Glucuronidation of Anticancer Prodrug PR-104A: Species Differences, Identification of Human UDP-glucuronosyltransferases and Implications for Therapy

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Abbreviations: PR-104A, 2-((2-bromoethyl)-2-[(2-hydroxyethyl) carbonyl]-4,6-dinitroanilino) ethyl methanesulfonate; AKR, aldo-keto reductase; AUC, area under the concentration-time curve; DLM, dog liver microsomes; D-SL, D-saccharic acid 1,4-lactone; HLM, human liver microsomes; IV, intravenous; M3G, morphine 3-O-glucuronide; MLM, mouse liver microsomes; MS/MS, tandem mass spectrometry; PK, pharmacokinetic; RLM, rat liver microsomes; S9, 9000g postmitochondrial supernatant; UDPGA, uridine 5′-diphosphoglucuronic acid; UHPLC, ultra high-performance liquid chromatography; UGT, UDP-glucuronosyltransferase.
ABSTRACT

PR-104, the phosphate ester of 3,5-dinitrobenzamide-2-mustard PR-104A, is currently in clinical trial as a hypoxia- and AKR1C3-activated prodrug for cancer therapy. Here, we investigate species (human, dog, rat, mouse) differences in metabolism to the corresponding O-glucuronide, PR-104G and identify the human UDP-glucuronosyltransferase (UGT) isoforms responsible. After intravenous PR-104, plasma AUC ratios (PR-104G/PR-104A) decreased in the order dog (2.3) > human (1.3) > mouse (0.03) > rat (0.005). The kinetics of UDPGA-dependent glucuronidation by liver microsomes in vitro fitted the single-enzyme Michaelis-Menten equation with similar $K_m$ (~ 150 µM) but differing $V_{\text{max}}$ (472, 88, 37 and 14 nmol/hr/mg for dog, human, rat and mouse respectively), suggesting that facile glucuronidation is responsible for the anomalously rapid clearance of PR-104A in dogs. In vitro-in vivo extrapolation of PR-104A glucuronidation kinetics is consistent with this being a major clearance pathway in humans. rUGT screening identified UGT2B7 as the only commercially available human isoform able to conjugate PR-104A, and UGT2B7 protein concentrations were highly correlated ($R=0.93$) with PR-104A glucuronidation by liver microsomes from 24 individuals. The active hydroxylamine metabolite of PR-104A, PR-104H, was also glucuronidated by UGT2B7, although with slightly lower specificity and much lower rates. UGT2B7 mRNA expression was highly variable in human tumor databases. Glucuronidation of PR-104A greatly suppressed nitroreduction by AKR1C3 and NADPH-supplemented anoxic human liver S9. In conclusion, PR-104A is glucuronidated by UGT2B7 with high specificity and appears to make a major contribution to clearance of PR-104A in humans, but also has the potential to confer resistance in some human tumors.
Introduction

Glucuronidation, catalyzed by microsomal uridine diphosphate (UDP)-glucuronosyltransferases (UGTs, EC 2.4.1.17), is a major clearance mechanism for drugs from all therapeutic classes (Court, 2004). Characterizing glucuronidation of new drugs is important because of inter-patient variability in UGT genotype and expression (Court, 2010), and because the resulting glucuronides may in some cases have increased biological activity (Ritter, 2000) or lead to altered pharmacokinetics and tissue distribution of the aglycone via the concerted action of glucuronide transporters and β-glucuronidases (Prijovich et al., 2009). In addition, drug-drug interactions due to UGT inhibition is of therapeutic concern (Williams et al., 2004), and overexpression of UGTs in tumors represents a potential mechanism of resistance to anticancer agents that are UGT substrates (Cummings et al., 2003; De Almagro et al., 2011).

Here we evaluate the role of glucuronidation in the biotransformation and elimination of a 3,5-dinitrobenzamide-2-mustard prodrug, PR-104A (Patterson et al., 2007; Gu et al., 2010), designed to target hypoxic regions of tumors. Hypoxia is a ubiquitous feature of tumors and a negative prognostic indicator in many contexts (Brown and Wilson, 2004; Hill et al., 2009). For these reasons tumor hypoxia is an attractive therapeutic target, and can be exploited by bioreductive prodrugs such as PR-104A that are metabolized to cytotoxins by pathways that are inhibited by oxygen. In the case of PR-104A, reduction of the 5-nitro group to the corresponding 5-hydroxylamine (PR-104H) and 5-amine (PR-104M) metabolites activates the latent nitrogen mustard moiety, resulting in DNA crosslinking and cytotoxicity in hypoxic cells (Patterson et al., 2007). Recently, the same reduction to PR-104H has been shown to be catalyzed under aerobic conditions by aldo-keto reductase (AKR) 1C3 in tumor cells with high expression of this enzyme (Guise et al., 2010). PR-104A is delivered to tumors by administration of the corresponding water-soluble phosphate ester, PR-104, which is currently in clinical trial in humans (Jameson et al., 2010) and is rapidly and extensively hydrolyzed to
PR-104A in mice (Patterson et al., 2007), rats (Patel et al., 2007) and humans (Jameson et al., 2010).

In a recent metabolite profiling study, we identified the β-O-glucuronide of PR-104A, PR-104G, as a major metabolite of PR-104 in humans and dogs, but not in rodents (Gu et al., 2010). A preliminary investigation of in vitro metabolism of PR-104A also demonstrated more extensive glucuronidation by dog and human than rodent liver microsomes (Helsby et al., 2008). This suggested the need for an in-depth evaluation of O-glucuronidation of PR-104A to better understand its excretion, toxicology and potential role in resistance in tumors. The objectives of the present study were 1) to investigate species (mouse, rat, dog and human) differences in glucuronidation of PR-104A in vivo; 2) to compare the kinetics of PR-104A glucuronidation in mouse, rat, dog and human in vitro; 3) to identify human UGTs responsible for glucuronidation of PR-104A; 4) to evaluate expression of the relevant UGTs in human tumors.
**Materials and Methods**

**Chemicals and Reagents**

PR-104, PR-104A, PR-104G and the corresponding diethyl analogue (cmpd 1; see Supplemental Figure S6A) were synthesized as previously (Gu et al., 2009; Gu et al., 2010), as was 2-chloro-N-(2-chloropropyl)-N-methyl-N-(2-nitrobenzyl)propan-1-ammonium chloride (SN25378) (Tercel et al., 1996) used as the internal standard for the quantification of PR-104G by HPLC. Uridine 5’-diphosphoglucuronic acid (UDPGA) triammonium salt, magnesium chloride, alamethicin and D-saccharic acid 1,4-lactone (D-SL, a specific β-glucuronidase inhibitor) were from Sigma-Aldrich (St. Louis, MO). Pooled CD-1 mouse, Sprague Dawley rat, Beagle dog, and human liver microsomes and 12 rUGT Supersomes (UGT1A1, -1A3, -1A4, -1A6-1A10, -2B4, -2B7, -2B15, and -2B17 expressed in baculovirus-infected insect cells) were purchased from BD Gentest (Woburn, MA). Recombinant human (rh)AKR1C3 was a gift from Dr Chris Squire, University of Auckland. HLM were also prepared from a human liver (HL-18), and protein content determined by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL) using bovine serum as standard. A set of characterized HLMs from 23 individuals was also purchased from XenoTech (Lenexa, KS). Acetonitrile (MeCN, LC-MS grade) was from Merck and all other reagents were of analytical grade.

