Mechanism of Action and Preclinical Antitumor Activity of the Novel Hypoxia-Activated DNA Cross-Linking Agent PR-104

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Abstract

Purpose: Hypoxia is a characteristic of solid tumors and a potentially important therapeutic target. Here, we characterize the mechanism of action and preclinical antitumor activity of a novel hypoxia-activated prodrug, the 3,5-dinitrobenzamide nitrogen mustard PR-104, which has recently entered clinical trials.

Experimental Design: Cytotoxicity in vitro was evaluated using 10 human tumor cell lines. SiHa cells were used to characterize metabolism under hypoxia, by liquid chromatography-mass spectrometry, and DNA damage by comet assay and γH2AX formation. Antitumor activity was evaluated in multiple xenograft models (PR-104 ± radiation or chemotherapy) by clonogenic assay 18 h after treatment or by tumor growth delay.

Results: The phosphate ester “pre-prodrug” PR-104 was well tolerated in mice and converted rapidly to the corresponding prodrug PR-104A. The cytotoxicity of PR-104A was increased 10- to 100-fold by hypoxia in vitro. Reduction to the major intracellular metabolite, hydroxylamine PR-104H, resulted in DNA cross-linking selectively under hypoxia. Reaction of PR-104H with chloride ion gave lipophilic cytotoxic metabolites potentially able to provide bystander effects. In tumor excision assays, PR-104 provided greater killing of hypoxic (radioresistant) and aerobic cells in xenografts (HT29, SiHa, and H460) than tirapazamine or conventional mustards at equivalent host toxicity. PR-104 showed single-agent activity in six of eight xenograft models and greater than additive antitumor activity in combination with drugs likely to spare hypoxic cells (gemcitabine with Panc-01 pancreatic tumors and docetaxel with 22RV1 prostate tumors).

Conclusions: PR-104 is a novel hypoxia-activated DNA cross-linking agent with marked activity against human tumor xenografts, both as monotherapy and combined with radiotherapy and chemotherapy.

Hypoxia is a uniquely attractive target in oncology for two reasons. The first is that hypoxic cells are obstacles to curative cancer therapy with all major treatment modalities. Hypoxia can compromise outcomes of surgery by increasing tumor metastasis (1–3). It is also a major cause of radioresistance because oxygen is a radiosensitizer, and multiple clinical studies have documented the importance of hypoxia determining local tumor control in radiotherapy (4–6). Hypoxia also contributes to chemoresistance through multiple mechanisms (7), including limitations on delivery of blood-borne drugs to hypoxic regions of tumors (8, 9). The second reason for targeting hypoxia is that it is a common feature of a wide variety of human tumors and is typically more severe in tumors than in normal tissues, thus providing a basis for tumor selectivity (10, 11).

Several strategies for exploiting tumor hypoxia are now in preclinical or clinical development (7), with the main focus on prodrugs that are activated by metabolic reduction under hypoxic conditions to form cytotoxins. Early efforts focused on quinone bioreductive drugs, such as porfiromycin (12), and 2-nitroimidazole–linked alkylating agents, such as CI-1010 (PD 144872, the R-enantiomer of RB 6145; ref. 13), although the latter caused irreversible retinal toxicity in preclinical species (14, 15) and did not proceed to clinical trial. Currently, the hypoxia-activated prodrugs most advanced clinically are N-oxides, such as tirapazamine (16) and banoxantone (17), but are still classified as investigational drugs.

We have identified a further class of hypoxia-activated prodrugs, dinitrobenzamide mustards (DNBM), which seem to offer advantages in preclinical models. DNBM prodrugs contain a latent nitrogen mustard moiety, which becomes activated when either of the nitro groups is reduced to the corresponding hydroxylamine or amine (18). This “electronic switch”
generates reactive nitrogen mustard metabolites selectively in hypoxic cells, resulting in hypoxia-selective cytotoxicity (19, 20). DBNM prodrugs have two notable features, shown for the prototype of this class, the 2,4-dinitrobenzamide dichloromustard SN 23862. The first is that its activation is confined to lower oxygen concentrations than for tirapazamine (21), which offers the potential for improved selectivity for severe (pathologic) hypoxia in tumors. The second is that its activated metabolites are able to diffuse locally in tumor tissue, providing an efficient bystander effect (killing of untargeted cancer cells; refs. 22, 23). We have shown that, unlike the DNBMAs, reductive activation of tirapazamine or the active form of CI-1010 does not provide bystander effects in hypoxic multicellular cultures (21). These features suggest that DBNM prodrugs have unique potential for exploiting tumor hypoxia selectively through the release of activated nitrogen mustards that can also kill adjacent cells at higher oxygen concentrations.

