In vivo activation of the hypoxia-targeted cytotoxin AQ4N in human tumor xenografts

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Abstract
AQ4N (banoxantrone) is a prodrug that, under hypoxic conditions, is enzymatically converted to a cytotoxic DNA-binding agent, AQ4. Incorporation of AQ4N into conventional chemoradiation protocols therefore targets both oxygenated and hypoxic regions of tumors, and potentially will increase the effectiveness of therapy. This current pharmacodynamic and efficacy study was designed to quantify tumor exposure to AQ4 following treatment with AQ4N, and to relate exposure to outcome of treatment. A single dose of 60 mg/kg AQ4N enhanced the response of RT112 (bladder) and Calu-6 (lung) xenografts to treatment with cisplatin and radiation therapy. AQ4N was also given to separate cohorts of tumor-bearing mice 24 hours before tumor excision for subsequent analysis of metabolite levels. AQ4 was detected by high performance liquid chromatography/mass spectrometry in all treated samples of RT112 and Calu-6 tumors at mean concentrations of 0.23 and 1.07 μg/g, respectively. These concentrations are comparable with those shown to be cytotoxic in vitro. AQ4-related nuclear fluorescence was observed in all treated tumors by confocal microscopy, which correlated with the high performance liquid chromatography/mass spectrometry data. The presence of the hypoxic marker Glut-1 was shown by immunohistochemistry in both Calu-6 tumors and RT112 tumors, and colocalization of AQ4 fluorescence and Glut-1 staining strongly suggested that AQ4N was activated in these putatively hypoxic areas. This is the first demonstration that AQ4N will increase the efficacy of chemoradiotherapy in preclinical models; the intratumoral levels of AQ4 found in this study are comparable with tumor AQ4 levels found in a recent phase I clinical study, which suggests that these levels could be potentially therapeutic.

Introduction
Tumor hypoxia is a contributing factor in the aggressiveness of tumors and poor response to treatment (1). Conventional therapies such as radiation and cytotoxic drugs preferentially target the well-oxygenated regions of tumors, but have limited effectiveness against poorly vascularized hypoxic compartments. This is due to direct effects, such as the limited penetration of drugs into hypoxic regions remote from the vasculature (2, 3). One approach to overcoming these resistance mechanisms is to use bioreductive agents designed to target the hypoxic regions directly, and a number of agents from this promising class of anticancer treatments are now under examination in the clinic (4). One of these agents, AQ4N (banoxantrone), is a well-tolerated prodrug that was designed to be activated into a cytotoxic agent in hypoxic regions to target this treatment-resistant fraction (5). The combination of AQ4N with conventional treatment modalities allows targeting of both well-oxygenated and hypoxic regions of tumors with enhanced therapeutic benefit. AQ4N has shown enhanced antitumor response to radiation (6, 7), cisplatin (7, 8), and other chemotherapeutic agents (7, 9) in syngeneic murine tumor models. Phase I clinical trials of AQ4N have been carried out when used alone in various malignancies (10) or in combination with radiotherapy in advanced esophageal carcinoma (11). AQ4N was well tolerated with no clinically significant toxicity at 768 mg/m² in the study of Papadopoulos et al. (10) and 447 mg/m² when combined with radiotherapy (11). AQ4N is currently under investigation in further clinical trials as a single agent, and in combination with radiotherapy and chemotherapy. AQ4N has shown predictable pharmacokinetics, and is well-tolerated when plasma levels exceed those known to give a therapeutic benefit in mice in combination with radiation (12).
AQ4N is a bis-N-oxide that is reduced via two sequential two-electron reductions to the tertiary amine, AQ4, which is a potent cytotoxic agent toward both aerobic and hypoxic cells (13). AQ4, but not AQ4N, intercalates in DNA with high affinity to generate a stable persistent complex that can inhibit topoisomerase II and cause DNA damage and cell death (14). AQ4, like other anthracines and anthracenediones, is fluorescent. This intrinsic fluorescence can be used to visualize the drug in vitro using a long wavelength excitation beam in laser-scanning confocal microscopy, revealing a predominantly nuclear localization, with a small amount of cytoplasmic fluorescence (15). In contrast, the prodrug AQ4N displays accumulation in peri-nuclear vesicles with a lower level of fluorescence, and is readily lost from cells by diffusion. A number of studies have shown that cytochrome P450 enzymes (CYP) and other haem-containing reductases, such as nitric oxide synthase, contribute to the bioreduction of AQ4N under anoxic conditions (16–18). The ability of S9 or microsome fractions to activate AQ4N to AQ4 ex vivo has been shown in human liver microsomes (16), activated macrophages (18), and murine tumors (19). Recent work has shown that AQ4N can be activated in vivo in tumor xenograft samples (3, 20), and in clinical samples from a phase I study (12). Here, we build on this work, and show detailed examination of subtumor distribution of AQ4 and direct colocalization with hypoxic regions of tumors. Because radiotherapy is increasingly being used in combination with conventional chemotherapy, we have evaluated the efficacy of AQ4N combined with radiation and cisplatin for the treatment of small cell lung cancer tumors and RT112 bladder cancer tumors grown as human xenografts in mice. We show that a dose of 60 mg/kg AQ4N can enhance chemo/radiotherapy and also show that the tumor exposure to AQ4 required for efficacy in these models is consistent with clinical exposures observed in phase I trials. This suggests that potentially therapeutic doses have been delivered to man with minimal toxicity.

