Impact of Tumor Blood Flow Modulation on Tumor Sensitivity to the Bioreductive Drug Banoxantrone[§]

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ABSTRACT

We investigated the hypoxia-dependent cytotoxicity of AQ4N (banoxantrone) using a panel of 13 cancer cell lines and studied its relationship to the expression of the quinone reductase DT-diaphorase (NQO1), which is widely found in cancer cells. We also investigated pharmacologic treatments that increase tumor hypoxia in vivo and their impact on AQ4N chemosensitivity in a solid tumor xenograft model. AQ4N showed ≥ 8 -fold higher cytotoxicity under hypoxia than normoxia in cultures of 9L rat gliosarcoma and H460 human non-small-cell lung carcinoma cells but not for 11 other human cancer cell lines. DT-diaphorase protein levels and AQ4N chemosensitivity were poorly correlated across the cancer cell line panel, and AQ4N chemosensitivity was not affected by DT-diaphorase inhibitors. The

vasodilator hydralazine decreased tumor perfusion and increased tumor hypoxia in 9L tumor xenografts, and to a lesser extent in H460 tumor xenografts. However, hydralazine did not increase AQ4N-dependent antitumor activity. Combination of AQ4N with the angiogenesis inhibitor axitinib, which increases 9L tumor hypoxia, transiently increased antitumor activity but with an increase in host toxicity. These findings indicate that the capacity to bioactivate AQ4N is not dependent on DT-diaphorase and is not widespread in cultured cancer cell lines. Moreover, the activation of AQ4N cytotoxicity in vivo requires tumor hypoxia that is more extensive or prolonged than can readily be achieved by vasodilation or by antiangiogenic drug treatment.

Introduction

Tumor angiogenesis gives rise to blood vessels that are tortuous, leaky, and irregular. Newly formed tumor blood vessels lack smooth muscle and have an incomplete endothelial lining and basement membrane (Brown and Giaccia, 1998). As a consequence, many solid tumors have low blood flow, poor oxygenation, and regions of chronic and acute hypoxia (Hockel and Vaupel, 2001; Vaupel and Mayer, 2007). Hypoxic tumors present a highly malignant phenotype (Hockel and Vaupel, 2001; Vaupel and Mayer, 2007) and are intrinsically resistant to ionizing radiation, whose cytotoxic effects are oxygen dependent (Nordsmark and Overgaard, 2000). Tumor hypoxia is also associated with resistance to many chemotherapeutic drugs (Vaupel and Mayer, 2007), which often target rapidly dividing cells, in particular those close to capillary beds. Tumor cells distant from blood vessels divide slowly, have low pH, and due to low perfusion are poorly exposed to many conventional chemotherapeutic drugs.

Bioreductive drugs are reduced to cytotoxic metabolites under hypoxic conditions, which gives them the potential circumvent the general chemoresistance associated with

a hypoxic tumor environment. The bioreductive drug AQ4N (banoxantrone; 1,4-bis[2-(dimethylamino)-*N*-ethylamino]-5,8-dihydroxyanthracene-9,10-dione) (Patterson and McKeown, 2000; Patterson, 2002; Atkinson et al., 2007) is an analog of mitoxantrone that undergoes a two-step, four-electron reduction in a hypoxic environment that initially forms the short-lived intermediate AQ4M and then the ultimate cytotoxic metabolite AQ4, a tertiary amine. AQ4 intercalates into DNA and inhibits topoisomerase II; moreover, it can passively diffuse into neighboring cells and exert bystander cytotoxicity (Patterson, 2002). AQ4N has a bis-*N*-oxide quinone structure with the potential to be reduced under hypoxic conditions by several cellular enzymes, including cytochrome P450 (McCarthy et al., 2003; Nishida et al., 2010) and inducible nitric oxide synthase (iNOS) (Nishida and Ortiz de Montellano, 2008; Mehibel et al., 2009). AQ4N is considered an ideal bioreductive drug in that it penetrates deep into tumor tissue (Tredan et al., 2009). Its active metabolite, AQ4, is stable, localizes to hypoxic tumor regions in both preclinical and clinical studies (Albertella et al., 2008; Williams et al., 2009), and binds to DNA even under normoxic conditions, yet can passively diffuse into neighboring tumor cells (Smith et al., 1997). In contrast, poor tumor penetration is achieved with the related drug mitoxantrone.

Tumor hypoxia can be modulated to increase the antitumor activity of bioreductive drugs such as AQ4N. Tumor hypoxia

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ABBREVIATIONS: AQ4N, banoxantrone; CPA, cyclophosphamide; DMEM, Dulbecco's modified Eagle's medium; DT-diaphorase, NAD(P)H dehydrogenase-quinone 1 (NQO1); FAA, flavone 8-acetic acid; FBS, fetal bovine serum; HCR, hypoxia cytotoxicity ratio; iNOS, inducible nitric oxide synthase; NAD(P)H, dehydrogenase; NQO1, quinone 1; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20.

can be increased by physically occluding blood flow to the tumor using a clamp (Denekamp et al., 1983) or by use of hydralazine, which vasodilates muscle, shunting blood from tumors into the surrounding muscle tissue thereby decreasing tumor blood perfusion via a steal effect (Belfi et al., 1994). Both of these approaches are reported to increase the antitumor activity of AQ4N in mouse tumor models (Patterson et al., 2000). A novel approach, investigated here, uses antiangiogenic drugs to increase tumor hypoxia. We tested this approach using axitinib, a vascular endothelial growth factor receptor-selective tyrosine kinase inhibitor (Hu-Lowe et al., 2008). Axitinib decreases tumor blood vessel patency, decreases microvessel density, and increases tumor hypoxia (Ma and Waxman, 2008). Axitinib initially causes tumor growth stasis in the case of 9L gliosarcoma xenografts, but when combined with cyclophosphamide (CPA), it decreases the entry of the liver-activated CPA metabolite 4-OH-CPA into the tumor cells, as well as antitumor innate immune cells, and compromises overall antitumor activity (Ma and Waxman, 2008; Doloff and Waxman, 2012). This limitation of antiangiogenesis may potentially be circumvented by using a bioreductive drug such as AQ4N, which can penetrate into hypoxic tumor regions (Williams et al., 2009).