**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 by the Animal Resources Unit (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 ad libitum UV treated Milli-RO water and a sterilized rodent diet (Harlan Teklad diet 2018s). Sprague Dawley 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 mice were 6-8 weeks of age (25-
30 g) and rats were 8-10 weeks (200-220 g). All animal studies were approved by the University of Auckland Animal Ethics Committee (approvals AEC R279 for mice and C363 for rats). Beagle dogs (7 - 8 months, 10-13kg) from Kangda Laboratory Animals S & T Co., Ltd. (Gaoyao, China) were housed individually at LAB Pre-Clinical Research International Inc. (Laval, Quebec, Canada) and fed Teklad Certified Canine Diet (#8727C). A 12-hour light/dark cycle was maintained in all animal studies. Human plasma samples were from three phase I clinical trial previously reported (Jameson et al., 2010)\(^1\)\(^2\). Patients were aged 28 to 79 years with body mass indices of 32.8 to 79.9 kg/m\(^2\).

**Dosing**

PR-104 was formulated as previously described (Gu et al., 2010). Mice and rats were dosed with PR-104 by i.v. bolus (326 mg/kg and 244 mg/kg respectively). Dogs were dosed at 15, 50 and 150 mg/kg by 10 min iv infusion on days 1, 8, 15 and 22) and humans (135 to 1400 mg/m\(^2\)) received PR-104 by a 60 min infusion. Blood samples were collected into EDTA tubes and deproteinized using acidified methanol as described (Gu et al., 2010)

**Determination of PR-104, PR-104A and PR-104G in plasma**

Mouse, rat and dog plasma samples were analyzed using a reported LC-MS method for PR-104 and PR-104A (Patel et al., 2007), with quantitation of PR-104G by absorbance detection (370 nm) at high concentration and by MS (m/z 675) at low concentration with reference to an external standard calibration curve. Human plasma samples were analyzed for all three analytes using an UHPLC-MS/MS assay (Gu and Wilson, 2009). Plasma concentrations were not corrected for the weak plasma protein binding of PR-104 and PR-104A (Patel et al., 2010). Non-compartmental pharmacokinetic parameters (AUC\(_{0-\text{inf}}\) and terminal half life, t\(_{1/2}\)) were calculated using WinNonlin software (version 5.0; Pharsight, Mountain View, CA).

**In vitro glucuronidation of PR-104A**

*In vitro* glucuronidation of PR-104A was evaluated in 96-well plates using 125 µl reaction volumes. Incubation conditions were optimized using HLMs for linear product formation with
respect to substrate concentrations (0-400 µM), microsomal protein concentrations (0.25, 0.5, 1, and 2 mg/ml), incubation time (0.5, 1, 2, and 3 h), and activation of microsomes by alamethicin (10, 25 and 50 µg/mg of protein). Final reaction conditions used 0.5 mg of protein/ml of microsomes, 0.1 M Tris-HCl buffer, pH 7.4, and alamethicin at 25 µg/mg of protein. After preincubation on ice for 10 min, MgCl₂ (5 mM), D-SL (5 mM), and substrate (PR-104A, 150 µM) were added and the mixture warmed to 37°C for 5 min. PR-104A stock solution was prepared in DMSO and diluted in water (final DMSO concentration <0.5%). The reaction was initiated by the addition of UDPGA (5 mM final concentration) and incubated at 37°C in a Thermomixer (Eppendorf, Hamburg, Germany). After 30 min a 100 µl aliquot was withdrawn and microsomal protein was precipitated by the addition of 100 µl of ice-cold MeCN (containing 0.1% formic acid) and internal standard (SN25378). After the removal of protein by centrifugation at 15,000g for 10 min at 4°C, samples were diluted with an equal volume of water and stored at -80°C until analysis.

**Analysis of PR-104G in in vitro incubations**

HPLC separation was carried out using an Agilent HP 1200 Rapid Resolution UHPLC system with photodiode array detector (Agilent Technologies). Samples were separated on a Zorbax SB-C18, 1.8 µ (50 × 3.0 mm, RRLC) with a 0.2 µm in-line filter which was maintained at 30 ºC. The mobile phase consisted of MeCN (A) and 45 mM formate buffer (B) with fast gradient elution at flow rate of 0.8 ml/min and run time of 4.5 min. The gradient profile was: 0 to 2 min, 20% B; 2 to 3.5 min, 20% to 80% B; 3.5 to 3.8 min, 80% B and 3.8 to 4 min, 80% to 20% B. The wavelength for quantitation of PR104G and PR-104A was 370nm, and internal standard (SN 25378) was quantified at 262 nm, each with a reference of 550 nm (bandwidth 50 nm). The injection volume was 75 µl and the autosampler was set at 4°C. Calibration curves were prepared by spiking PR-104G into blank HLMs to give a final concentration range of 0.3-100 µM. Intra- and inter- assay precision (expressed as relative standard deviation (R.S.D.)) and accuracy (expressed as percentage of the nominal value) were determined by analysis of replicates (n=3) of spiked samples (1 and 10 µM PR-104G) in
three separate assays. Stabilities of PR-104G (10 µM) and PR-104A (100 µM) under incubation conditions as described above but without UDPGA were tested in HLMs at 37°C for up to 0.5 h.

**UGT reaction screening**

UGT reaction screening of PR-104A, its non-alkylating analogue (cmpd 1) and PR-104H was performed with 12 commercially available rhUGTs, insect cell control (negative control) microsomes, and pooled HLM using a constant amount of microsomal protein (0.5 mg/ml) and one concentration of PR-104A (150 µM), cmpd 1 (150 µM) or PR-104H (250 µM). Incubation conditions were the same as above using an incubation time of 30 min for PR-104A and cmpd 1, and 15 min for the less stable PR-104H. Incubations without UDPGA or without substrate were included as controls. The incubation samples were extracted and analyzed for PR-104G as described above and confirmed with the more sensitive UHPLC-MS/MS method reported previously (Gu and Wilson, 2009). The glucuronides of cmpd 1 and PR-104H were quantified using the same method, with photodiode array detection at 254 nm at high concentrations and the MS/MS transitions (m/z 503 > 327 and 661 > 485 respectively) at low concentrations. This semi-quantitation was carried out based on the assumption that the extinction coefficients of the glucuronides are same as their corresponding aglycones.