Materials and Methods

Cells and Reagents

RT112 bladder carcinoma and Calu-6 non–small cell lung carcinoma cells were obtained from the European Collection of Cell Cultures (RT112) and the American Type Tissue Collection (Calu-6). All were maintained in RPMI 1640 (Life Technologies Bethesda Research Laboratories) supplemented with 10% FCS (LabTech International) and 2 mmol/L glutamine (Invitrogen Life Technologies). AQ4N, banoxantrone (1,4-Bis[2-(dimethylamino)ethy]laminio)-5,8-dihydroxyanthracene-9,10-dione bis-N-oxide), AQ4M (1,4-Bis[2-(dimethylamino)ethyl]aminio)5,8-dihydroxyanthracene-9,10-dione mono-N-oxide), and AQ4 (1,4-Bis[2-(dimethylamino)ethyl]aminio)5,8-dihydroxyanthracene-9,10-dione) were provided by KuDOS Pharmaceuticals (Cambridge).

Colony Formation Assays

One thousand cells were seeded in each well of a six-well plate and allowed to attach overnight. Drugs were added to wells in triplicate to give final concentrations as indicated. Cells were exposed to drugs for 1 h before replacement with normal media. Cells were allowed to grow for a further 6 to 10 d to form colonies, after which time medium was removed and replaced with modified Giemsa stain for 1 h to fix and stain the colonies. Colonies were counted automatically using a Colony Counter (Oxford Optronics). Survival curves were generated and concentrations required to kill 90% of cells (IC90) were calculated using the four-parameter sigmoidal analysis of the Xlfit program (IDBS). Data are calculated from the mean values of two independent experiments.

Xenograft Studies

Tumor Initiation. For initiation of tumor xenografts, RT112 cells were prepared at 5 × 10^5 per mL in serum-free RPMI and Calu-6 cells at a concentration of 2 × 10^6 per mL in a 1:1 mix of serum-free RPMI and phenol red–free MatriGel (BD Biosciences). A volume (0.1 mL) of cell suspension was implanted intradermally on the back of female nu/nu cba mice of age 10 to 12 wk. Once a palpable tumor formed, volume measurements were taken daily.

Ex vivo Studies with Xenograft Tissues. Untreated Calu-6 (n = 3) and RT112 (n = 4) tumors were excised, snap frozen in liquid nitrogen, and stored at −80°C. The tumors were disaggregated, lysed, and S9 fractions prepared as described previously (21). AQ4N (12.5 μmol/L) was then incubated with the tumor lysates under nitrogen at 37°C in 4-mL amber glass vials (Chromacol) sealed with subaseal (Aldrich). AQ4N, AQ4M, and AQ4 were then analyzed by chromatography (see below) and their concentrations calculated from peak height ratios and comparison with calibration curves that were prepared by spiking heat-inactivated lysate preparations with known amounts of metabolite (0–25 μmol/L).