Here, we report the mechanism of action and nonclinical antitumor activity of a new DBNM prodrug, PR-104 (see Fig. 4A for structure), which we have optimized for hypoxic selectivity and in vivo antitumor activity. PR-104 combines two key aspects identified by structure-activity relationship studies in the DNBM series; unlike the 2,4-dinitro-5-mustard SN 23862, PR-104 is a 3,5-dinitrobenzamide-2-mustard, which is more readily reduced in hypoxic cells, and its asymmetrical nitrogen mustard contains more reactive leaving groups (bromide and mesylate rather than chloride). We show that PR-104, a water-soluble phosphate “pre-prodrug,” is converted efficiently to the more lipophilic DBNM alcohol PR-104A, which is a hypoxia-selective DNA cross-linking agent and cytotoxin. PR-104 shows a higher therapeutic ratio than tirapazamine for killing hypoxic cells in human tumor xenografts but also efficiently kills aerobic cells in tumors as shown by its marked single-agent (monotherapy) activity. PR-104 is currently in a phase I clinical trial, which commenced in January 2006.

Materials and Methods

Compounds. PR-104 [2-((2-bromoethyl)-2-[[2-hydroxyethyl](amino[carboxyl])-4,6-dinitroaminolino)ethy methanesulfonate phosphate ester] was synthesized, as the free acid, from PR-104A as described (24). The batches used in this study varied in purity from 93% to 98% for PR-104 and 96% to 100% for PR-104A based on high-performance liquid chromatography (HPLC) with absorbance detection (254 nm). The studies on combination with doctaxel and gemcitabine used good manufacturing practices grade PR-104, lot 309-01-001 (purity 97%). Tirapazamine was synthesized in this laboratory (25). Excipient-free chlorambucil, melphanal, cisplatin, and cyclophosphamide were purchased from Sigma-Aldrich, and doctaxel (Aventis Pharma) and gemcitabine, melphalan, cisplatin, and cyclophosphamide were synthesized as described (26). The IC50 was determined by interpolation as the drug concentration reducing staining to 50% of controls on the same plate.

Clonogenic cell killing in single-cell suspensions and spheroids. HCT116 multicellular spheroids were grown in spinner flasks for 10 days (diameter, ~900 μm) and single cells were prepared by dissociating with 0.25% trypsin/EDTA (Life Technologies, Invitrogen). Single cells and intact spheroids were exposed to PR-104A for 4 h as magnetically stirred suspensions (10 ml/bottle), at the same average cell density, in αMEM with 10% fetal bovine serum under flowing 5% CO2 in air or N2 in a 37°C water bath. Drug-treated spheroids were dissociated as above, and cells were washed by centrifugation and plated to determine clonogenic survival.

PR-104A metabolism in stirred cell suspensions. Subconfluent SiHa monolayers in T-175 flasks were harvested (trypsin/EDTA) to prepare suspensions (2.5 × 10⁶/ml) in αMEM (8-10 ml), which were exposed to PR-104A as above. Samples were removed at intervals, chilled, and reoxidized by pipetting rapidly on ice and centrifuged (11,000 × g for 30 s in a prechilled rotor). The extracellular medium and extracted cell pellets (50 μl ice-cold methanol per pellet of 2 × 10⁶ cells, vortexed for 10 s) were frozen at -80°C for subsequent HPLC. Viability of cells by trypan blue exclusion was in the range 89% to 95% in all samples.

HPLC, mass spectrometry, and bioassay of cellular metabolites. Methanol extracts of SiHa cell pellets were centrifuged (13,000 × g for 5 min) and diluted 1:2 with ammonium formate buffer (45 mmol/L, pH 4.5), and samples (100 μl) were analyzed by HPLC with photodiode array and electrospray/single-stage quadrupole mass spectrometer detectors (Agilent 1100/MSD model D, Agilent Technologies) as detailed elsewhere. The isotopic point for conversion of PR-104A to PR-104H was shown to be 254 nm (data not shown); all metabolites were therefore quantified assuming extinction coefficients equal to PR-104A at this wavelength. Intracellular drug concentrations were calculated using the mean intracellular water volume of SiHa cells determined by Coulter pulse height analysis (mean, 1,776 fl), calibrated against HT29 cells (27). In addition, extracellular medium samples were fractionated by HPLC using an acetonitrile/water gradient and Agilent 1100 fraction collector and bioassayed against UV4 cells (21). Brieﬂy, the eluate was diluted 15-fold into UV4 cultures in 96-well plates and cell densities were determined by sulforhodamine B staining 4 days later.