We used a panel of tumor cell lines to investigate the hypoxia-dependent cytotoxicity of AQ4N and the impact of the two-electron quinone reductase DT-diaphorase (NQO1) (Siegel et al., 2012), which is widely expressed in tumor cells (Fitzsimmons et al., 1996). DT-diaphorase reduction of quinones can increase cytotoxicity (Beall et al., 1995), but in some instances can be protective under hypoxic conditions (Plumb et al., 1994). We also investigated pharmacologic treatments that increase tumor hypoxia in vivo and their impact on AQ4N chemosensitivity in a solid tumor xenograft model. Our findings show that increases in the hypoxic fraction of 9L gliosarcoma xenografts can readily be achieved using the vasodilator hydralazine or the antiangiogenic agent axitinib, but these treatments failed to give a sustained increase in AQ4N antitumor activity.

Materials and Methods

Chemicals and Antibodies. Axitinib was obtained from Pfizer Global Research and Development (Groton, CT). AQ4N (29.7 mg/ml stock solution) was obtained from Novacea, Inc. (South San Francisco, CA). Crystal violet, formaldehyde, hydralazine, NADH, menadione, dithiothreitol, TEMED, ammonium persulfate, dimethylsulfoxide, Scott's Tap Water (cat. S5134) and dicumarol were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography grade methanol was purchased from J.T. Baker (Phillipsburg, NJ). Paraformaldehyde solution (16%; methanol-free) was purchased from Electron Microscopy Sciences (Hatfield, PA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Polyethylene glycol 400 was purchased from Hampton Research (Aliso Viejo, CA). Mayer's Hematoxylin (HK100-20) was purchased from Biogenex (San Ramon, CA). Normal horse serum, avidin/biotin blocking kit, biotinylated horse anti-mouse antibody (BA-2000), Vectastain Elite ABC Kit, VIP peroxidase substrate, and VectaMount were purchased from Vector Laboratories (Burlingame, CA). Hypoxyprobe-1 kit, including pimonidazole, was purchased from Chemicon (Temecula, CA). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 culture medium were purchased from Invitrogen (Carlsbad, CA). Minimum Eagle's medium and McCoy's 5A culture medium were purchased from ATCC (Manassas, VA). Flavone 8-acetic acid (FAA; NSC-347518) was obtained from the NCI

Developmental Therapeutics Program (Frederick, MD). Dicumarol and FAA were dissolved in dimethylsulfoxide at room temperature and prepared fresh for each experiment. Acrylamide/bis-acrylamide (30% wt/vol) was purchased from National Diagnostics (Atlanta, GA). Primary antibody to DT-diaphorase (goat polyclonal antibody IC-191 [sc-16464]) and horseradish peroxidase-conjugated donkey-anti-goat secondary antibody (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines. Cell lines were authenticated by and obtained from the following sources. Rat gliosarcoma cell line 9L and its derivative, 9L/2B11, which expresses cytochrome P450 2B11, were described previously elsewhere (Jounaidi et al., 2006). Human tumor cell lines BxPC-3 (renal), FaDu (head and neck), and HT-29 (colon) were purchased from ATCC. Other human tumor cell lines (non-small cell lung carcinoma (NSCLC): A549, H460, and H522; breast: MCF-7 and T47D; brain: U251; prostate: PC3 and DU-145; colon: KM-12 and Colo-205) were obtained from Dr. Dominic Scudiero (National Cancer Institute, Frederick, MD). Cell cultures were grown in the following media, all supplemented with penicillin/streptomycin: DMEM + 10% FBS (9L and 9L/2B11 cells), minimum Eagle's medium + 10% FBS (FaDu cells), McCoy's 5A medium + 10% FBS (HT-29 cells), RPMI medium + 10% FBS (BxPC-3 cells), and RPMI medium + 5% FBS (all other cell lines).

Growth Inhibition Assay. AQ4N dissolved in 0.9% NaCl at 65 mM (~30 mg/ml) was diluted to 10 mM in phosphate-buffered saline (PBS) and stored at 4°C. Tumor cells were seeded in triplicate wells of 48-well plates at densities ranging from 4×10^3 to 1×10^4 cells/well in 0.5 ml of culture medium, depending on the cell line's doubling time, and were grown for 24 hours under normoxia. Cells were then treated for 24 hours with AQ4N (0 to 0.5 mM) in 1 ml of culture medium under normoxia or under hypoxia (0.1% O₂). Hypoxia was maintained using a Pro-Ox2 system (Biospherix, LaConia, NY) with CO₂ maintained at 5%. Wells were rinsed once with PBS, then cultured in drug-free media under normoxia for an additional 3 days. Cells were washed twice with PBS on ice and stained for 20 minutes with crystal violet solution (1.25 g of crystal violet in 50 ml of 37% formaldehyde mixed with 450 ml of methanol and stored at room temperature). The plates were rinsed 3 times in cold water and vacuum dried overnight. The stain was eluted with 70% ethanol/well for 1 hour (1 ml/well) and read at 595 nm on a UV SpectraMax384 plate reader (Molecular Devices Corporation, Sunnyvale, CA) in NUNC 96-well Maxisorp U-shaped plates (VWR International, Bridgeport, NJ).

Cell killing was determined after normalization to drug-free controls and subtraction of background absorbance (Schwartz and Waxman, 2001). EC₅₀ values were calculated using a nonlinear regression sigmoidal dose-response (variable slope) model as implemented in GraphPad Prism 4.0 software (GraphPad Inc., San Diego, CA). The absolute EC₅₀ values varied somewhat from experiment to experiment, but the hypoxia cytotoxicity ratios (HCR values) and the overall shapes of the dose-response curves were consistent. HCR is defined as the ratio of EC₅₀ for cell killing under normoxia to EC₅₀ for cell killing under hypoxia.

To study the effect of DT-diaphorase inhibition on chemosensitivity to AQ4N, 9L and H460 cells were seeded in 48-well plates at 4×10^3 and 6×10^3 cells/well, respectively, and were cultured overnight under normoxic conditions. The cells were pretreated for 4 hours under normoxia with either dicumarol or FAA, followed by 24 hours of further treatment in the presence of AQ4N, combined with either dicumarol or FAA, under either normoxia or hypoxia. Drug-containing culture medium was removed and replaced with drug-free medium. Cell growth continued for 3 days under normoxia, after which cell survival was assayed by crystal violet staining.