In vivo Efficacy Studies. Tumor-bearing mice were randomly assigned into treatment groups once a tumor volume of 240 to 280 mm^3 had been achieved. AQ4N was provided as a dihydrochloride salt (C_{22}H_{28}N_{4}O_{6}.2HCl). This was prepared in sterile double-distilled water to yield a 60 mg/kg (135 μmol/kg) dose of free AQ4N. Cisplatin (2 mg/kg) was prepared in 0.9% physiologic saline. Both drugs were given by i.p. injection at 0.1 mL per 10 g mouse weight. Tumors were locally irradiated with 230 kV X-rays (dose rate, 2 Gy/min). During this procedure, the nonanaesthetized mice were turned round half way through radiotherapy to facilitate a uniform dose across the tumor. In the current study, radiation was administrated as 2-Gy fractions (once daily). In combined treatment protocols, AQ4N was administrated 30 min before radiation treatment and cisplatin 6 h after. AQ4N was given once weekly and cisplatin once per experiment. The experimental end point was a relative tumor volume four times that at the start of treatment (RTV4). Sample sizes were based on the number of tumors per group required to detect a 50% change in radiation response where this had been previously determined (power, 0.8; significance level, 0.05). All procedures were carried out in accordance with local regulations and institutional guidelines.
with the Scientific Procedures Act 1986 by approved protocols (Home Office Project Licences 40-1770 and 40/2328) following Institutional guidelines.

Analysis of Tumor Samples—Sample Preparation

Mice bearing RT112 or Calu-6 tumors of ~600 mm³ in size were randomly assigned into two groups (n = 3/group) that received either vehicle or 60 mg/kg AQ4N by i.p. injection. After 24 h, tumors were excised and split into three pieces. Two pieces were dip washed in saline, blotted dry and snap frozen in liquid nitrogen, and stored at −80°C until analysis. The third piece was fixed in formalin before embedding in paraffin for generation of tissue sections.

Analysis of Tumor Samples—AQ4N and AQ4 Determination

Extraction From Tumor Xenografts. Frozen tumor samples were weighed and a 2.5× volume of ice-cold homogenization solution (0.2% H₂O₂ in MeCN) was added. Samples were then homogenized immediately using a pellet pestle, and centrifuged at 10,000 g for 5 min. Subsequently, 200 μL of the supernatant was evaporated to dryness and then reconstituted in 100 μL of mobile phase A [5% acetonitrile 95% ammonium formate (0.05 mol/L; pH 3.6)]. Samples were centrifuged at 10,000 g for 3 min and 20 μL injected into the high performance liquid chromatograph (HPLC). Stock standards of AQ4N and AQ4 were prepared in DMSO (10 mg/mL) and diluted in H₂O. Calibration curves were prepared from 0 to 2 μg/mL by spiking drug-free tumor homogenates (H460 human tumor xenografts) with the appropriate concentrations of AQ4N or AQ4.

HPLC/Mass Spectrometry Analysis. Chromatographic analysis of AQ4N was adapted from the methods described previously (22, 23). Briefly, a Hichrom RPB column (25 cm × 4.6 mm id, ser no HIRPB-6186, Hichrom Ltd) was used for the separation with a flow rate of 1.4 mL/min. Mobile phase A was 5% acetonitrile 95% ammonium formate (0.05 mol/L; pH 3.6) and mobile phase B was 50% acetonitrile 50% ammonium formate. A Waters Alliance 2695 (Milford) quaternary pump chromatography system was programmed to run a gradient of 30% B to 56% B over 10 min and then return to initial conditions for 10 min to equilibrate before the next injection. A Waters 996 Photodiode Array Detector (λ₁ = 240 nm, λ₂ = 612 nm) with Masslynx V3.4 software (Micromass Ltd) was used for spectral analysis of the peaks of interest. The 2695 is attached to a Waters ZMD (Micromass Ltd) quadrupole mass spectrometer with the solvent flow split 1:10 before mass spectral analysis.