**Enzyme kinetics for the glucuronidation of PR-104A**

Apparent enzyme kinetic parameters (K_m and V_max) for the glucuronidation of PR-104A were determined with liver microsomes from mouse, rat, dog, human and rUGT2B7 using various concentration of PR-104A (0-400 µM) at fixed concentrations of 0.5 mg of protein/ml of microsomes, MgCl_2 (5 mM), alamethicin (25 µg/mg of protein), D-SL (5 mM), and UDPGA (5 mM) in duplicate 125 µl samples. Incubation conditions were the same as for the UGT screen above. After incubation, the samples were extracted by acidified acetonitrile containing internal standard as described above, and the concentrations of PR-104G were...
quantified by UHPLC. Apparent $K_m$ and $V_{max}$ were estimated by fitting the Michaelis-Menten equation using SigmaPlot (Systat Software Inc.): $V = (V_{max} \times S) / (K_m + S)$, where $K_m$ is the Michaelis-Menten constant, $V_{max}$ is the maximum velocity, and $S$ is the substrate concentration, using a nonlinear least squares regression method. Incubations without UDPGA or without substrate or with boiled HLMs were also performed.

**Prediction of hepatic clearance by in vitro - in vivo extrapolation**

Microsomal intrinsic clearance, $CL_{int}$, was calculated as $V_{max}/K_m$ and subsequently scaled to the whole liver using the reported scaling factors for microsomal protein per gram of liver (Barter et al., 2007), liver weight and hepatic blood flow (Davies and Morris, 1993) for each species. In vivo hepatic clearance, $CL_H$, was then predicted using a dispersion model (Iwatsubo et al., 1997).

$$F_H = \frac{4a}{[1 + a]^2 e^{-(a+1)/2D_N} - [(1 - a)^2 e^{-(a+1)/2D_N}]^{-1}}$$

$D_N$, the dispersion number, was taken as 0.17 (Roberts and Rowland, 1986) and

$$a = (1 + 4 R_N D_N)^{1/2}; R_N, \text{ the efficiency, is given by } R_N = \frac{f_{CL_{int}}}{Q_H}.$$ The fraction of drug unbound in blood was evaluated as, $f_u = f_{up}/R_B$, where $R_B$ is the blood to plasma concentration ratio (assuming a value of 1 for this study) and $f_{up}$ is the fraction unbound in plasma.

**Western blot analysis of UGT2B7 protein levels and correlation analysis**

Laemmli sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol was added to HLM samples. Samples were heated at 95°C for 5 min to denature protein and centrifuged for 3 min. An aliquot of supernatant containing 15 µg of protein from 24 individuals or 2 µg of protein from a UGT2B7 microsomal standard (BD Gentest) were loaded onto NuPAGE® 4-12% Bis-Tris gels (1.5 mm × 15 wells, Invitrogen), electrophoresed at 150 V for 1h and transferred to nitrocellulose membrane and immunostained as described (Gu et al., 2009).
but using, rabbit α-UGT2B7 polyclonal primary antibody (BD Gentest, 1:5,000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, 1:10,000 dilution). Bands were visualized by enhanced chemiluminescence using an ImageReader LAS-3000 (Fujifilm) and densities were determined using ImageJ software (version 1.37). Pearson product moment correlation (SigmaStat 3.5) was used to describe relationships between glucuronidation activities and levels of UGT2B7 expression.

Reduction of PR-104G by human liver S9 and AKR1C3

Reduction of PR-104A and PR-104G to their corresponding hydroxylamines was compared under both aerobic and anoxic conditions in human liver S9 as previously reported for PR-104A (Gu et al., 2010). In brief, Reactions comprising hepatic S9 (2 mg of protein/ml), PR-104A or PR-104G (100 µM), and cofactor (NADPH, 1 mM) in sodium/potassium phosphate buffer (67 mM, pH 7.4) with 5 mM MgCl₂ and 1 mM EDTA and were incubated for 20 min at 37°C under air or in an anaerobic chamber. Reduction by purified recombinant AKR1C3 (2 µM) was evaluated as previously described for PR-104A (Guise et al., 2010), using 0.1 mM NADPH and 0.5 mg BSA/ml. Reactions were terminated, and metabolites were extracted by addition of 0.1 ml of ice-cold acidified methanol with centrifugation at 12,000g for 5 min. The supernatant was diluted into an equal volume of water and analyzed by LC/MS/MS as above.

Expression of UGT2B7 mRNA in human tumors

Abundance of UGT2B7 transcripts was assessed using Oncomine (www.oncomine.org) to interrogate two multi-tumor expression databases. The Su database (Su et al., 2001) utilizes the UGT2B7 probe 41376_i_at with the U95A-Av2 GeneChip array (Affymetrix Inc), while the Ramaswamy database (Ramaswamy et al., 2001) utilizes the UGT2B7 probe J05428_at with the Hu6800 and Hu35KsubA GeneChips (Affymetrix Inc). The latter database includes representative normal tissues. Values for liver and hepatocellular carcinoma were also obtained from the Chen database (Chen et al., 2002).
Results

Species difference in pharmacokinetics of PR-104G after PR-104 dosing

The concentration-time profiles of PR-104, PR-104A and PR-104G (for structures see Fig. 1A) after i.v dosing mice, rats, dogs and humans with PR-104 are shown in Fig. 1B-E. PR-104G concentrations were low relative to PR-104A in rodents (Fig. 1B,C), the AUC of PR-104G being only approximately 3% and 0.5% of that for PR-104A in mice and rats respectively (Table 1). In contrast, concentrations of PR-104G were at least as high as PR-104A in dogs (Fig. 1D) and humans (Fig. 1E; see Supplemental Figure S1A for additional dose levels for dogs and humans). At doses approximating the maximum tolerated dose, the AUC of PR-104G was 127% of that for PR-104A in humans, and 233% in dogs (Table 1). In dogs the pharmacokinetics of PR-104, PR-104A and PR-104G after the 4th dose in a weekly x 4 schedule of PR-104 at 150 mg/kg was indistinguishable from that after the first dose (Supplemental Figure S1A). In dogs the AUC of PR-104A increased linearly with dose while the AUC of PR-104G appeared to be non-linear with dose (Supplemental Figure S1B). This non-linearity was not evident in humans, although as shown in Supplemental Figure S2 the ability to detect any such effects was compromised by high inter-individual variability which was greater for PR-104G than PR-104A.

Development of an assay for PR-104A glucuronidation in liver microsomes

Given the large differences in plasma PR-104G/PR-104A ratios between species, we developed a method for comparing rates of PR-104A glucuronidation by liver microsomes from the same species. The UHPLC method, for which chromatograms are illustrated in Supplemental Figure S3, provided good resolution of PR-104G, internal standard and PR-104A (retention times 1.86, 2.58 and 3.07 min respectively), free of interference from endogenous peaks. Incubation of PR-104A in the absence of UDPGA showed <5% loss over 30 min. Intra- and inter-assay precision and accuracy were acceptable for quantification of PR-104G spiked into the optimized liver microsome incubation system (as
defined below) at 1 or 10 µM, using absorbance detection at 370 nm (Supplemental Table S1).