DT-Diaphorase Expression. Tumor cell lines were seeded overnight for 24 hours at 37°C at 2×10^6 cells/10-cm dish and cultured under normoxic conditions. The cell culture was then continued another 24 hours under normoxia or under hypoxia (0.1% O₂). Cells were washed, and extracts were prepared in 50 mM KPi buffer (pH 7.4) with 1 mM EDTA and 20% glycerol containing 0.1 mM

phenylmethylsulfonyl fluoride and 0.1% dithiothreitol by sonication (5 × 5 second pulses at 20% power) using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), followed by centrifugation at 15,000g for 20 minutes at 4°C. The cell supernatant (40 µg protein, determined using the Dc Protein Assay kit [cat. no. 500-0112]; Bio-Rad Laboratories, Hercules, CA) was used for Western blot analysis of DT-diaphorase protein on a 12.5% SDS-acrylamide gel. Nitrocellulose membranes were blocked with 5% fat-free milk in Tris-buffered saline/Tween 20 (TBST) buffer (1 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) and were probed for 1 hour at room temperature with rabbit polyclonal anti-DT-diaphorase (1:1500) in TBST. The blot was then washed in TBST and incubated with goat-anti-rabbit-horseradish peroxidase secondary antibody (1:5000) in TBST. The blot was washed 3 × 5 minutes, and DT-diaphorase protein was visualized with Amersham enhanced chemiluminescence detection reagent (cat. no. RPN 2106; Piscataway, NJ) and exposed to Kodak X-OMAT blue film XB-1.

Tumor Growth Studies. Six-week-old immunodeficient male Fox Chase ICR *scid* mice (24–26 g) and male NCr nude (nu/nu) mice (21–23 g) were purchased from Taconic Farms (Germantown, NY) and housed in the Boston University Laboratory of Animal Care Facility. All animal studies were performed in accordance with protocols approved by the Boston University Institutional Animal Care and Use Committee. Mice were euthanized if they approached authorized tumor size limits specified in the approved animal protocol. Autoclaved cages containing food and water were changed once a week. Body weights were measured every 1 to 3 days, depending on the drug administration route.

Mice were implanted with tumor cells grown to 70% to 80% confluence in DMEM containing 10% FBS (9L cells) or in RPMI containing 5% FBS (H460 cells), trypsinized, and resuspended in serum-free media at a concentration of 8×10^6 cells/ml, then kept on ice until injection. 9L cells (either 2 or 4×10^6) and H460 cells (6×10^6) in a volume of 0.2 mL were injected subcutaneously (s.c.) into each flank (two tumors per mouse) using a 28-gauge needle. Tumor sizes (length $L \times$ width W) were measured every 3 to 4 days using digital calipers (VWR International, Radnor, PA), and tumor volumes were calculated using the equation $V = (\pi/6) \times (L \times W)^{3/2}$. When tumors reached a mean volume of 250–750 mm³ (9L, as specified) or 3500 mm³ (H460), mice were treated with AQ4N diluted in PBS (100, 125, or 150 mg/kg, and 0.15 ml injected i.p. per mouse, as specified), either with or without hydralazine treatment (10 mg/kg, i.p.) given 15 minutes later. In some cases, mice were treated with pimonidazole (100 mg/kg, i.p.) followed 15 minutes later by either hydralazine (10 mg/kg, i.p. in PBS) or PBS. Where indicated, Hoechst 33342 (15 mg/kg, i.v.) was administered 44 minutes after pimonidazole treatment, and the mice were euthanized 1 minute later. The 45 minutes of total time from pimonidazole injection to tumor harvest is sufficient for pimonidazole to circulate through the body and provide a reliable assay for hypoxia induced by hydralazine (Ljungkvist et al., 2000; Olive et al., 2000; Shin et al., 2007).

AQ4N Toxicity in Nude Mice. Non-tumor-bearing male NCr nude mice (6 weeks old, 21–23 g) were initially administered AQ4N (100 mg/kg) by i.v. or i.p. injection to determine AQ4N toxicity, as indicated by body weight loss ($n = 6$ mice/group). The i.v. treatment group was then given AQ4N at 125 mg/kg by i.v. injection every 7 days. Mice initially given 100 mg/kg AQ4N by i.p. injection were subsequently treated with 125 mg/kg i.p. AQ4N every 2 weeks. Body weight was monitored relative to the initial treatment day, and the data are presented as mean $\pm$ S.E.

Effect of Axitinib and AQ4N on 9L Tumor Growth. Axitinib was suspended at 5 mg/ml in polyethylene glycol 400 and sonicated in a water bath at room temperature for 20 minutes to obtain a fine suspension. The pH was adjusted to ~2.0–3.0 using 0.1 N HCl followed by a second sonication. A final 3:7 (v/v) ratio of polyethylene glycol 400:H₂O was obtained by adding acidified water (pH 2.0–3.0) (Ma and Waxman, 2009). The injection-ready solution was prepared every 4 to 5 days and stored at 4°C in the dark. Axitinib was

administered to tumor-bearing mice daily by i.p. injection at 25 mg/kg body weight and in a volume of 5 µl/g body weight. Control mice received daily i.p. injection of vehicle (30% polyethylene glycol 400/70% acidified water, pH 2–3) in a volume of 5 µl/g body weight. To test the impact of persistent hypoxia induced by daily axitinib treatment, mice were treated with 1) vehicle control, daily for 24 days; 2) axitinib (25 mg/kg, i.p.), daily for 24 days; 3) AQ4N (125 mg/kg, i.v.) once on day 5; or 4) AQ4N + axitinib in combination, with AQ4N administered 4 hours after axitinib on days 5, 12, and 19. Tumor growth was measured as described earlier.

Tumor Cryosectioning and Immunohistochemical Staining. Fresh tumor tissue was snap frozen in dry ice–chilled 2-methylbutane and stored at –80°C. Tumor cryosections (6 µm, 3–4 sections/slide) were prepared using a Leica 1800 cryostat. Slides were scanned for Hoechst 33342 fluorescence then stained for pimonidazole. Cryosections were rinsed with PBS for 30 seconds, then fixed with 1% paraformaldehyde in PBS at room temperature for 30 minutes. After a PBS wash (5 minutes), slides were incubated with permeabilization solution (1% Triton X-100 (v/v) and 1% sodium citrate (w/v) in 1× PBS) for 5 minutes on ice followed by a second PBS wash (5 minutes). Slides were blocked 20 minutes at room temperature with PBS containing 2% normal horse serum. The slides were then incubated for 1 hour at 37°C with Hypoxyprobe-1 (primary antibody) diluted 1:50 followed by two 5-minute PBS washes. Slides were incubated for 1 hour at 37°C with biotinylated anti-mouse secondary antibody diluted to 7.5 µg/mL (1:200), followed by three 5-minute PBS washes.