Single-cell gel electrophoresis (comet assay). SiHa cell suspensions (10⁶ cells/ml in αMEM) were exposed to PR-104A under aerobic and hypoxic conditions as above. The effect on DNA breakage induced by cobalt-60 γ-irradiation (10 Gy) was assayed using the alkaline comet assay as previously (28), except that images were analyzed to determine tail moments using Komet v5.0 software (Kinetic Imaging Ltd.). γH2AX assays. Following drug treatment of stirred cell suspensions as above, cells were grown in as monolayers for 24 h and harvested with trypsin/EDTA. Cytosins were ﬁxed with paraformaldehyde and stained with 4,6-diamidino-2-phenylindole and then for γH2AX using a phosphorylated-speciﬁc mouse monoclonal antibody (Upstate Biotechnology) as described (29) but using an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes). Slides were viewed with a Leica DMR microscope using a 100× oil immersion objective and a cooled color Nikon digital sight camera. Image
fluorescence was quantified using ImageJ software (version 1.37). 4',6-Diamidino-2-phenylindole–stained nuclei were outlined and overlaid on pH2AX images using Adobe Photoshop (version 5.0 LE). For flow cytometry, cells were fixed in 70% ethanol, rehydrated, and incubated with the above pH2AX antibody (1:500 dilution, 2 h) and secondary (1:400 dilution, 1 h) at room temperature. Cells were resuspended in 1 mL PBS containing 100 µg/mL RNase and 20 µg/mL propidium iodide and analyzed using a Becton Dickinson FACScan with CellQuest software using forward scatter to gate out debris.

**Animals, dosing, and toxicology.** Specific pathogen-free homozygous nude (C.B-17SCID/Slc®) mice (Charles River Laboratories) were bred by the Animal Resources Unit (University of Auckland), housed in Techniplast microisolator cages, and fed Harlan Teklad diet 2018i. Animals were identified by ear tags and weighed 18 to 28 g at the time of experiments. All animal studies were approved by the University of Auckland Animal Ethics Committee (approvals R279 and C337). PR-104 free acid was dissolved in PBS + 1 equivalent NaHCO3 or the clinical formulation (PR-104 sodium salt lyophilized with mannitol) was reconstituted in 2 mL water and diluted in PBS. Chlorambucil was dissolved in 0.5 mmol/L NaHCO3 (pH 8.5), tirapazamine in 0.9% NaCl solution + 5% DMSO, melphalan, cyclophosphamide, cisplatin, and gemcitabine in 0.9% NaCl solution, and doxetaxel in the manufacturer’s diluent. Final concentrations of PR-104, tirapazamine, chlorambucil, and melphalan were determined by spectrophotometry. Dosing solutions were prepared fresh, held at room temperature in amber vials, and used within 3 h. Maximum tolerated dose (MTD) values were determined using 1.33-fold dose escalations. Animals were randomized to treatment groups (five to nine mice per group) when tumors reached treatment size (mean, 233; SD, 87 mm3). Tumor size and body weight were determined thrice weekly. Tumor volume was calculated as \( \pi \left( L \times w^2 \right)/6 \), where \( L \) is the major axis and \( w \) is the perpendicular minor axis. Animals were culled 100 days after start of treatment or when mean tumor diameter exceeded 15 mm. Treatment efficacy was assessed by comparing survival with controls using the log-rank test or by post hoc ANOVA (Holm-Sidak test) when multiple groups were compared (SigmaStat v3.10). In addition, the median time for tumors to increase in volume 4-fold relative to pretreatment volume (RTV-4) was determined, and the specific growth delay (SGD) was calculated as the percentage increase in RTV-4 for treated versus control. This variable normalizes for differences in tumor volume at treatment and for differences in control tumor growth rate between cell lines. Long-term controls (time to end point, >100 days) were assigned an RTV-4 value of 100 days, and significance of drug effects was tested using the Mann-Whitney U test (SigmaStat v3.10).

**Results**

**Hypoxia-selective cytotoxicity of PR-104A.** The antiproliferative potency of PR-104A was compared with chlorambucil and tirapazamine by determining IC50 values in a panel of 10 human carcinoma cell lines following 4-h drug exposures under aerobic and hypoxic conditions (Fig. 1A and B). Under hypoxia (<10 ppm O2 gas phase), PR-104 varied in potency between cell lines, with the lowest IC50 (0.51 µmol/L) in H460 non-small cell lung cancer cells and highest (7.3 µmol/L) in PC3 prostate cells. Its potency was slightly greater than tirapazamine in most cell lines and up to 10-fold greater than chlorambucil (Fig. 1A). The hypoxic selectivity of PR-104A, measured as the ratio of IC50 values under aerobic and hypoxic conditions (Fig. 1B), was ~100-fold for HCT116, C33A, and H1299 cells and ~10-fold for the other cell lines. Tirapazamine showed consistently high hypoxic selectivity (~100-fold) in all cell lines, whereas chlorambucil lacked hypoxic selectivity. The hypoxic selectivity of PR-104A was confirmed by clonogenic assay against stirred suspensions of HCT116 cells, which indicated a similar hypoxic cytotoxicity ratio (30-fold) whether cells were exposed in multicellular spheroids or as single cells from enzymatically dissociated spheroids under equivalent ambient conditions (Fig. 1C).