Reaction conditions for UDPGA-dependent glucuronidation of PR-104A by human liver microsomes (HLM), based on the above UHPLC assay for PR-104A, were optimized as shown in Supplemental Figure S4A-C. Final optimized reaction conditions were 0.5 mg/ml protein and 25 µg/mg protein of alamethicin, 5 µM UDPGA and 5 µM of β-glucuronidase inhibitor D-saccharic acid 1,4-lactone, with an incubation time of 0.5 h at 37°C. Under these conditions PR-104G was the only significant reaction product (Supplemental Figure S3B), and was stable when spiked into the reaction mixture (without PR-104A) with <10% loss over 30 min (data not shown). An initial evaluation of PR-104A concentration dependence, under these conditions, with an in-house HLM preparation from a single individual (HL-18) gave simple Michaelis-Menten kinetics (r=0.99), with an apparent $K_m$ of 158 µM and $V_{max}$ of 2.0 nmol/min/mg protein with 95% confidence intervals (CI) of 131-185 µM and 1.9-2.2 nmol/min/mg respectively (Supplemental Figure S4D).

**Enzyme kinetics of in vitro glucuronidation in liver microsomes**

Enzyme kinetic parameters for the glucuronidation of PR-104A by pooled HLM were compared with liver microsomes from mice, rats and dogs under the above conditions. For all species, the kinetics fitted the single-enzyme Michaelis-Menten equation (Fig. 2), with similar $K_m$ (~ 150 µM) but with large differences in velocities. Kinetic parameters are summarized in Table 2, and demonstrate decreasing $V_{max}$ values in the order dog>>human>rat>mouse.

**In vitro-in vivo correlations for PR-104A elimination by glucuronidation**

Microsomal $CL_{int}$ values determined for PR-104G formation from the above in vitro kinetic studies were extrapolated to PR-104A hepatic clearances from blood by glucuronidation using a dispersion model. Protein binding of PR-104A in HLM was <5% under these conditions (Supplemental Table S2) and therefore ignored in the extrapolation. The
predicted *in vivo* hepatic clearance \((CL_H)\) values for glucuronidation of PR-104A for this model (Table 3) were within 17% of those for well-stirred and parallel tube models (Obach et al., 1997) (data not shown) in all four species. For humans, the predicted \(CL_H\) was 20-fold lower than the measured PR-104A clearance (assuming PR-104 is quantitatively metabolized to PR-104A; i.e. PR-104A dose = PR-104 dose), while for mice, rats and dogs this ratio was 40, 11 and 8-fold respectively (Table 3).

**Reaction phenotyping**

A PR-104A concentration of 150 µM, close to the \(K_m\), was used to screen 12 commercially available recombinant human UGTs expressed in insect cells under the above optimized reaction conditions. No glucuronidation of PR-104A was observed in the presence of control insect cell microsomes (Fig. 3A and Fig. S5) or any of the isoforms tested with the exception of UGT2B7, which gave a similar rate of formation of PR-104G to pooled HLM (Fig. 3A). The lack of PR-104G formation by other UGTs using absorbance detection (LOD 0.1 µM) was confirmed by MS/MS detection (LLOQ of 1 nM), indicating very high selectivity for UGT2B7. The kinetics of *in vitro* glucuronidation of PR-104A with rUGT2B7 were well fitted by Michaelis-Menten kinetics (Fig. 3B), although with an apparent \(K_m\) and \(V_{max}\) values lower than for pooled HLM (Table 2). A non-alkylating analogue of PR-104A in which a diethylamino moiety replaces the nitrogen mustard unit (cmpd 1) was also evaluated in the UGT screen. An O-glucuronide analogous to PR-104G was detected by absorbance (Supplemental Figure S6A) and MS/MS \((m/z 503 > 327)\) in a HLM pool. Quantitation by UHPLC with absorbance detection, assuming an unchanged extinction coefficient relative to the aglycone (consistent with the similarity in absorption spectra, Supplemental Figure S6A), demonstrated a similar rate of glucuronidation by HLM and rUGT2B7, but the reaction was also catalyzed at lower rate by UGT1A9 (Supplemental Figure S6B).

**PR-104A glucuronidation in panel of individual HLMs**
The relationship between UGT2B7 expression and PR-104A glucuronidation was tested further using a panel of HLMs for which UGT2B7 activity has been characterized by the manufacturer using conjugation of morphine to its 3-\text{-}O-glucuronide (M3G) as a probe (Table S2). The glucuronidation rate of PR-104A was determined as above at a substrate concentration of 150 µM (Supplemental Table S3). Expression of UGT2B7 protein in the same set of HLMs was then compared by western blotting (Supplemental Figure S7 and Supplemental Table S3). Immunoblotting showed strong labelling of UGT2B7 with an apparent molecular mass of ~55 kDa with ~3-fold variation across the 24 individual HLMs (Supplemental Figure S7A,B). There was a significant correlation between morphine glucuronidation and UGT2B7 content estimated from the immunoblots ($r=0.60$, Fig. 4A) similar to the previously reported correlation coefficient ($r=0.5$, $P<0.001$) between morphine glucuronidation and immunoquantified UGT2B7 protein content (Court et al., 2003). However, there was an even stronger correlation ($r=0.93; P<10^{-10}$) between PR-104A glucuronidation activity and UGT2B7 content across the 24 HLMs (Fig. 4B). The higher correlation coefficient for PR-104A is consistent with evidence that morphine glucuronidation is not exclusive to UGT2B7 (Court et al., 2003), and supports the hypothesis that UGT2B7 is the only enzyme capable of glucuronidating PR-104A in humans.

**In vitro glucuronidation of PR-104H**

The above demonstration of facile glucuronidation of PR-104A by UGT2B7 led us to ask whether the main active metabolite in humans (Gu et al., 2010), PR-104H, is also a substrate for glucuronidation. Incubation of PR-104H with a HLM pool showed this to be highly unstable as expected (52% loss during 15 min incubation), but gave a polar HPLC peak which was characterized as the corresponding glucuronide (PR-104HG) by HPLC with photodiode array detection and MS/MS. Thus the UV spectra of PR-104H and the PR-104HG were very similar (Supplemental Figure S8A), consistent with the similarity of the spectra of Cmpd 1 and its glucuronide (Supplemental Figure S6A), or PR-104A and PR-104G (Gu et al., 2010). In addition, PR-104HG showed an MS fragmentation pattern.
consistent with the proposed structure (Supplemental Figure S8B) and analogous to that for PR-104G (Gu et al., 2010). Incubation of PR-104H with 12 recombinant human UGTs demonstrated that this reaction is again catalyzed predominantly by UGT2B7, although at apparent rates only ~ 0.5% of that for PR-104A. Unlike PR-104A, a trace of the glucuronide was also detected with UGT2B4 by MS detection (Fig. 5A).