Slides were then incubated with Vectastain Elite ABC complex for 30 minutes at room temperature followed by two 5-minute PBS washes. Peroxidase substrate (VIP) was added, and color development was terminated by immersing the slides in tap water. Slides were counterstained with Mayer's hematoxylin for 15 seconds, followed by 3 ddH₂O rinses, then one wash with 5% acetic acid for 1 minute, followed by Scott's Tap Water 2 × 2 minutes, then 95% ethanol for 2 minutes. The slides were dehydrated by sequential washing with 95% ethanol (twice for 2 minutes), 100% ethanol (twice for 2 minutes), and 100% xylene (twice for 3 minutes) and sealed with VectaMount. Negative control slides were incubated with primary antibody solution without primary antibody. Immunostained tumor sections were examined using an Olympus BX51 bright-field light microscope. Hypoxyprobe staining was observed at 10× magnification. Counterstained slides were observed at 4.2× and 20× magnification on an Olympus FSX 100 BioImaging Navigator microscope.

Hoechst 33342 Perfusion. Hoechst 33342, a DNA-binding fluorescent dye that is used to monitor tumor blood flow and perfusion (Ljungkvist et al., 2000), was used to determine the effect of hydralazine on 9L and H460 tumor perfusion. Stock solutions of 16 mM Hoechst 33342 in PBS were stored at 4°C in the dark. Tumor-bearing mice were given a tail vein injection of 15 mg of Hoechst 33342/kg body weight (40–55 µl/mouse; injection using a 29 G insulin syringe) and then were euthanized 1 minute later. Mice were injected with pimonidazole 45 minutes before euthanasia, followed 15 minutes later with either hydralazine or PBS (vehicle) injection, followed 29 minutes later by Hoechst 33342 injection. Tissue samples were collected and processed for cryosectioning as described earlier. Tumors were analyzed using a fluorescent microscope and captured with an Olympus MagnaFire digital camera at 20× magnification. The Hoechst positive staining area of each tumor was quantified using National Institutes of Health (NIH) Image J software based on data collected from at least 20 images per tumor using methods described previously elsewhere (Ma and Waxman, 2009), and the data are expressed as a percentage of the image area, mean $\pm$ S.E., based on $n = 4$ –7 tumors per group.

Statistical Analysis. Results are expressed as mean $\pm$ S.E. and are based on the indicated number of tumor or tissue samples per group. Statistical significance was assessed by two-tailed Student's t test, as indicated, using GraphPad Prism 4.0 software and $P \leq 0.05$.

Results

Hypoxia Increases AQ4N Chemosensitivity in 9L and H460 Cells but Not in 10 Other Tumor Cell Lines.

A panel of 13 human and rat tumor cell lines was treated with AQ4N (0–1 mM) under both normoxic (~20% O₂) and hypoxic (0.1% O₂) conditions. Dose-response curves for AQ4N cytotoxicity were shifted to the left under hypoxia as compared with normoxia in the case of 9L and H460 cells (Figs. 1, A and B), with a ninefold sensitization to cell killing under hypoxia observed at the EC₅₀ concentration (i.e., hypoxia cytotoxicity ratio (HCR) = 9; Table 1). The dose-response curves were much steeper under normoxia, such that the degree of chemosensitization by hypoxia was greatest at low AQ4N concentrations and ultimately was eliminated at high AQ4N concentrations. This suggests that AQ4N kills cells by a different mechanism under hypoxia compared with normoxia. A549 and U251 cells gave HCR values of ~3, and all other cell lines tested showed HCR values <1.5 (Table 1), with the shapes of the dose-response curves being far more similar for normoxia versus hypoxia (e.g., Du145 and MCF-7 cells; Figs. 1, C and D). The intrinsic sensitivity of the cells to AQ4N under normoxia varied widely, ranging from ~3 μM (A549 and U251 cells) to ~130–160 μM (FaDu and HT29 cells). BxPC-3 and PC3 cells were insensitive to AQ4N at the highest concentrations tested (250 and 500 μM, respectively). 9L/2B11 cells, which express the drug-metabolizing P450 enzyme 2B11, showed the same chemosensitivity to AQ4N as 9L cells,

under both normoxia and hypoxia (Table 1), indicating that P450 2B11 does not activate AQ4N.

DT-Diaphorase Does Not Determine the Cell Line Dependence of AQ4N Sensitivity. AQ4N is a derivative of mitoxantrone, which has a quinone structure that is reduced by DT-diaphorase (Fisher et al., 1992). We therefore investigated whether DT-diaphorase could contribute to AQ4N bioactivation and chemosensitivity. Western blot analysis of the tumor cell line panel (Fig. 2A) showed that DT-diaphorase levels ranged from high (H460, DU145, A549, and FaDu cells), to intermediate (9L, Colo-205, HT-29, U251, and BxPC-3), to low (KM12, H522, and PC3 cells), with no DT-diaphorase protein detected in T47D and MDA-MB-231 cells. DT-diaphorase levels were similar in cells grown under hypoxia and normoxia (Fig. 2A; Table 1). Moreover, there was no correlation between DT-diaphorase expression (Fig. 2A) and AQ4N chemosensitivity (Table 1). For example, FaDu cells showed very low AQ4N sensitivity but high DT-diaphorase expression. Moreover, 9L and HT-29 cells showed similar levels of DT-diaphorase protein, but HT-29 cells were ~30-fold less sensitive to AQ4N under normoxia, and ~140-fold less sensitive under hypoxia. Similarly, MCF-7 and U251 cells had similar DT-diaphorase protein levels, but U251 cells were 20- to 40-fold more sensitive to AQ4N. Finally, BxPC-3 cells expressed moderate levels of DT-diaphorase but were insensitive to AQ4N (Fig. 2A versus Table 1). Furthermore, the DT-diaphorase inhibitors dicumarol (35 μM) (Preusch et al., 1991) and FAA (20 or 100 μM) (Phillips, 1999) did not have

Fig. 1. Effect of AQ4N on the growth of cultured cancer cell lines. (A) 9L, (B) H460, (C) DU145, and (D) MCF-7 cells were seeded in 48-well plates at densities of 4.0, 6.6, 6.0, and 7.5 × 10³ cells/well, respectively, for 24 hours under normoxia. Cells were then treated with AQ4N (0–0.25 mM) for 24 hours under normoxia or 0.1% O₂. The culture medium was then changed to drug-free medium, and the cells were additionally cultured for 3 days under normoxia. Relative cell survival was determined by crystal violet staining of the cells remaining at the end of the experiment (A595 nm). Data shown are based on a single experiment for each cell line (mean ± S.D. for triplicate samples) and are representative of at least three independent determinations for each cell line, as summarized in Table 4.

