997), demonstrates antitumor activity in vivo (Borner et al., 2000), is neurotoxic (Goren et al., 1986; Lewis and Meanwell, 1990), 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 may therefore 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 semi-mustard, and the subsequent dealkylation (M15) and thiol conjugation products (M16-M18) lack alkylating potential and are thus not expected to be of toxicological significance. Similarly, 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 species including humans, especially a conjugate of PR-104A in which cysteine has displaced the bromine. The cysteiny1 and N-acetylcy steinyl 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 semi-mustards and intermolecular cyclization products in rodents as demonstrated 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 (Dirven et al., 1996) (Colvin et al., 1993). The abundant metabolites resulting from GSH conjugation in all species suggest that PR-104 will not be immune from this resistance mechanism, although local release of the active metabolites in tumors has the potentially to overwhelm local detoxification pathways.

The biodistribution of the nitroreduction products of PR-104A in mice has been reported recently (Gu and Wilson, 2009). With amine PR-104M (M23) at higher concentrations than hydroxylamine PR-104H (M22) in plasma and all 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. The total circulating reduced metabolites, normalized for PR-104A, is about 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} \sim 0.14 and 0.1 \mu M respectively), although these samples were stored for longer periods than human and rodent samples before analysis so 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 semi-mustard (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 SN-38 (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 GSH conjugation is prominent in all species, glucuronidation is essentially restricted to humans and dogs while \(N\)-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


Footnotes

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Numbered footnotes:


Disclosure Statement:

Yongchuan Gu and Graham J. Atwell have no conflicts of interest to disclose. William R. Wilson is a founding scientist, stockholder and consultant to Proacta, Inc.
Figure Legends

Fig. 1. Synthetic schemes for reference standards. (A) The β-glucuronide of PR-104A, PR-104G (M1); (B). The semi-mustard metabolites of PR-104A (M2 and M3).

Fig. 2. Excretion of PR-104 in mice. (A) Total radioactivity in urine and feces after an i.v. dose of [3H]PR-104 (326 mg/kg). Values are mean ± SEM for 3 mice. (B) A representative radioactivity (dashed line) and absorbance (370 nm, solid line) HPLC profile for urine collected in the same experiment (0-4 hr fraction). (C) Cumulative excretion of metabolites in urine (0-8hr) from radioactivity determinations, as % of injected dose. (D) Metabolites in bile 0.5 h post-dose (326 mg/kg, pooled gallbladder samples from 4 mice).

Fig. 3. Excretion of PR-104 and metabolites in rat and human urine. (A) Representative chromatogram of rat urine, 1-2 h after an i.v. dose of 244 mg/kg. (B) Representative chromatogram of human urine (90 min post infusion, dose 135 mg/m²). (C) Metabolite profiles in human urine (135 mg/m², 4 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 (R²=0.90) and PR-104G (R²=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.

Fig. 5 Proposed metabolic pathways of PR-104 in mice, rats, dogs, and humans. GS = glutathione, CyS = cysteine, NAC = N-acetylcysteine, Gluc = glucuronic acid

Fig. 6 Plasma concentration-time profiles of PR-104 metabolites. (A) mice dosed at 975 mg/m² (326 mg/kg), mean ± SEM for 3 mice; and (B) humans dosed at 1100 mg/m², mean ± SEM for 10 subjects.
**Tables**

Table 1: Metabolites identified in mouse, rat, dog and human samples collected after administration of a single iv dose of PR-104. 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.

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<td>Oxidative N-dealkylation / CySH conjugate</td>
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<td></td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M13</td>
<td></td>
<td>360</td>
<td>460</td>
<td>-</td>
<td>Oxidative N-dealkylation / NAC conjugation</td>
<td>-/+</td>
<td>-/+</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M14</td>
<td></td>
<td>380</td>
<td>403</td>
<td>Br</td>
<td>Intramolecular alkylation</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M15</td>
<td></td>
<td>365</td>
<td>297</td>
<td>-</td>
<td>Intramolecular alkylation / Oxidative N-dealkylation</td>
<td>-/+</td>
<td>-/+</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M16</td>
<td></td>
<td>345</td>
<td>630</td>
<td>-</td>
<td>Intramolecular alkylation / GSH conjugate</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M17</td>
<td></td>
<td>345</td>
<td>444</td>
<td>-</td>
<td>Intramolecular alkylation / CySH conjugate</td>
<td>-/+</td>
<td>-/+</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M18</td>
<td></td>
<td>345</td>
<td>486</td>
<td>-</td>
<td>Intramolecular alkylation / NAC conjugation</td>
<td>-/+</td>
<td>-/+</td>
<td></td>
<td>-/+</td>
</tr>
<tr>
<td>M19</td>
<td></td>
<td>380</td>
<td>421</td>
<td>Br</td>
<td>Hydrolysis</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M20</td>
<td></td>
<td>370</td>
<td>455</td>
<td>Cl</td>
<td>Nucleophilic displacement</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>-/-</td>
</tr>
<tr>
<td>M21</td>
<td></td>
<td>370</td>
<td>373</td>
<td>-</td>
<td>Oxidation/hydrolysis</td>
<td>-/-</td>
<td>-/+</td>
<td></td>
<td>-/+</td>
</tr>
<tr>
<td>M22</td>
<td>PR-104H</td>
<td>240</td>
<td>485</td>
<td>Br</td>
<td>Nitroreduction</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>+++/++</td>
</tr>
<tr>
<td>M23</td>
<td>PR-104M</td>
<td>240</td>
<td>469</td>
<td>Br</td>
<td>Nitroreduction</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td>+++/++</td>
</tr>
</tbody>
</table>