**Reductive metabolism of PR-104A.** We evaluated metabolism of PR-104A in aerobic and hypoxic suspensions of SiHa cells by liquid chromatography-mass spectrometry; a summary of the identified metabolites is provided in Fig. 2A, a representative chromatogram in Fig. 2B, and the time course for intracellular metabolites (extracted with methanol) and extracellular metabolites (by direct analysis of medium) in Fig. 2C. PR-104A was lost from the extracellular medium at a faster rate in hypoxic cultures (first-order rate constant, 0.23 ± 0.01 h−1 versus 0.045 ± 0.005 h−1 under aerobic conditions; data not shown). The major intracellular metabolite under hypoxia was the hydroxylamine PR-104H (base peak m/z 485 by positive mode electrospray ionization, corresponding to the M+H+ ion, with an isotope pattern showing 1 Br; representative mass spectra are provided in Supplementary Fig. S1 and variables are summarized in Supplementary Table S2). Zinc dust reduction of PR-104A gave a species identical to the PR-104H metabolite (by retention time, absorbance, and mass spectrum). The 1H-13C gradient-enhanced heteronuclear multiple bond correlation nuclear magnetic resonance spectrum of synthetic PR-104H (see Supplementary Fig. S2), showing coupling to 13C resonances of PR-104A, showed clear correlation to long-range heteronuclear coupling peaks.
between the nitro group N and proton of the CH para to the carboxamide and couplings of the hydroxylamine N with both CH protons. This unambiguously identified the position of the hydroxylamine as para to the nitrogen mustard. PR-104H reached its maximum concentration at 1 h and was present at 10- to 20-fold lower concentrations in aerobic cells (Fig. 2C).

A complex set of minor products (compounds 1-4) in SiHa cells, detected only under hypoxia, was formed with slightly slower kinetics. These had absorption spectra very similar to PR-104H and mass spectra consistent with displacement of either the bromo or mesylate leaving group of PR-104H by chloride or hydroxide ion, giving 1 (m/z 425; 1 Cl + 1 Br isotope pattern), 2 (m/z 441; 1 Cl), 3 (m/z 381; 2 Cl), and 4 (m/z 407; 1 Br). A common hydrolysis product of 1 to 3, compound 5 (m/z 363; 1 Cl), was detected but not resolved chromatographically from 9. In addition, a metabolite in hypoxic cells formed with similar kinetics to PR-104H and had a mass spectrum (m/z 469; 1 Br) consistent with the corresponding amine (6). Its identity was confirmed by its presence as a minor product in the zinc dust reduction of PR-104A and its slow autoxidation to PR-104H. Its nucleophilic displacement products 7 (m/z 365; 2 Cl), not chromatographically resolved from 1, and 8 (m/z 347; 1 Cl) were also detected.

In addition, two metabolites that we interpret as arising from reduction of the nitro group ortho to the mustard moiety were detected only under hypoxia. Compound 9 had a mass spectrum (m/z 373; 1 Br) consistent with cyclization via intramolecular alkylation of the ortho hydroxylamine by the mesylate leaving group of the mustard to form a tetrahydroquinoxaline [as reported following ortho nitroreduction of the dinitrobenzamide chloromustard SN 23862 (21, 30)]. The corresponding hydrolysis product 10 (m/z 311, no halogens) showed an absorbance spectrum distinct from the para nitro-reduction products (Supplementary Fig. S1) and increased linearly with time consistent with its formation as a stable end product.

Certain of the reduced metabolites were also detected in extracellular medium (Fig. 2C, right). The extracellular metabolite profile was biased, relative to that within cells, in favor of the more lipophilic metabolites. Thus, the mesylate-containing PR-104H was present at ~100-fold lower concentrations than in the cells, and the corresponding amine 6 was not detected, whereas the more lipophilic chlorodisplacement products 1 and 3 and the tetrahydroquinoxaline 10 were relatively prominent. This is consistent with more efficient passive diffusion of lipophilic reduction products out of the cells.

We investigated the bioactivity of reduction products in extracellular medium, after incubation of hypoxic SiHa cells with PR-104A for 3 h, by assaying the HPLC fractions for inhibition of proliferation of the ERCC1 mutant UV4 (Fig. 2B, bottom). This showed two new peaks of bioactivity, in addition to PR-104A. The earlier eluting peak corresponded to the dichlorohydroxylamine 3 and the later peak to the bromohydroxylamine/chlorohydroxylamine 1 and/or the dichloroamine 7 (which were not resolved).

PR-104A is a hypoxia-selective DNA-damaging agent. DNA cross-linking in SiHa cells following exposure to PR-104A was shown by single-cell gel electrophoresis (comet assay), which showed little or no effect of drug only (data not shown) but greater suppression of radiation-induced DNA single-strand breaks (i.e., greater interstrand cross-linking) under hypoxic than aerobic conditions (Fig. 3A). We also showed that UV41 cells, which are defective in DNA interstrand cross-link repair by virtue of mutation of XPF/ERCC4 (31), are hypersensitive to the major hypoxic metabolite of PR-104A, the para hydroxylamine PR-104H (Fig. 3B). The hypersensitivity of UV41 relative
to 41cER40.1, a human XPF transfectant restoring 90% of XPF activity to UV41 (32), was 21-fold under aerobic conditions and 28-fold under hypoxia. The UV41/41cER40.1 differentials for PR-104H were similar to those for chlorambucil (Fig. 3B), consistent with cytotoxicity of PR-104H being due predominantly to DNA cross-linking.