TABLE 1

AQ4N sensitivity of cancer cell lines to AQ4N under normoxia and hypoxia.

AQ4N cytotoxicity was determined under either normoxia or hypoxia (0.1% O₂) and EC₅₀ (μM) values determined as described under *Materials and Methods*. EC₅₀ and HCR (hypoxia cytotoxicity ratio) values shown are mean ± S.D. based on *n* = 3–8 independent experiments for each cell line. DT-diaphorase activity data were obtained from published sources (Fitzsimmons et al., 1996; Doherty et al., 1998; Dehn et al., 2006; Begleiter et al., 2009). Cell lines are grouped into four groups (A–D) based on their level of DT-diaphorase protein (Fig. 2A), as shown here: high, intermediate, low, and no DT-diaphorase expression (top to bottom). Dashes indicate that data are not available. Activity data generally parallel the protein levels shown in Fig. 2.

Tumor Cell Line	Tumor Type	EC ₅₀ (μM) normoxia	EC ₅₀ (μM) hypoxia	DT-diaphorase activity	Hypoxia Cytotoxicity Ratio
<i>nmoles/mg/min</i>					
High DT-diaphorase protein					
H460	Lung	5.76 ± 3.41	0.69 ± 0.64	5012	9.12 ± 2.37
DU145	Prostate	40.3 ± 3.98	34.3 ± 20.3	2511	1.41 ± 0.25
A549	Lung	2.83 ± 1.55	0.87 ± 0.24	1548	3.26 ± 1.9
FaDu	Head and neck	132.4 ± 74.8	91.5 ± 11.2	510	1.41 ± 0.79
Intermediate DT-diaphorase protein					
9L	Brain	5.04 ± 2.37	1.08 ± 1.62	—	9.05 ± 3.22
9L/2B11	Brain	4.99 ± 3.5	1.55 ± 2.37	—	7.72 ± 4.0
Colo-205	Colon	32.67 ± 10.5	33.84 ± 33.2	1000	2.28 ± 2.25
HT-29	Colon	157.5 ± 49.6	154.5 ± 54	550	1.0 ± 0.34
U251	Brain	3.17 ± 0.41	1.09 ± 0.47	446	3.19 ± 0.93
BxPC-3	Pancreatic	>250	>250	400	—
MCF-7	Breast	62.4 ± 6.13	43.6 ± 7.76	63.1	1.45 ± 0.04
Low DT-diaphorase protein					
KM12	Colon	—	—	398	—
H522	Lung	44.7 ± 25.5	28.9 ± 19	—	1.34 ± 0.53
PC3	Prostate	>500	>500	251	—
No DT-diaphorase protein					
T47D	Breast	—	—	50	—
MDA-MB-231	Breast	—	—	2.5	—

a significant effect on the chemosensitivity of 9L cells to AQ4N under either normoxic or hypoxic culture conditions (Fig. 2, B–E). Together, these findings indicate that DT-diaphorase does not contribute significantly to AQ4N cytotoxicity.

Hydralazine Increases Tumor Hypoxia and Decreases Tumor Perfusion. Hydralazine was administered to *scid* mice bearing 9L or H460 tumors to induce tumor hypoxia. A substantial decrease in tumor blood flow was achieved, as

Fig. 2. DT-diaphorase protein levels and contribution to AQ4N cytotoxicity in human and rat cancer cell lines. (A) Western blot analysis of DT-diaphorase protein in whole-cell lysates prepared from each of the indicated cell lines, grown under conditions of normoxia or hypoxia (0.1% O₂) for 24 hours, as described under *Materials and Methods*. For each cell line, the first sample shown is from cells grown under normoxia, and the second sample is from cells grown under hypoxia, except for 9L/2B11 cells (marked by *), where the order is reversed. MB231, MDA-MB-231 breast cancer cell line. Rat DT-diaphorase protein, present in rat 9L and 9L/2B11 cells, migrates at a higher apparent molecular weight than human DT-diaphorase protein (other lanes), as expected (Beall et al., 1994). (B–E) Effect of DT-diaphorase inhibitors dicumarol (Dicum) and FAA pretreatment (4 hours) on AQ4N sensitivity under normoxia (B and D) or hypoxia (C and E) in the continued presence or absence of dicumarol (B and C) or FAA (D and E) for an additional 24 hours. The culture medium was then changed to drug-free medium, and the cells were cultured for an additional 3 days. EC₅₀ values are specified in the inset on each graph. Data shown are mean values ± S.D. (*n* = 3).

indicated by the decrease in uptake and staining by Hoechst 33342 in both tumor models (Fig. 3A). The suppression by hydralazine of tumor blood flow (Hoechst staining) was observed over a 3-fold range in 9L tumor size (unpublished data). The decrease in tumor blood flow was more complete in 9L tumors and was associated with an increase in tumor hypoxia, as judged by the increase in pimonidazole staining (Fig. 3B). 9L tumors are highly vascularized (Ma and Waxman, 2007) and show little hypoxia in the absence of hydralazine treatment. In contrast, in the H460 tumors, which contained extensive hypoxic regions before the hydralazine treatment, hydralazine decreased tumor blood flow by only ~50% (Fig. 3A), and it had only a small effect on tumor hypoxia (Fig. 3B). H460 tumors are highly vascularized (Mukhopadhyay et al., 2002; Navab et al., 2009) but have heterogeneous regions of hypoxia (Lam et al., 2009), in agreement with our findings.