† maximal UV absorbance  * plasma/urine  ‡ plasma only
Table 2 Pharmacokinetic parameters of PR-104 metabolites in mouse and human plasma after i.v. administration of 975 mg/m² (bolus) and 1100 mg/m² (60 min infusion) respectively

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse</th>
<th>Human †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC₀-∞ (µmol h L⁻¹)</td>
<td>t₁/₂ (min)</td>
</tr>
<tr>
<td>PR-104</td>
<td>80.7</td>
<td>3.4</td>
</tr>
<tr>
<td>PR-104A</td>
<td>81.6</td>
<td>16.8</td>
</tr>
<tr>
<td>PR-104H (M22)</td>
<td>3.0</td>
<td>23.9</td>
</tr>
<tr>
<td>PR-104M (M23)</td>
<td>10.2</td>
<td>20.5</td>
</tr>
<tr>
<td>PR-104S1 (M8)</td>
<td>19.3</td>
<td>24.7</td>
</tr>
<tr>
<td>PR-104G (M1)</td>
<td>2.7</td>
<td>13.1</td>
</tr>
</tbody>
</table>

† 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.
Table 3. *In vitro* hepatic S9 metabolism of PR-104A. Values are mean ± SEM for triplicate samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxygen</th>
<th>Cofactor</th>
<th>PR-104H (pmol/mg/min)</th>
<th>PR-104M (pmol/mg/min)</th>
<th>PR-104S1 (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Oxic</td>
<td>NADH</td>
<td>1.61 ± 0.06</td>
<td>ND†</td>
<td>2.28 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NADPH</td>
<td>4.85 ± 0.35</td>
<td>0.415 ± 0.037</td>
<td>12.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>4.10 ± 0.05</td>
<td>0.616 ± 0.016</td>
<td>15.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Anoxic</td>
<td>NADH</td>
<td>21.8 ± 0.8</td>
<td>26.1 ± 1.3</td>
<td>0.218 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NADPH</td>
<td>110 ± 15</td>
<td>142 ± 24</td>
<td>0.207 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>102 ± 5</td>
<td>154 ± 8</td>
<td>0.208 ± 0.008</td>
</tr>
<tr>
<td>Human</td>
<td>Oxic</td>
<td>NADH</td>
<td>2.99 ± 0.05</td>
<td>0.086 ± 0.013</td>
<td>8.17 ± 0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NADPH</td>
<td>6.16 ± 0.12</td>
<td>0.922 ± 0.107</td>
<td>35.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>6.15 ± 0.14</td>
<td>1.07 ± 0.12</td>
<td>37.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Anoxic</td>
<td>NADH</td>
<td>53.9 ± 1.4</td>
<td>11.2 ± 0.8</td>
<td>0.257 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NADPH</td>
<td>284 ± 22</td>
<td>52.1 ± 0.9</td>
<td>0.257 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>282 ± 27</td>
<td>54.8 ± 5.6</td>
<td>0.247 ± 0.004</td>
</tr>
</tbody>
</table>

† Not detectable
Fig 1

A

\[
\begin{align*}
\text{PR-104A} & \quad \text{MeOOC} \quad \text{AcO} \quad \text{AcO} \\
\text{1} & \quad \text{OSO}_2\text{Me} \\
\text{2} & \quad \text{LiOH} \\
\text{M1 (PR-104G)} & \\
\end{align*}
\]

B

\[
\begin{align*}
\text{3} & \quad \text{aqueous HX} \\
\text{4} & \quad \text{X} = \text{OSO}_2\text{Me} \\
\text{M8 (PR-104S1)} & \\
\text{M10 (PR-104S2)} & \quad \text{X} = \text{Br}
\end{align*}
\]
Fig 2