Incubation of SiHa cells following exposure to PR-104A resulted in phosphorylation of Ser 139 of histone H2AX (γH2AX), which was more prominent following hypoxic than aerobic exposure (Fig. 3C). Image analysis showed a 2.5-fold increase in the geometric mean integrated fluorescence per nucleus after hypoxic versus aerobic exposure. A similar differential was shown by flow cytometry (Fig. 3D). Chlorambucil also caused H2AX phosphorylation but in an oxygen-independent manner (Fig. 3D). The γH2AX response to both drugs occurred with delayed kinetics, reaching a maximum at ~24 h and was accompanied by accumulation of cells with an S-phase DNA content (data not shown). Thus, γH2AX induction may be due, in part, to arrest of replication forks at DNA interstrand cross-links.

PR-104 is well tolerated in mice and rapidly converted to PR-104A. PR-104A had limited aqueous solubility (1.66 mmol/L in culture medium); a water-soluble phosphate ester, PR-104, was therefore prepared as a pro-drug to release PR-104 via systemic phosphatases (Fig. 4A). Titration of the PR-104–free acid with one equivalent of sodium bicarbonate gave an
aqueous solubility of >200 mmol/L. The MTD of PR-104 as a single i.p. dose in nude mice was 1.33 mmol/kg i.p., equivalent to 770 mg/kg of the free acid (molecular weight, 579.28). MTD values for the other agents investigated (in mmol/kg) were 0.750 for cyclophosphamide, 0.237 for chlorambucil, 0.0422 for melphalan, 0.178 for tirapazamine, 0.0316 for cisplatin, 0.10 for docetaxel, and 1.0 for gemcitabine.

Facile conversion of the phosphate, PR-104, to its alcohol PR-104A (identified by retention time, absorbance spectrum, and mass spectrum) in mice was shown by liquid chromatography-mass spectrometry of plasma (Fig. 4B). Following i.v. dosing, PR-104 was cleared with an initial half-life of ~3 min; PR-104A was the major species under the curve of PR-104A after i.p. PR-104 (62 μmol-h/L) was 78% of that after i.v. PR-104 (area under the curve, 80 μmol-h/L). I.p. dosing was therefore used for therapeutic studies in mice.

Histopathology after an ultimately lethal dose of PR-104 identified mucosal cell degeneration/regeneration in the small intestines, particularly the ileum, as the dominant finding in seven of eight mice. This was usually associated with mild to moderate mucosal cell hypertrophy, although no overall loss of mucosal epithelium was found. In addition, a moderate decrease in bone marrow cellularity was seen in all animals, particularly the erythroid series and megakaryocytes. There were no other pathology findings, and in animals surviving to the end of MTD experiments (28 days), no loss of photoreceptor cells in the retina was evident, which contrasts our findings with tirapazamine and CI-1010 (15).

Aerobic and hypoxic cell killing by PR-104 in human tumor xenografts. We first assessed the antitumor activity of PR-104 by excision assay, determining clonogenic cell survival 18 h after giving the drug alone or following a single dose of ionizing radiation (15-20 Gy) to sterilize aerobic tumor cells (Fig. 5A). Using doses at 75% (SiHa and H460) or 100% (HT29) of MTD, PR-104 was active as monotherapy against SiHa, HT29, and H460 xenografts (each \( P < 0.01 \)), but neither chlorambucil nor tirapazamine provided significant single-agent activity. PR-104 showed even greater activity when combined with radiation, with cell killing at or beyond the dynamic range of the assay for all three tumor types. Tirapazamine showed modest but statistically significant (\( P < 0.01 \)) activity after radiation in all three tumors, whereas chlorambucil failed to reach significance for SiHa and H460. These results indicate that PR-104 has marked activity against both radiobiologically hypoxic and aerobic cells in SiHa, HT29, and H460 tumor xenografts at well-tolerated single doses. Further studies with the SiHa tumor, a well-characterized model with respect to hypoxic cell content (33, 34), using PR-104 at 0.266 mmol/kg (20% of its MTD), confirmed its activity after irradiation (\( P < 0.001 \)) and showed that the three reference nitrogen mustards lacked activity against hypoxic cells at 20% of their respective MTD values (Fig. 5B).