AQ4N Is More Toxic to *scid* Mice Than Nude Mice. Our initial efforts to evaluate the effects of hydralazine on

AQ4N activity against 9L tumors grown in *scid* mice (Supplemental Fig. 1A) were hampered by the severe toxicity of AQ4N (Supplemental Fig. 1B). 9L tumor-bearing *scid* mice treated with AQ4N (150 mg/kg AQ4N, i.p.) rapidly lost body weight, became frail and dehydrated, and died within 8 days. Subsequent studies were therefore performed on nude mice, where AQ4N (100 mg/kg and 125 mg/kg) was noticeably less toxic, and where toxicity could be further reduced by i.v. AQ4N delivery (i.v. AQ4N: 8% body weight loss over 3 days with recovery complete within 7 days versus i.p. AQ4N: 15% body weight loss over 7 days with a second week required for recovery; Supplemental Fig. 2, solid line versus dashed line). The body weight loss associated with i.v. AQ4N treatment is consistent with an earlier report (Lalani et al., 2007). AQ4N was best tolerated when given by weekly i.v. injection, at either 100 or 125 mg/kg, with no additional toxicity apparent when the dose of AQ4N was increased to 125 mg/kg (Supplemental Fig. 2, treatments beginning day 29–30). Subsequent studies were therefore performed on nude mice given AQ4N by i.v. injection at 125 mg/kg.

Hydralazine Does Not Increase AQ4N Antitumor Activity. Given the finding that hydralazine renders 9L tumors hypoxic (Fig. 3B), we hypothesized that hydralazine might increase AQ4N antitumor activity. 9L tumor growth was not significantly affected by periodic treatment with hydralazine (Fig. 4A). AQ4N (125 mg/kg, i.v.) induced a small

Fig. 3. Hydralazine increases hypoxia in 9L tumors more extensively than in H460 tumors, and decreases tumor blood perfusion in both models. 9L and H460 tumors were grown s.c. in *scid* mice to sizes of 750 mm³ and 3,500 mm³, respectively. The mice were then injected with pimonidazole (100 mg/kg, i.p.) followed by PBS or hydralazine (10 mg/kg, i.p.) 15 minutes later. Hoechst 33342 (15 mg/kg, i.v.) was injected 29 minutes later, and after 1 minute the mice were euthanized. (A) Hoechst staining indicated that hydralazine decreased tumor blood perfusion in 9L tumors (15.9 ± 8.1 versus 2.9 ± 1.5, *n* = 7 tumors) and in H460 tumors (19.7 ± 3.5 versus 10.1 ± 2.2, *n* = 4 tumors) compared with controls. Values shown are mean ± S.E. for the indicated number of tumors per group, showing a decrease of nearly 2-fold in the Hoechst staining in H460 tumors and a decrease of >5-fold in 9L tumors; however, the difference did not meet *P* < 0.005 significance by one-tail and two-tail *t* test. (B) Immunostaining for hypoxia-specific dye pimonidazole (purple) and counterstaining with hematoxylin (blue) revealed a substantial increase in tumor hypoxia in 9L (*n* = 7) but not H460 tumors (*n* = 4), as evident from the representative Olympus BX51 microscopic photos taken at 4.2× and 20× magnification, as indicated.

Fig. 4. Hydralazine does not increase AQ4N antitumor activity against 9L tumor xenografts in nude mice. (A) Tumor volumes. (B) Body weight profiles. 9L tumors grown s.c. in male NCr nude mice were administered vehicle (PBS, i.p.), hydralazine (Hyd) (10 mg/kg, i.p.), AQ4N (125 mg/kg, i.v.), or AQ4N followed by hydralazine 15 minutes later. Drug treatment was on days 0, 9, 15, and 21 (arrows along x-axis). Error bars represent mean ± S.E. for tumor volume or body weight based on *n* = 5–6 mice (10–12 tumors) per treatment group at the beginning of drug treatment. Decreases in mice and tumor number during the course of treatment are indicated by the decline in number of tumors, shown in parentheses.

antitumor response, but no further increase in antitumor activity was seen when AQ4N was combined with hydralazine, even after several treatment cycles (Fig. 4A). Body weight profiles showed increases in weight, as expected from the increase in tumor burden (Fig. 4B). The cumulative toxicity of AQ4N under these conditions resulted in several deaths after the fourth treatment cycle. Given the increase in 9L tumor hypoxia induced by hydralazine and the effectiveness of the same schedule of hydralazine treatment in prior studies (Patterson et al., 2000), the lack of an effect of hydralazine suggests that prolonged or more extensive hypoxia is required to potentiate AQ4N activity in this tumor model.

Antiangiogenesis-Induced Hypoxia Transiently Increases AQ4N Antitumor Activity. Axitinib, a vascular endothelial growth factor receptor-selective angiogenesis inhibitor, increases 9L tumor hypoxia within 4 days, with further increases seen after 9 days of axitinib treatment (Ma and Waxman, 2007; Ma and Waxman, 2008). Therefore, we used axitinib-treated 9L tumors as a model to study the effects of persistent hypoxia on AQ4N activity. Nude mice bearing 9L tumor xenografts were treated with AQ4N alone or with AQ4N in combination with daily axitinib treatment, which was initiated 5 days before AQ4N treatment (neoadjuvant axitinib treatment schedule). AQ4N alone had no significant antitumor effect, while axitinib alone led to strong tumor growth inhibition (Fig. 5A). AQ4N in combination with axitinib resulted in a short-term delay of tumor growth compared with that effected by axitinib alone (days 12–24; Fig. 5A), but this transient increase in antitumor activity was accompanied by a marked increase in host toxicity (Fig. 5B). Moreover, rapid tumor growth resumed 8 days after cessation of axitinib treatment on day 24, consistent with an earlier report (Mancuso et al., 2006). The toxicity of axitinib seen in nude mice was greater than that seen previously using the same 9L tumor model in *scid* mice (Ma and Waxman, 2008), as manifested by dehydration and the persistent suppression of body weight gain. Furthermore, the combination of axitinib with AQ4N resulted in a substantial increase in host toxicity (Fig. 5B).

Discussion

AQ4N and other bioreductive drugs are reduced to cytotoxic metabolites in a hypoxic environment, enabling them to preferentially kill hypoxic tumor cells, which are often resistant to radiation and traditional chemotherapeutic drugs. In the case of AQ4N, di-*N*-oxides mask its tertiary amino groups and inhibit DNA binding (Patterson and McKeown, 2000) until it undergoes a two-step, four-electron reduction to the di-amino derivative AQ4, which binds DNA tightly and exerts cytotoxicity (Wilson et al., 1996). AQ4 is more cytotoxic than AQ4N toward many tumor cell lines—as high as 380-fold more cytotoxic in leukemia cells—when tested under normoxic conditions (Alters et al., 2005; Cleland et al., 2005; Lalani et al., 2007). Moreover, the cytotoxicity of AQ4N toward V79 cells under anoxic conditions can be increased ~100-fold by incubation in the presence of liver microsomal enzymes that convert AQ4N to AQ4 (Patterson, 1993). However, few reports investigate whether AQ4N is more active against cultured cancer cells under hypoxic compared with normoxic conditions (Mehibel et al., 2009).