A. Cumulative excretion (%) over different time periods:
- 0-4hr
- 0-8hr
- 0-24hr
- 0-48hr

B. HPLC separations for UV@370nm and Radioactivity:
- Peaks labeled M11, M12, M13, M14, M15, PR-104, and others.

C. Pie chart showing distribution of metabolites:
- M8: (4.9±0.4)%
- M13: (2.7±0.3)%
- M12: (6.0±0.4)%
- M16: (1.2±0.1)%
- M17: (7.0±0.2)%
- M18: (1.3±0.1)%
- M19: (0.7±0.1)%
- PR-104: (4.6±0.2)%
- PR-104A: (4.6±0.2)%
- M3: (6.1±0.5)%
- M1: (0.8±0.1)%
- Others: (2.9±0.4)%

D. HPLC separations showing peaks at different times:
- Peaks labeled M1, M2, M5, M6, M8, PR-104, and PR-104A.
Fig 3

A

mAU

0 5 10 15 20 min

M17 M18

M13

M8

PR-104A

M19 M20

M21

B

mAU

0 5 10 15 20 min

M1

M3

M17 M18

M12

M8

PR-104A

C

PR104A

(1.0±0.2)%

M3(3.0±0.7)%

M1

(11.8±2.0)%

Others*

(7.5±1.5)%

D

PR-104A,G (nmol)

0 100 200 300 400 500 600 700 800

0 200 400 600 800

Dose level (mg/m²)

0 200 400 600 800 1000 1200 1400 1600

PR-104A

PR-104G

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Fig 4
Fig 5

((M1) PR-104G)  

Glucuronidation  

Thiol conjugation  

Reduction  

N-dealkylation  

Cyclization  

Carboxyl diol  

(M2) X=GS, Y=OSO₂Me  

(M3) X=CyS, Y=OSO₂Me  

(M4)  

(M5) X=NAC, Y=OSO₂Me  

(M6) X=Br, Y=GS  

(M7) X=Br, Y=CyS  

Thiol adducts  

(M8) X=OSO₂Me  

(M9)  

(M10) X=Br  

(M11) X=GS  

(M12) X=CyS  

(M13) X=NAC  

(M14) X=EtBr  

(M15) X=H  

(M16) X=EtGS  

(M17) X=EtCyS  

(M18) X=EtNAC
Fig 6

A

B

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Supplement Fig. S1: Absorbance and mass spectra of PR-104 metabolites

A. PR-104A

Intens. 33.

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

Drug Metab Dispos

Yongchuan Gu, Graham J. Atwell and William R. Wilson
B. PR-104G (M1)
C. M2

\[ \text{+MS2 (726.0), 4.7-6.6 min} \]

\[ \text{[M+H-75]^+} \]

\[ \text{[M+H-129]^+} \]

\[ \text{[M+H-96]^+} \]
D. M3

[Chemical structures and mass spectra images are present, showing peaks and molecular formulas.]

[Graphs and molecular structures are depicted, including mass spectrometry data at specific m/z values.]
E. M4

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)
F. M5
I. PR-104S1 (M8)
J. M9

![Graphs and diagrams showing mass spectra and molecular structures](image-url)
K. PR-104S2 (M10)
L. M11

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)
M. M12

Counts vs. Mass-to-Charge (m/z)

Counts vs. Mass-to-Charge (m/z)
N. M13

Counts vs. Mass-to-Charge (m/z)
O. M14
P. M15

Counts vs. Mass-to-Charge (m/z)

+MS2 (297.0)

\[ [\text{M}+\text{H-HNO}_2]^+ \]
\[ [\text{M}+\text{H-C}_2\text{H}_6\text{NO}_3]^+ \]
\[ [\text{M}+\text{H-C}_2\text{H}_8\text{O}_2]^+ \]
Q. M16
R. M17

+ Product Ion (5.14 min) (444.0 -> **) mouse urine.

Counts vs. Mass-to-Charge (m/z)

+MS2 (444.0)

120.0

250.2

329.2

357.2

[M+H-148]^+

[M+H-87]^+

N

O

NO2

O2N

NH2

HO

S

R. M17
S. M18

+ Product Ion (6.35 min) (486.0 -> **) mouse urine

Counts vs. Mass-to-Charge (m/z)

+MS2 (486.0)
V. M21

[Graphs and diagrams showing mass spectrometry data and molecular structures.]
Fig. S2: Representative chromatograms of rat urine collected 0-1 h (A) or 2-3 h (B) after dosing with PR-104 at 244 mg/kg IV.