Antitumor activity of PR-104 monotherapy in tumor growth delay assays. The notable activity of PR-104 as a single agent by tumor excision assay led us to evaluate its activity using a tumor growth delay end point. To test whether PR-104 monotherapy is schedule dependent, we compared a daily (qd/C214) versus weekly (qw/C23) schedule against the chemoresistant H460 xenograft model using the same total...
We therefore hypothesized that addition of PR-104 to drugs with suboptimal extravascular transport properties would improve treatment outcome. Gemcitabine has been reported to be least effective against cells in or adjacent to hypoxic regions of tumors (35). We therefore tested the combination of gemcitabine and PR-104 against the human pancreatic xenograft model Panc-01 (Fig. 6C). Gemcitabine was active as a single agent, increasing median survival by 11 days ($P < 0.001$, log-rank test; SGD of 61%; $P = 0.001$). PR-104 possessed comparable single-agent activity in this line (median survival, 11 days; $P < 0.001$, log-rank test; SGD, 30%; $P = 0.024$). The combination provided therapeutic activity greater than either agent alone, with a median survival of 32 days ($P < 0.001$, log-rank test) with tumor regression in eight of nine animals (SGD, 152%; $P < 0.001$; tumor growth curves are shown in Supplementary Fig. S4A). Post hoc ANOVA (Holm-Sidak method) confirmed the survival probability for the combination group as significantly different from either single agent ($P < 0.01$).

Finally, we evaluated docetaxel using the androgen-refractory human prostate xenograft 22Rv1. The high molecular weight and target avidity of taxanes is likely to limit their tissue penetration (36, 37). Moderate antitumor activity was seen for either docetaxel or PR-104 alone, with median survival increases of 14.5 and 17 days ($P < 0.001$), respectively, and corresponding SGD of 122% ($P = 0.014$) and 156% ($P = 0.001$). Coadministration of docetaxel and PR-104 provided a 68-day improvement in median survival ($P < 0.001$) with tumor regression in nine of nine animals (tumor growth curves are shown in Supplementary Fig. S4B), three of which (33%) failed to regrow by day 100 (SGD, 689%; $P < 0.001$). Post hoc ANOVA confirmed the significance of the combined agent therapeutic gain ($P < 0.01$).

**Discussion**

This study describes a nitrogen mustard pro-drug, PR-104, designed to target tumor hypoxia through its selective metabolism to an activated DNA cross-linking agent. We show that PR-104 is a two-stage prodrug system; PR-104 itself is a water-soluble phosphate ester, readily formulated at high concentrations, which is rapidly hydrolyzed in vivo to the less soluble alcohol metabolite PR-104A (Fig. 4). The latter is sufficiently lipophilic to penetrate through multiple layers of tumor cells, required to reach hypoxic target cells, as shown by its selective cytotoxicity against intact and dissociated multicellular spheroids (Fig. 1C). The alcohol PR-104A is a hypoxia-activated prodrg, shown by its selective metabolic reduction (Fig. 2), DNA damage (Fig. 3), and cytotoxicity (Fig. 1) under hypoxic conditions.

The key metabolite from PR-104A in hypoxic cells, PR-104H, was identified as the hydroxylamine resulting from reduction of the nitro group to the mustard moiety. Steady-state concentrations of PR-104H in hypoxic SiHa cells were 10- to 20-fold higher under hypoxic than aerobic conditions (Fig. 2C), similar to the hypoxic cytotoxicity differential in this cell line (Fig. 1B). This is consistent with cytotoxicity occurring predominantly through this pathway under both aerobic and hypoxic conditions. Tetrahydroquinoxaline metabolites from ortho nitroreduction and intramolecular alkylation [as reported previously for the prototype DNBM SN 23862 (21, 30)] were
also detected in SiHa cells (compounds 9 and 10 in Fig. 2A). The ortho nitroreduction pathway generates a monofunctional mustard and is unlikely to contribute to cytotoxicity (30), but its stable end product 10 may be a useful biomarker for hypoxic activation of PR-104A.

We also detected multiple products arising from PR-104H (and the minor amine metabolite 6) by replacement of the mustard leaving groups with Cl- or OH- (compounds 1-5, 7, and 8). The absence of analogous products from PR-104A itself, despite its much higher concentration, is clear evidence that reduction of the nitro group activates the mustard nitrogen moiety to nucleophilic displacement. This confirms the original design concept for hypoxic activation of nitroaromatic mustards, which was to exploit the large change in electron density on the mustard nitrogen afforded by the biotransformation of an electron-withdrawing nitro group to an electron-donating hydroxylamine or amine (38). This electronic switch presumably contributes to the greater cytotoxicity of the reduced extracellular metabolites 1, 3, and 7 than PR-104A, relative to their molar concentrations, in the bioassay study (Fig. 2B).