Fig. 5. Antitumor activity of AQ4N-axitinib combination in 9L tumor xenografts. Male NCr nude mice were injected with 2×10^6 9L cells/site (s.c.) and axitinib treatment was initiated when the tumors reached ~ 350 mm³. Blue arrows below the x-axis: days that AQ4N (125 mg/kg, i.v.) was administered; horizontal red lines below the x-axis: days of axitinib administration (25 mg/kg, i.p., daily); horizontal black lines below the x-axis: days of vehicle administration (PBS; see *Materials and Methods*). Mice were treated with either vehicle or axitinib (Ax) daily for 24 days. Axitinib treatment was inadvertently skipped on day 12; a double dose given on day 13 with no apparent negative impact on tumor growth or host toxicity (c.f., Ma and Waxman, 2008). Numbers in parentheses indicate the number of remaining mice in the combination treatment group at the indicated time points. (A) Tumor volume, showing that the combination of AQ4N with axitinib significantly retards tumor growth when compared with axitinib treatment alone from days 12–24 ($P < 0.05$, Student's paired *t* test) and that the tumor growth rate accelerates rapidly upon cessation of axitinib treatment. The modest antitumor activity of AQ4N alone seen in Fig. 4 was not apparent here, where tumors were larger at the time of first AQ4N injection (day 5). (B) Body weight profiles, highlighting the toxicity of the axitinib + AQ4N combination therapy. Data shown are mean $\pm$ S.E. values based on $n = 6$ –8 mice (12–16 tumors) per group at the beginning of drug treatment.

Our present study investigated the tumor cell line-dependence of AQ4N cytotoxicity under hypoxia compared with normoxia, whether the quinone reductase DT-diaphorase contributes to AQ4N bioactivation, and whether the antitumor activity of AQ4N can be increased by combination with drugs that increase tumor hypoxia.

Investigation of the hypoxia-dependence of AQ4N cytotoxicity identified only two cell lines (9L gliosarcoma and H460 non-small-cell lung carcinoma) that displayed a substantial increase in AQ4N toxicity (~9-fold) under hypoxia. Two other cell lines (A549 lung carcinoma and U251 glioblastoma) exhibited sensitivity to AQ4N similar to 9L and H460 cells in normoxia, but showed only a 3-fold increase in chemosensitivity under hypoxia. Thus, strong hypoxia-dependent bioreductive activation of AQ4N is not a common feature of cancer cell lines. Indeed, several of the cell lines tested, notably BxPC-3, HT-29, PC3, and FaDu cells, were insensitive to AQ4N under both normoxia and hypoxia,

consistent with an earlier report for PC3 and BxPC-3 cells under normoxia (Lalani et al., 2007). BxPC-3 cells are intrinsically sensitive to the active metabolite AQ4 in vitro, and they respond favorably to AQ4N when grown as solid tumors in vivo (Lalani et al., 2007), indicating that AQ4N activation is not deficient in BxPC-3 tumors in vivo, in contrast to our findings in culture. Conceivably, enzymes active in AQ4N bioactivation, such as cytochromes P450 2S1 and 2W1 (Nishida et al., 2010), might be more highly expressed in the context of the tumor environment in vivo; alternatively, AQ4N activation might occur in tumor-associated host cells such as macrophages, which have the capacity to activate AQ4N by iNOS (Mehibel et al., 2009). Conversely, whereas cultured 9L cells responded well to AQ4N, the 9L tumor xenografts were largely unresponsive (Figs. 4, 5), perhaps due to a deficiency in vivo in the enzymes that reduce AQ4N to AQ4. The poor activity of AQ4N against 9L tumors is not simply due to their insensitivity to anthraquinones, as indicated by 9L tumor sensitivity to mitoxantrone (DiMeco et al., 2002; Saini et al., 2004).

Certain cytochrome P450 enzymes (McErlane et al., 2005; Nishida et al., 2010; Xiao et al., 2011) and iNOS (Fitzpatrick et al., 2008; Nishida and Ortiz de Montellano, 2008; Mehibel et al., 2009) can activate AQ4N via a two-step reduction of the di-*N*-oxide to the di-amino compound AQ4. Other pathways of reductive metabolism demonstrated for related quinones include one-electron reduction to a semiquinone radical and two-electron reduction to a hydroquinone, both of which can be cytotoxic (Nguyen and Gutierrez, 1990; Ross et al., 1996). Disproportionation and comproportionation of the quinones and their reduced forms may also occur (Ross et al., 1996; Bailey et al., 1998). As DT-diaphorase (NQO1) is an active catalyst of quinone reduction (Siegel et al., 2012), we investigated its role in AQ4N activation. We found DT-diaphorase was widely expressed across our cancer cell panel, where its protein levels were unaffected by hypoxia. However, we saw no correlation between chemosensitivity to AQ4N and endogenous DT-diaphorase protein levels across the cell line panel. Although DT-diaphorase activity was not directly measured in the present study, DT-diaphorase protein and activity levels were well correlated across the 60-tumor cell line NCI-60 panel (Fitzsimmons et al., 1996). Moreover, we found that DT-diaphorase inhibitors had no impact on normoxic or hypoxic sensitivity to AQ4N, supporting the conclusion that DT-diaphorase does not make a significant contribution to AQ4N bioactivation. DT-diaphorase does, however, activate other bioreductive drugs (Beall et al., 1994; Fitzsimmons et al., 1996; Nolan et al., 2010).

Further work is needed to identify the enzymes that activate AQ4N in tumors in vivo. Bioactivation of AQ4N could be catalyzed by different sets of enzymes under normoxia versus hypoxia, as shown for PR-104, which is reduced by P450 reductase (Guise et al., 2007), methionine synthase reductase, diflavin oxidoreductase 1, and iNOS (Guise et al., 2012) under hypoxia, but undergoes reduction catalyzed by aldo-ketoreductase 1C3 (Guise et al., 2010) under normoxic conditions.