**Metabolic reduction of PR-104G by hypoxic reductases and AKR1C3**

PR-104A is known to be reduced to the hydroxylamine PR-104H by AKR1C3 (Guise et al., 2010), independently of oxygen, and by one-electron reductases such as NADPH:cytochrome P450 oxidoreductase under anoxia (Guise et al., 2007). We tested whether PR-104G is also a substrate for metabolic reduction, which is a potential alternative route of formation of PR-104HG. We confirmed the NADPH-dependent aerobic reduction of PR-104A to PR-104H by purified recombinant AKR1C3, and its inhibition by naproxen (Fig. 5B). PR-104G was also reduced to the corresponding hydroxylamine, PR-104HG, by AKR1C3 although at a 250-fold lower rate than for PR-104A (Fig. 5B). In human liver S9 preparations, we confirmed the NADPH-dependent reduction of PR-104A to PR-104H and its strong enhancement under anoxia (Gu et al., 2010), and showed similar anoxia-selective reduction of PR-104G to PR-104HG although the anoxic rate was 7.6-fold lower for PR-104G than PR-104A (Fig. 5C).

**Expression of UGT2B7 mRNA in human tumors and normal tissues**

The above observation that enzymatic nitroreduction of PR-104G is much less facile than for PR-104A demonstrates that glucuronidation will suppress its metabolic activation, and that high UGT2B7 activity is consequently a potential mechanism of intrinsic tumor cell resistance to PR-104A. We therefore examined UGT2B7 transcript abundance in two multi-tumor mRNA expression databases and in a liver cancer database, through Oncomine, and compared with levels in normal tissues (Fig. 6). UGT2B7 was highly expressed in normal kidney and liver, as expected, and poorly in blood leukocytes (Fig. 6A). UGT2B7 transcripts
were more abundant than the median transcript in almost all renal cell and hepatocellular
carcinomas although at lower levels than the corresponding normal tissues. In contrast,
UGT2B7 expression was elevated in ovarian carcinomas relative to normal ovary, and was
lowest in leukemias and lymphomas (Fig. 6B,C).
Discussion

The metabolite profile of the bioreductive prodrug PR-104 differs significantly between humans and preclinical species, with the O-glucuronide PR-104G much more prominent in humans and dogs than rodents (Gu et al., 2010). In a preliminary investigation, PR-104A was also shown to be more extensively glucuronidated by dog and human than rodent liver microsomes in vitro (Helsby et al., 2008). Here we confirm and extend these observations with a quantitative comparison of PR-104A glucuronidation in vitro and in vivo in four species. The in vivo studies clearly demonstrate much higher exposure to PR-104G, relative to PR-104A, in dogs and humans than rats and mice (Table 1), with AUC ratios (PR-104G/PR-104A) in the order of 100-fold lower in rodents than the other species.

Given that PR-104G is not a significant metabolite in mouse urine or bile or in rat urine (Gu et al., 2010), its low plasma concentrations appear to reflect slow formation in rodents rather than rapid clearance relative to the other species. This conclusion is broadly consistent with the species differences in kinetics of in vitro glucuronidation of PR-104A in liver microsomes from each species in the present study (CL_{int} dog>human>rat>mouse; Table 2) although the differences are less dramatic than in vivo, with rat and human liver microsomes showing similar kinetics at low PR-104A concentrations. It is noteworthy that a recent population PK model for PR-104 and PR-104A, using a single structural (compartmental) model identified 2.5-fold faster clearance of PR-104A in dogs than expected for ¾ power body weight scaling relative to humans, with 0.78-fold and 0.63-fold slower clearance in rats and mice respectively compared to humans (Patel et al., 2010). Thus the facile glucuronidation of PR-104A in dogs may account for its relatively high clearance in this species.

The high concentrations of PR-104G in human plasma (Fig. 1E) also suggest that glucuronidation may be a major mechanism of PR-104A clearance in humans, although in the absence of information on clearance of the glucuronide itself these high concentrations could reflect slow elimination rather than (or in addition to) rapid formation. In this regard, extrapolation of in vitro HLM glucuronidation kinetics (CL_{int}) to in vivo hepatic clearance...
The calculated CLH (CLH) is informative (Table 3). Although the predicted CLH is 20-fold lower than the measured whole body blood clearance (CL), significant extra-hepatic glucuronidation can be expected for UGT2B7 substrates (Knights and Miners, 2010) (as reflected in the expression profile shown in Fig. 6A). In addition, it is well known that in vitro glucuronidation kinetics underpredicts in vivo rates, typically by an order of magnitude (Miners et al., 2006). Thus the in vitro-in vivo extrapolation for PR-104A is consistent with glucuronidation being a major mechanism of clearance in humans, and the dominant mechanism in dogs for which the CL/predicted CLH ratio is only 8 (Table 3). The extremely low concentrations of PR-104G in vivo in rodents are less readily explained by the in vitro kinetics for these species.

PR-104A appears to be a remarkably specific substrate for the UGT2B7 isoform in humans, as demonstrated by the complete absence of glucuronidation by 11 other commercially available rhUGTs (Fig. 3A), even using very sensitive MS/MS detection for PR-104G. The strong correlation between UGT2B7 protein levels and PR-104A glucuronidation kinetics in HLM from 24 individuals (Fig. 4B) suggests that there are no other major human hepatic UGTs for which PR-104A is a substrate. This specificity is further supported by the finding that UGT2B7 levels correlate even more strongly with glucuronidation of PR-104A than morphine (Fig. 4A), the latter being catalyzed predominantly by UGT2B7 but with a significant contribution from UGT1A9 and 2B4 (Court et al., 2003). Given that UGT2B7 is strongly expressed in kidney (Knights and Miners, 2010), renal glucuronidation may serve as a local clearance mechanism for PR-104A. Consistent with this, only ~1% of total PR-104 dose was excreted in human urine in as PR-104A, while 13% was excreted as PR-104G (Gu et al., 2010). We also note that the specificity of PR-104A for UGT2B7 suggests that PR-104A, or an analogue such as cmpd 1, may be a useful probe for UGT2B7 activity.

The major cytotoxic metabolite from PR-104A is the hydroxylamine PR-104H in which the nitrogen mustard moiety is activated, resulting in DNA interstrand crosslinking (Patterson et al., 2007; Gu et al., 2009). PR-104H was also a highly selective UGT2B7 substrate, although a trace of glucuronidation by UGT2B4 was detected (Fig. 5A). Absolute rates of
glucuronidation by pooled HLM or UGT2B7-enriched microsomes were at least 100-fold lower than for PR-104A or cmpd 1, but it is not clear to what extent this is influenced by the chemical instability of PR-104H (and presumably of its glucuronide PR-104HG). We also considered the alternate route to formation of PR-104HG (Fig. 7), namely reduction of PR-104G, and showed this to be detectable but minor (250-fold less than for PR-104A) with purified recombinant AKR1C3 (Fig. 5B). Similarly, reduction by aerobic human liver S9 was much more facile for PR-104A than PR-104H (Fig. 5C). Somewhat surprisingly, given the dominant role of AKR1C3 in aerobic reduction of PR-104A in human tumor cell lines (Guise et al., 2010) and its sensitivity to naproxen (Gu et al., 2011), the latter caused only marginal (although statistically significant) inhibition of PR-104A reduction in aerobic human liver S9 (Fig. 5C). Reduction of PR-104G to PR-104HG by anoxic human liver S9 preparations was more readily detectable but was again slower than for PR-104A (Fig. 5C). Taken together these results suggest that reduction of PR-104G to PR-104HG is likely to be a minor pathway at best.