The preliminary investigation of DNA damage by PR-104A reported here suggests that DNA interstrand cross-linking is the major mechanism of cytotoxicity. PR-104A forms cross-links in SiHa cells selectively under hypoxia (Fig. 3A), and its major hypoxic metabolite PR-104H shows hypoxia-independent selective toxicity to UV41 cells defective in DNA interstrand cross-link repair (Fig. 3B). The quantitative relationship between cytotoxicity and cross-link formation has yet to be established, but the latter is a potential response biomarker with utility during clinical development of nitrogen mustard prodrugs (39). Of note, Ser139 phosphorylation of histone H2AX to form γH2AX, a well-established biomarker of double-strand break formation (40), was shown with both PR-104A and chlorambucil, with a greater response to PR-104 but not chlorambucil under hypoxia. To our knowledge, γH2AX induction by nitrogen mustards has not previously been reported in tumor cells. Further studies are needed to determine whether this reflects collapse of replication forks at cross-links and whether γH2AX has potential as a pharmacodynamic biomarker. A possible limitation in this context is that the enhancement in γH2AX response to PR-104A by hypoxia (Fig. 3C and D) seemed to be less than that for cytotoxicity (Fig. 1C), which may reflect complicating effects on cell cycle progression and replication fork arrest.

It is noteworthy that PR-104H itself makes little contribution to bioactivity in the extracellular medium, relative to the more lipophilic metabolites in which Cl (hydrophobicity substituent constant p = 0.71) replaces the mesylate group (p = -0.88). This is consistent with the relatively low cytotoxic potency of exogenously added PR-104H (slightly less than chlorambucil) when added to UV41 cultures (Fig. 3B). We infer that the cytotoxicity of the extracellular metabolites of PR-104 reflects their membrane transport properties as well as their reactivities. The picture that emerges is of a small family of oxygen-insensitive activated metabolites of PR-104A in hypoxic cells, with a range of tissue diffusion properties, and that the more lipophilic metabolites, such as 1, 3, and 7, are likely to be the dominant mediators of bystander effects in tumors.

Frankly toxic doses of PR-104 (1.78 mmol/kg; i.p.) in athymic CD-1 mice identified gastrointestinal toxicity and bone marrow hypocellularity as probable dose-limiting toxicities. No retinal changes were evident, indicating this physiologically hypoxic normal tissue is insensitive to PR-104A [unlike tirapazamine and CI-1010 (15)], consistent with recent studies showing that the O2 concentration for 50% inhibition PR-104A cytotoxicity in SiHa cultures is 10-fold lower than the threshold of 0.2% for tirapazamine-induced radiosensitization of hypoxic tumor xenografts (57).
lower than for tirapazamine.\(^2\) This requirement for severe hypoxia for PR-104A activation may contribute to its excellent in vivo tolerance at high dose.

I.p. administration of the phosphate pre-prodrug PR-104 to mice, at 42% of its MTD, provided area under the curve values for PR-104A in plasma of mice (62 pmol-h/L) well in excess of that required for hypoxic cytotoxicity in a human tumor cell line panel in vitro (Fig. 1A; area under the curve values at IC\(_{50}\) from 2.1 to 30 pmol-h/L). Consistent with this, PR-104 showed marked activity against hypoxic cells in multiple human tumor xenograft models (Fig. 5). A striking aspect of the data is that PR-104 was much more active than tirapazamine against hypoxic cells (i.e., when given after a large dose of radiation to sterilize aerobic cells) in the three tumor models in which these agents were compared (SiHa, HT29, and H460; Fig. 5), yet PR-104A was no more potent and was less hypoxia selective than tirapazamine against these same cell lines in vitro (Fig. 1A and B). This disparity may reflect, in part, the limited ability of tirapazamine to penetrate into hypoxic regions of tumors (9), along with the 7.5-fold higher molar dose of PR-104 achievable in mice. PR-104 was also much more active than conventional nitrogen mustards (melphalan, chlorambucil, and cyclophosphamide) at equivalent fractions of their respective MTDs in killing hypoxic cells in SiHa tumors (Fig. 5B).

Further, PR-104 (but not tirapazamine) showed substantial activity as monotherapy using either excision assays (Fig. 5) or tumor growth delay end points (Table 1; Fig. 6). This shows that PR-104 kills the aerobic subpopulation as well as hypoxic cells in these tumors. It is not yet clear to what extent this reflects the operation of a bystander effect (hypoxic metabolites diffusing into adjoining aerobic regions) or whether the very high systemic exposures to PR-104A that can be achieved are sufficient to kill aerobic cells directly. The importance of direct activity against aerobic cells is suggested by an apparent correlation between aerobic sensitivity in vitro (Fig. 1) and monotherapy antitumor activity (Table 1); the three lines showing the largest tumor responses (H460, SiHa, and HT29) had high hypoxic IC\(_{50}\) values and low aerobic IC\(_{50}\) values relative to the two cell lines that were less responsive in vitro (H1299 and C33A). Single-agent activity has also been noted for VPN40541 (41), an analogue of KS119W that is a nitroaromatic prodrug of a sulfonylhydrazine DNA cross-linking agent (42). The ability of nitrogen mustards, and other cross-linking agents with long-lived DNA lesions, to cause cell cycle–independent cytotoxicity may underlie their utility in killing slowly cycling hypoxic cells in tumors. This is also consistent with the apparent schedule independence of PR-104A as monotherapy, which showed similar activity when the same total dose was given on a daily or weekly schedule. The single-agent activity of PR-104 against H460 tumors refractory to docetaxel, cisplatin, gemcitabine, and cyclophosphamide (Figs. 5A and 6A and B) is particularly striking and suggests that intratumor activation of nitrogen mustards using hypoxia targeting can overcome the treatment-refractory nature of this tumor (43, 44).