AQ4N cytotoxicity toward sensitive tumor cell lines was characterized by dose-response curves that were shifted to the left under hypoxia, as expected, but showed distinctly lower slopes under hypoxia compared with normoxia (Fig. 1). To a first approximation, at concentrations of AQ4N below its

K_m for bioactivation, a 10-fold increase in AQ4N concentration would be expected to result in a 10-fold increase in AQ4N activation, and correspondingly, a 10-fold decrease in cell survival; thus, a plot of log [survival] versus log [dose] would ideally have a slope of 1.0, as shown for “Hypoxic Expected” in the theoretical curve shown in Fig. 6 (dotted line). The slopes measured under hypoxia in our experiments were close to 1.0 for DU145 and MCF7 cells, but were substantially below 1.0 for 9L and H460 cells (Fig. 1); that is, for those cell lines, cell survival was greater than expected. These nonparallel dose-response curves indicate that the hypoxia cytotoxicity ratio (HCR) commonly used to characterize the hypoxia dependence of bioreductive activity can only be used to approximate relative hypoxic sensitivity in the case of AQ4N. The discrepant shapes of the dose-response curves under hypoxia versus normoxia can be explained if, for example, AQ4N activation is a multistep process wherein AQ4N competitively inhibits a downstream activation step. Under normoxic conditions, we observed the expected dose-response curves of slope 1.0 (Fig. 6, dashed line) for 9L and MCF-7 cells, but the slope increased to ~2.0 for H460 and DU145 cells, indicating more killing than expected. This latter finding suggests a higher order dependency on AQ4N concentration, for example, with multiple AQ4N molecules participating in the activation reaction.

The maximum HCR values for AQ4N reported here for a panel of cancer cell lines (Table 1) and, previously, for macrophages (Mehibel et al., 2009) are up to ~10-fold, which is considered moderate for a bioreductive drug. HCR values >100 are reported for AQ4N under anoxic conditions and in the presence of liver microsomes (Patterson, 1993), suggesting AQ4N activity is limited at the bioactivation step. In contrast, other bioreductive drugs such as tirapazamine display HCR values of 100 or more (Brown, 2000). The low HCR values observed here could be an intrinsic property of AQ4N. Alternatively, the low HCR of AQ4N could reflect the prolonged (24-hour) AQ4N exposure under hypoxia in our experiments, which may inhibit cell proliferation and thereby decrease the intrinsic sensitivity of the cells to the active metabolite AQ4, which targets topoisomerase II and is expected to be preferentially active against cycling cells. The increased effectiveness of AQ4N in vivo may reflect the high affinity of AQ4 for DNA binding (Patterson and McKeown, 2000), or the increased expression in vivo of enzymes active in AQ4N bioreduction, as discussed previously.

Tumor hypoxia can be modulated in several ways, including physical occlusion of blood flow to the tumor, vasodilation of extratumoral tissues (e.g., muscle) to divert blood away from the tumor, and, as investigated here, treatment with

Fig. 6. Model for impact of AQ4N on tumor cell sensitivity under normoxic and hypoxic conditions.

antiangiogenic drugs. We found that the vasodilator hydralazine increased tumor hypoxia in both 9L and H460 xenografts, consistent with findings in other tumor models (Patterson et al., 2000; Shin et al., 2007). We also found that hydralazine was ineffective as a monotherapy, consistent with results with MAC26 tumors (Patterson et al., 2000). However, hydralazine did not increase AQ4N-dependent antitumor activity, perhaps because the increase in tumor hypoxia is only transient (Honess and Bleehen, 1992; Horsman et al., 1992). The lack of an effect was most likely not due to the reduced blood flow decreasing tumor uptake of AQ4N, as the same schedule of hydralazine treatment was previously shown elsewhere to effectively increase AQ4N exposure, as indicated by an increase in AQ4N antitumor activity (Wilson et al., 1996; Patterson et al., 2000).

Here, we investigated the effects of axitinib, which induces hypoxia in 9L tumors within 4 days (Ma and Waxman, 2008), to determine whether more persistent hypoxia would potentiate AQ4N activity. In our studies, AQ4N was delivered by i.v. injection to minimize the toxicity seen with i.p. AQ4N treatment. When using this novel antiangiogenic + bioreductive drug combination, we observed a transient improvement in antitumor activity compared with axitinib treatment alone, but this activity increase was associated with a considerable increase in host toxicity (Fig. 5). Conceivably, the combination of AQ4N with antiangiogenesis that we propose might be more effective when applied to other bioreductive drugs, or in the case of AQ4N by treatment at a lower dose but at a greater frequency of administration or in combination with radiation (Lalani et al., 2007; Williams et al., 2009). We did not directly assay AQ4N penetration to the flank tumors, but we used pimonidazole staining to verify that hydralazine treatment increases tumor hypoxia significantly; this, in turn, is expected to increase tumor accumulation of AQ4, as has been shown in several tumor models (Atkinson et al., 2007; Albertella et al., 2008; Williams et al., 2009). In contrast to AQ4N, mitoxantrone has poor tumor penetration, but the combination of AQ4N with mitoxantrone can result in more effective antitumor activity due to AQ4N targeting hypoxic regions and mitoxantrone targeting less hypoxic regions (Tredan et al., 2009).

The apparent systemic toxicity of AQ4N for *scid* mice reported here and previously by others was associated with anemia (Nijmeijer et al., 2005), and it was not seen in immunocompetent mice (Patterson et al., 2000; Gallagher et al., 2001; Lalani et al., 2007; Tredan et al., 2009). Toxicities associated with AQ4N treatment of mice, rats, monkeys, and humans include atrophy of the lymph nodes, spleen, and thymus as well as lymphopenia, leucopenia, cytopenia, and anemia (Cleland et al., 2005; Nijmeijer et al., 2005; Steward et al., 2007). AQ4N can be detected in the spleen, small intestine, and tumors of *scid* mice up to 2 weeks after treatment (Cleland et al., 2005), indicating that it persists even after treatment is halted. The reason for the strain dependence of AQ4N toxicity and, in particular, the high sensitivity of *scid* mice, where both B cells and T cells are deficient, is unknown. The route of AQ4N administration also has a substantial effect on host toxicity (Supplemental Fig. 2), with greater toxicity seen when AQ4N is administered intraperitoneally compared with intravenously, suggesting that AQ4N toxicity is increased by first-pass metabolism.

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Authorship Contributions

Participated in research design: Manley, Waxman.

Conducted experiments: Manley.

Performed data analysis: Manley.

Wrote or contributed to the writing of the manuscript: Manley, Waxman.

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