The summary of the identified pathways, in Fig. 7, points to a potential role of the nuclear factor erythroid-2 related factor-2 (Nrf2) transcription factor as a determinant of PR-104A biotransformation. This model is broadly analogous to that for Nrf2 co-regulation of UGTs and two-electron reductases in the metabolism of 9,10 phenanthraquinone (Taguchi et al., 2008). In the context of PR-104A, the Keap1/Nrf2/ARE transcriptional response pathway is known to regulate AKR1C3 expression and PR-104A reduction in some cells (Guise et al., 2010). Nrf2 also regulates expression of the UGT2B7*1/*1 allele although not its *2 allele (Nakamura et al., 2008). In addition, the Keap1/Nrf2/ARE pathway is likely to be modulated by hypoxia given that Nrf2 is activated through oxidation of its repressor Keap1 by reactive oxygen species (elevated by cycling hypoxia) and independently by PERK-dependent phosphorylation (Cullinan and Diehl, 2006) which is elevated by the unfolded protein response under hypoxia (Wouters and Koritzinsky, 2008). However, understanding of the significance of these pathways is currently limited by a lack of information on PR-104G and PR-104HG transporter specificity and intracellular levels of the glucuronides.
A linked question is to what extent glucuronidation represents a detoxification mechanism for PR-104A and PR-104H; unlike conjugation with glutathione (Gu et al., 2010), glucuronidation modifies the alcohol side chain rather than the nitrogen mustard moiety and is thus not expected to have a direct effect on alkylating reactivity. However, the glucuronidation pathway sequesters the aglycone in a form less amenable to reductive activation (whilst more available for efflux if appropriate transporters are expressed). This suggests that tumor expression of UGT2B7 could be a mechanism of intrinsic or acquired resistance to PR-104A, although further studies will be required to evaluate this. Overexpression of UGT1A6 (De Almagro et al., 2011) and UGT1A9 (Cummings et al., 2003) has been shown to confer resistance to methotrexate and SN-38 respectively in cell culture, but we are not aware of evidence for a role of UGT2B isozymes in resistance to anticancer agents. In this regard the high and variable expression of UGT2B7 mRNA in human tumors, especially in renal, hepatocellular and ovarian carcinomas (Fig. 6B,C) is of note. In contrast leukemias and lymphomas show lower expression, supporting the rationale for current clinical trials of PR-104 in human leukemia (NCI trial identifier NCT01037556) which have been initiated because of evidence for hypoxia\(^3\) and AKR1C3 expression (Birtwistle et al., 2009) in leukemic bone marrow.

Given the apparent prominence of glucuronidation in clearance of PR-104A in humans, and the much larger variation in PR-104G than PR-104A AUC between individuals (Supplemental Figure S2), it will also be important to determine whether variation in normal tissue expression (Fig. 6A) or activity of UGT2B7 (Fig. 4B), whether because of difference in genotype (as for epirubicin\(^4\)) or enzyme induction, might contribute to inter-patient variability in PR-104 pharmacokinetics.

Finally, we note that the high concentrations of circulating PR-104G in human plasma after dosing with PR-104 raise the question whether PR-104G itself might act as a PR-104A prodrug via β-glucuronidase-catalyzed regeneration of the aglycone (Fig. 7). PR-104G is a substrate for \textit{E. coli} β-glucuronidase (A. Hogg, K.O. Hicks and W.R. Wilson, unpublished...
data), suggesting that PR-104A could be regenerated in the gastrointestinal tract subsequent to biliary excretion, although gastrointestinal toxicity has not been observed in humans or other species to date. Further, there is longstanding interest in the possibility of selective activation of glucuronides in tumors that express $\beta$-glucuronidases (Connors and Whisson, 1966) especially given that elevated activity is found in necrotic regions of tumors (Juan et al., 2009). We are currently evaluating whether such hydrolysis of PR-104G may contribute to the antitumor activity of PR-104 in some human tumor xenografts, and whether $\beta$-glucuronidase expression in tumors may increase their sensitivity to PR-104 in humans.
Acknowledgements

We thank Xenotech LLC for providing data on morphine glucuronidation by the human liver microsomes evaluated in Fig. 4A and Table S2, Graham Atwell for supply of authentic PR-104G and Proacta Inc for plasma samples from dogs and humans.

Authorship contributions

*Participated in research design:* Gu, Tingle and Wilson

*Conducted experiments and data analysis:* Gu

*Wrote or contributed to the writing of the manuscript:* Gu, Tingle and Wilson
References


Footnotes

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Numbered footnotes:


2 Jameson MB, McKeage MJ, Ramanathan RK, Rajendran J, Gu Y, Wilson WR, Melink TJ and Tchekmedyan NS. Final results of a phase Ib trial of PR104, a pre-prodrug of the bioreductive prodrug PR104A, in combination with gemcitabine (G) or docetaxel (D) in patients with advanced solid tumors. J Clin Oncol 28 (15s); Abstr 2554. 2010. ASCO Meeting abstract

Figure Legends

Fig. 1 (A) Structures of PR-104, PR-104A and PR-104G (gluc = glucuronide), and plasma pharmacokinetics after i.v. administration of PR-104 to (B) mice at 326 mg/kg (3-6 mice per time point), (C) rats at 244 mg/kg (n=6), (D) dogs at 150 mg/kg (n=6) and (E) humans at 1100 mg/m² (approximately 30 mg/kg, n=12). Values are means and errors are SEM.

Fig 2 Enzyme kinetics of glucuronidation of PR-104A in liver microsomes from (A) mouse, (B) rat, (C) dog and (D) human. Individual values for duplicate incubations are shown. Inserts: Eadie-Hofstee plots. Goodness of fits r>0.99 for all.

Fig 3 Reaction phenotyping for glucuronidation of PR-104A and its diethyl analogue cmpd 1. (A) Formation of PR-104G from PR-104A (150 µM) by pooled HLM and rhUGTs in insect microsomes by absorbance detection at 370 nm. Values are means and errors are SEM for triplicate samples. ND: below limit of detection (0.1 µM, equivalent to 6.7 pmol/min/mg protein). (B) Enzyme kinetics for glucuronidation of PR-104A by rUGT2B7. Individual values for duplicate incubations are shown. Insert: Eadie-Hofstee plot. Goodness of fit r>0.99.

Fig 4. Relationship between UGT2B7 expression and glucuronidation of morphine and PR-104A in HLMs. Each point is for an individual subject. Lines are linear regressions. (A) Relationship of morphine glucuronidation rates (1 mM morphine, 10 min, 0.1 mg/ml protein, as determined by the supplier) and UGT2B7 protein levels. (B) Relationship between PR-104A glucuronidation rates (150 µM, 30 min, 0.5 mg/ml protein) and UGT2B7 protein levels.