Despite this notable monotherapy activity, the therapeutic advantage of hypoxia-activated prodrugs is expected to be greatest in combination with agents that spare hypoxic cells as reported in preclinical models for tirapazamine (45), CI-1010 (13), banoxantrone (AQ4N; ref. 17), and NLCQ-1 (46) with
radiation or cytotoxic chemotherapy. The supra-additive activity of PR-104 when combined with gemcitabine and with docetaxel in two different tumor models (Fig. 6B and C) points to this potential. The use of gemcitabine and docetaxel in the treatment of severely hypoxic tumors, such as carcinoma of the pancreas (47, 48) and prostate (5, 49, 50), suggests that clinical benefit could be derived from the addition of a hypoxia-activated prodrug, such as PR-104, to standard of care. Overall, the nonclinical studies reported here show that PR-104 has marked activity against multiple human tumor xenograft models. This therapeutic activity is seen when PR-104 is used as monotherapy or in combination with agents for which hypoxic cells are likely to limit therapeutic response (illustrated by radiotherapy and docetaxel or gemcitabine chemotherapy). Our working model is that the efficacy of PR-104 reflects two key features we have sought to design into the DNBM class of hypoxia-activated prodrugs. The first is an efficient bystander effect, the possibility of which is suggested by identification of cytotoxic, oxygen-stable lipophilic extracellular metabolites of PR-104A in hypoxic tumor cell cultures (Fig. 2). Analogous metabolites of earlier DNBM prodrugs have been shown to give efficient bystander effects when reduced by Escherichia coli NTR (22, 23) or by endogenous reductases in tumor cells (21). The second feature is the restriction of metabolic activation to severe (pathologic) hypoxia to minimize activation in physiologically hypoxic normal tissues. We have recently shown that the oxygen concentrations required to inhibit cytotoxicity of PR-104A in SiHa cultures are 10-fold lower than for tirapazamine. This combination of effective inhibition of activation at normal tissue oxygen tensions with a bystander effect provides an attractive paradigm for exploiting tumor hypoxia. Clinical evaluation of PR-104, now in progress, will determine whether this promise translates into effective cancer therapy.

### Acknowledgments

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### Table 1. Monotherapy (single agent) activity of i.p. PR-104 using a q4d × 3 schedule (1.07 mmol/kg/dose) against human tumor xenografts in CD-1 nude mice

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Tissue origin</th>
<th>Tumor volume, mean ± SD (mm³)</th>
<th>Median life extension (days)</th>
<th>Long-term controls*</th>
<th>Log rank (P)</th>
<th>SGD (%) ¹</th>
<th>Mann-Whitney test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460</td>
<td>Lung</td>
<td>218 ± 126</td>
<td>35</td>
<td>1/12</td>
<td>&lt;0.001</td>
<td>400</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>H1299</td>
<td>Lung</td>
<td>310 ± 61</td>
<td>8</td>
<td>2/6</td>
<td>0.012</td>
<td>40</td>
<td>0.527</td>
</tr>
<tr>
<td>HT29</td>
<td>Colon</td>
<td>296 ± 51</td>
<td>39</td>
<td>0/6</td>
<td>0.002</td>
<td>192</td>
<td>0.004</td>
</tr>
<tr>
<td>SiHa</td>
<td>Cervix</td>
<td>254 ± 33</td>
<td>&gt;77</td>
<td>3/6</td>
<td>0.012</td>
<td>531</td>
<td>0.009</td>
</tr>
<tr>
<td>C33A</td>
<td>Cervix</td>
<td>252 ± 45</td>
<td>7</td>
<td>0/7</td>
<td>0.056</td>
<td>53</td>
<td>0.006</td>
</tr>
<tr>
<td>MiaPaCa-2</td>
<td>Pancreas</td>
<td>329 ± 65</td>
<td>12</td>
<td>0/5</td>
<td>0.514</td>
<td>81</td>
<td>0.151</td>
</tr>
<tr>
<td>Panc-01</td>
<td>Pancreas</td>
<td>165 ± 46</td>
<td>21</td>
<td>0/8</td>
<td>&lt;0.001</td>
<td>94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A2780</td>
<td>Ovary</td>
<td>254 ± 80</td>
<td>26</td>
<td>1/15</td>
<td>&lt;0.001</td>
<td>460</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Tumors nonpalpable or below end point size (mean diameter, 15 mm) at 100 d.

GD (median tumor growth delay as a % of median time for controls to reach end point; see Materials and Methods).

Pooled analysis of two experiments.

### References


Mechanism of Action and Preclinical Antitumor Activity of the Novel Hypoxia-Activated DNA Cross-Linking Agent PR-104

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