Fig 5 (A) Enzyme screening of glucuronidation of PR-104H. Values are mean and range for duplicate determinations. ND: not detected by either absorbance or MS. (B) In vitro reduction of PR-104G and PR-104A (each 100 µM) with NADPH (1 mM) by rhAKR1C3 (0.5 mg/ml) under aerobic conditions (NADPH 1 mM, 30 min) with and without naproxen (NAP, 300 µM). Values are mean and SEM for triplicate determinations. (C) In vitro hepatic S9 (2 mg/ml) reductive metabolism of PR-104G and PR-104A (each 100 µM) under oxic and anoxic
condition (NADPH 1 mM, 30 min) with and without naproxen (NAP, 300 µM). Values are mean and SEM for triplicate determinations.

Fig 6. Box and whisker plots of UGT2B7 transcript abundance by mRNA microarray analysis of human tissues, expressed relative to the median transcript for each sample. Boxes span the 25-75 percentile, and whiskers the 10-90 percentile, with outliers as points. (A) Normal tissues from the Ramaswamy database (Ramaswamy et al., 2001) and liver (not available in Ramaswamy) from the Chen database (Chen et al., 2002). (B) Tumors from the Ramaswamy database, including HCC from the Chen database. (C) Tumors from the Su database (Su et al., 2001). Numbers of samples are shown in parentheses. Abbreviations: AML, acute myeloid leukemia; BAC, breast adenocarcinoma; BC, bladder cancer; BDC, breast ductal carcinoma; B-ALL; B-cell acute lymphoblastic leukemia; CCRC, clear cell renal carcinoma; CRAC, colorectal adenocarcinoma; DLBCL, diffuse large B cell lymphoma; EAC, endometrial adenocarcinoma; FL, Follicular lymphoma; GBM, glioblastoma multiforme; GAC, gastroesophageal adenocarcinoma; GC, germinal center; HCC, hepatocellular carcinoma; LungAC, lung adenocarcinoma; Med, medulloblastoma; Mel, melanoma; Meso, pleural mesothelioma; OvAC, ovarian adenocarcinoma; OvPSC, ovarian papillary serous cytadenocarcinoma; Panc, pancreas; PancAC; pancreatic adenocarcinoma; PrAC, prostate adenocarcinoma; PrC, prostate carcinoma; RCC; renal cell carcinoma; SCLC; squamous cell lung cancer; T-ALL, T-cell acute lymphoblastic leukemia; TCC, transitional cell carcinoma (bladder).

Fig 7 Pathways of Nrf2-regulated biotransformation of PR-104A via glucuronidation and reduction, and potential reactivation of PR-104G by β-glucuronidase. 1eR: one-electron reductases
**Table 1** Pharmacokinetic parameters of PR-104A and PR-104G in mice, rats, dogs and humans. Mean with SE in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose (mg/kg)</strong></td>
<td>326</td>
<td>244</td>
<td>150</td>
<td>30†</td>
</tr>
<tr>
<td><strong>Dose (µmol/kg)</strong></td>
<td>562</td>
<td>421</td>
<td>259</td>
<td>51†</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;PR-104A&lt;/sub&gt;(µM h)</strong></td>
<td>82.5</td>
<td>147 (5)</td>
<td>33.1 (1.16)</td>
<td>33.5 (4.1)</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;PR-104G&lt;/sub&gt;(µM h)</strong></td>
<td>2.92</td>
<td>0.75 (0.08)</td>
<td>77.2 (0.2)</td>
<td>45.0 (14.7)</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;PR-104G&lt;/sub&gt;/AUC&lt;sub&gt;PR-104A&lt;/sub&gt;(%)</strong></td>
<td>3.33</td>
<td>0.51 (0.05)</td>
<td>233 (8)</td>
<td>127 (30)</td>
</tr>
</tbody>
</table>

†1100 mg/m².
Table 2 *In vitro* enzyme kinetics of PR-104A glucuronidation (estimate ± SE).

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
<th>rUGT2B7</th>
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<tbody>
<tr>
<td><strong>In vitro microsomal metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>155.4±13.7</td>
<td>97.4±6.2</td>
<td>157.1±7.6</td>
<td>178.2±11.9</td>
<td>63.4±3.4</td>
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<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>0.24±0.01</td>
<td>0.61±0.01</td>
<td>7.87±0.17</td>
<td>1.47±0.05</td>
<td>0.58±0.01</td>
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<tr>
<td>$CL_{int}$ (µl/min/mg)</td>
<td>1.54</td>
<td>6.26</td>
<td>50.1</td>
<td>8.25</td>
<td>-</td>
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</tbody>
</table>
Table 3  *In vitro-in vivo* extrapolation of glucuronidation of PR-104A and comparison with whole body clearance.

<table>
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<th>Species</th>
<th>Mouse</th>
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<th>Dog</th>
<th>Human</th>
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<tbody>
<tr>
<td>MPPGL (mg/g liver) †</td>
<td>49</td>
<td>49</td>
<td>60</td>
<td>41</td>
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<tr>
<td>Liver weight (g/kg) *</td>
<td>87.5</td>
<td>44.0</td>
<td>32.0</td>
<td>25.7</td>
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<tr>
<td>QH (ml/min/kg) *</td>
<td>90.0</td>
<td>55.2</td>
<td>30.9</td>
<td>20.7</td>
</tr>
<tr>
<td>f_u (20 µM PR-104A)§</td>
<td>0.67</td>
<td>0.60</td>
<td>0.64</td>
<td>0.40</td>
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<tr>
<td>Predicted CLH (ml/min/kg)</td>
<td>4.29</td>
<td>7.41</td>
<td>24.8</td>
<td>3.16</td>
</tr>
<tr>
<td>AUC PR-104A (µM h)‡</td>
<td>55.1</td>
<td>88.3</td>
<td>21.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Whole body CL PR-104A (ml/min/kg) ††</td>
<td>170</td>
<td>79.4</td>
<td>203</td>
<td>63.4</td>
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<tr>
<td>Whole body CL / Predicted CLH</td>
<td>40</td>
<td>11</td>
<td>8</td>
<td>20</td>
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</table>

† Microsomal protein per gram liver. Averaged values reported in Barter et al., 2007.

* Values from Davies and Morris, 1993.

§ Unbound fraction in plasma, using values from Patel et al., 2010

‡ Calculated for unbound PR-104A from total drug AUC values at doses near the maximum tolerated dose as shown in Table 1.

†† Calculated from dose/AUC assuming 100% conversion of PR-104 to PR-104A (i.e. PR-104A dose = PR-104 dose).
Fig 1

A

\[
\begin{align*}
\text{NO}_2 \\
\text{H} \\
\text{O} \\
\text{Br} \\
\text{OSO}_2\text{Me}
\end{align*}
\]

PR-104: R=PO_4^2-  
PR-104A: R=H  
PR-104G: R=gluc

B

Mouse

Time (min)

Concentration (µM)

0 30 60 90 120

0.1 1 10 100

PR-104  
PR-104A  
PR-104G

C

Rat

Time (min)

Concentration (µM)

0 30 60 90 120 150 180

0.1 1 10 100

PR-104  
PR-104A  
PR-104G

D

Dog

Time (min)

Concentration (µM)

0 30 60 90 120

0.1 1 10 100

PR-104  
PR-104A  
PR-104G

E

Human

Time (min)

Concentration (µM)

0 30 60 90 120 150 180 210 240 270 300

0.1 1 10 100

PR-104  
PR-104A  
PR-104G
**Fig 3**

**Graph A:**
- Formation of glucuronide PR-104G (pmol/min/mg protein)
- Variants: 1A1, 1A3, 1A4, 1A6, 1A8, 1A10, 2B4, 2B15, HLM-pooled, Insect control, Boiled HLM
- ND values indicated

**Graph B:**
- PR-104A Concentration (µM) vs. V (pmol/min/mg protein)
- V/[S] vs. V
- Data points and curves indicating reaction kinetics
Fig 4

A

Glucuronidation of morphine (pmol/mg/min) vs. UGT 2B7 protein (µg)

- R = 0.60
- P < 0.01

B

Glucuronidation of PR-104A (pmol/mg/min) vs. UGT 2B7 protein (µg)

- R = 0.93
- P < 10^-10
Fig 5

A

Hydroxylamine formation (pmol/min/mg protein)

PR-104A PR-104G

Hydroxylamine formation (pmol/min/mg protein)

PR-104A PR-104G

Hydroxylamine formation (pmol/min/mg protein)

B

C

AKR1C3 AKR1C3+NAP

Hydroxylamine formation (pmol/min/mg protein)

PR-104A PR-104G

Hydroxylamine formation (pmol/min/mg protein)

PR-104A PR-104G

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Figure S1: Plasma pharmacokinetics of PR-104 and metabolites in dogs, following a 10 min i.v. infusion using a qwx4 schedule following 1st dose (day 1, solid lines) of PR-104 at 15 (squares), 50 (triangles) and 150 (circles) mg/kg (n=6 for each dose level) and 4th dose (day 22, dashed lines) at 150 mg/kg. The day 1 data at dose of 150 mg/kg is redrawn from Fig. 1D. B: Dose-dependent AUC of plasma PR-104G and PR-104A in dogs after i.v. infusion of PR-104, based on non-compartmental analysis.
Figure S2: Dose-dependence of AUC for PR-104A and PR-104G in humans after a 60 min i.v. infusion of PR-104, based on non-compartmental analysis of the data of Fig. 1E and additional dose levels from the same phase I studies. Box and whisker plots. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. A. PR-104A; B. PR-104G.
Figure S3: Representative HPLC chromatograms for the glucuronidation of PR-104A \textit{in vitro}. A: blank (boiled HLM) overlaid with boiled HLM spiked with 0.3 $\mu$M PR-104G (LLOQ), internal standard (IS) and 30 $\mu$M PR-104A, at 370 nm. B: After 20 min incubation of pooled HLM with 150 $\mu$M PR-104A using the optimized conditions described in the main text.
Figure S4: Optimization of incubation conditions for glucuronidation of PR-104A, with respect to (A) protein concentration, (B) incubation time, (C) alamethicin concentration, and (D) PR-104A concentration in HLM. 100 µM PR-104A, 0.5 mg/ml protein, 50 µg/mg alamethicin with 1 h incubation were used for in A, B and C unless the condition is used as a variable. Data are presented as individual values of duplicate incubations.
Figure S5: Representative HPLC chromatograms, monitored at 370 nm, for the glucuronidation of PR-104A by insect cell microsomes, using reaction conditions optimized for HLM (0.5 mg/ml protein concentration, 0.5 h incubation time and 25 µg/mg protein of alamethicin). A: insect cell control. B: UGT2B7-expressing supersomes.
Figure S6: (A) Normalized absorption spectra of the diethyl analogue of PR-104A (1) and its major product 2 in UDPGA-supplemented pooled HLM by on-line photodiode array detection. (B) Formation of glucuronide 2 by rhUGTs using absorbance detection at 370 nm. ND: not detected.
Figure S7 Immunoblot analysis of UGT2B7 enzyme using anti-UGT2B7 antibody recognising the 55-kDa form. Total protein/lane was 2 μg for the UGT2B7 standard and 15 μg for the HLMs. The sizes of the molecular mass markers, in kDa, are indicated on the left.
Figure S8 (A) Normalized absorbance spectra of PR-104H and PR-104HG.

(B) Product ion mass spectrum of the parent molecular ion of PR-104HG (m/z 661).
### Table S1 Intra- and inter-assay precision and accuracy for PR-104G in HLM incubation mixture.

<table>
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<th>Nominal (µM)</th>
<th>Intra-assay</th>
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<th>Inter-assay</th>
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<tr>
<td></td>
<td>Measured†</td>
<td>Accuracy‡</td>
<td>Precision§</td>
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<td>10</td>
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<td>8.90</td>
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<td>101</td>
<td>9.57</td>
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</table>

†By UHPLC with absorbance detection at 370 nm.
‡Measured value as % of nominal value.
§Relative standard deviation.
Table S2 Protein binding of PR-104A in HLM (0.5 mg/ml) using equilibrium dialysis using Cuprophan flat sheet membrane (MW cut-off 10,000 Da) and a DIANORM® equilibrium dialyser (GD-5, DIACHEMA AG) at 12 rpm. PR-104A was added to the buffer only (Tris) or HLM compartment at the indicated concentrations. Total PR-104A concentrations were determined by HPLC after 3 hr incubation following deproteinization with 3 volumes of acidified methanol.

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Initial conc (Tris) µM</th>
<th>Initial conc (HLM) µM</th>
<th>Final conc (Tris) µM</th>
<th>Final conc (HLM) µM</th>
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<tbody>
<tr>
<td>20</td>
<td>150</td>
<td>150</td>
<td>144.3</td>
<td>152.4</td>
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<tr>
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Table S3 Glucuronidation of morphine and PR-104A, and immuno-quantitation of UGT2B7, for HLMs from different donors

<table>
<thead>
<tr>
<th>HLM ID</th>
<th>Morphine † pmol/mg/min</th>
<th>PR-104A ‡ pmol/mg/min</th>
<th>UGT2B7 § (µg)</th>
<th>HLM ID</th>
<th>Morphine pmol/mg/min</th>
<th>PR-104A pmol/mg/min</th>
<th>UGT2B7 (µg)</th>
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<td>2.03</td>
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† Morphine glucuronidation (as morphine-3-glucuronide) data are from XenoTech. The reactions were conducted at a substrate concentration of 1 mM with 10 min incubation and 0.1 mg/ml protein.

‡ PR-104A glucuronidation was determined as described in Materials and Methods.

§ UGT2B7 quantitation (relative to rUGT2B7) is based on band intensity (determined by ImageJ) calibrated with the XenoTech rUGT2B7 microsomal standard.