Analysis of Tumor Samples—Confocal Microscopy. To capture AQ4 fluorescence, frozen tumor pieces were set in OCT cryomedium (Raymond A Lamb) at −80°C. Ten-micrometer-thick cryosections were collected onto APES-coated glass slides, which were allowed to air dry and then stored at −80°C until use. Slides were defrosted, analyzed, and images captured with a Zeiss LSM510 confocal system attached to an Axiovert 200M inverted microscope using LSM510 Software (all from Zeiss). Specimens were scanned using a red diode laser with appropriate excitation (wavelength, 633 nm) and emission (650 nm LP) filters, at 50% power and an iris aperture of 110 μm. Specimens were examined using a 63× Plan-Apochromat/1.4 oil differential interference contrast microscopy objective lens. Optical sections of 512 × 512 pixels were captured at 1.60 μs pixel time and digital magnification of 1.0 using Kalman (n = 4) scanning settings. The percentage of cells containing strong nuclear fluorescence was estimated according to the following semiquantitative scoring metric: 0, 0% (no staining); 1, 1% to 5%; 2, 5% to 15%; 3, 15% to 30%; 4, >30%. For colocalization with Glut1, sections were subsequently fixed in ice-cold acetone and nonspecific antibody binding sites were blocked using 10% goat serum in PBS containing 0.1% bovine serum albumin and incubated for 1 h at room temperature. Following washing in PBS, goat anti-rabbit IgG TRITC antibody (Sigma Aldrich) was applied at a concentration of 1:150 in PBS containing 0.1% Tween 20 supplemented with 0.1% bovine serum albumin and incubated for 1 h at room temperature protected from light. Tumor sections were then reimaged using the Leica TCS SP5 upright multiphoton microscope. Regions previously showing AQ4 staining were relocated using the saved positions and automated stage. For simultaneous detection of AQ4 and Glut1, the protocol was modified to use increased concentrations of both primary and secondary antibodies (1:100), reduced antibody incubation times (5 min), and brief 10-s PBS washes to minimize the wash-out of AQ4 during the immunostaining procedure.

Analysis of Tumor Samples—Glut1

Five-micrometer sections of paraffin-embedded tumors were dewaxed by standard histologic techniques. Primary antibody incubations were performed with rabbit polyclonal Glut1 antibody A3536 ( Dako) at 1/200 dilution in PBS containing 10%FCS/0.5% Tween20 for 1 h at room temperature. Secondary antibody incubations were performed with anti-rabbit Alexa Fluor 488 (Invitrogen Molecular Probes) at 1/1,000 dilution, and slides were counter-stained with 4',6-diamidino-2-phenylindole. The proportion of cells expressing Glut1 throughout all tumor sections was estimated using the semiquantitative scoring system as described for the confocal microscopy above. Slides were scored blind by two independent observers.

Statistical Analyses

The significance of the impact of AQ4N on the outcome of radiotherapy and chemoradiotherapy was evaluated using Mann-Whitney U tests. All other comparisons were made using paired t tests. Data cited within the text are mean values ± SEM.

Results

AQ4N Enhances Radiotherapy and Combined Cisplatin-Radiotherapy Response

The impact of AQ4N treatment on the response of Calu-6 (lung tumor) and RT112 (bladder tumor) xenografts to radiation alone or radiation plus cisplatin was evaluated in a schedule designed to simulate a standard clinical protocol.
Radiotherapy was administered as five daily fractions of 2 Gy. AQ4N (60 mg/kg) was administered on day 1, 30 minutes before the first dose of radiation. Cisplatin (2 mg/kg) was administered once, 6 hours after the first dose of radiation. Previous studies using syngeneic murine models showed that a 6-hour interval between AQ4N and cisplatin administration did not cause any increase in bone marrow toxicity compared with that induced by cisplatin alone (8).

All experimental protocols in both xenograft models were well tolerated, as indicated by lack of gross morbidity, or body weight loss. Addition of a single dose of AQ4N to 5 × 2 Gy radiation gave a moderately enhanced growth delay of ∼5 days in both models (Fig. 1A and B), which was a significant improvement on the response to radiotherapy alone in the Calu-6 model (P = 0.03). The addition of cisplatin to AQ4N/radiation further enhanced tumor response. Compared with radiation alone, this combination resulted in a significant increase in growth delay of 10 days (P = 0.02) for Calu-6 tumors. In RT112 tumors, there was a more divergent response to the combination of cisplatin, AQ4N, and radiation. The average improvement in growth delay compared with radiation alone was 15 days, and one treated tumor completely regressed (Fig. 1A). At the doses used here, AQ4N did not significantly modify cisplatin response in the absence of radiation (data not shown). Growth delays induced by 2 mg/kg cisplatin alone were 3 ± 1 and 2 ± 2 days for Calu-6 and RT112 tumors, respectively, suggesting a positive interaction between the treatment modalities in the triple combination. Overall, the data clearly show that 60 mg/kg AQ4N can enhance tumor response to combination therapy in vivo.

The beneficial effect of combination of AQ4N with cisplatin and radiation in a 2-week dosing schedule was further examined in the Calu-6 model, incorporating repeat doses of AQ4N (2 × 60 mg/kg on day 1 and 8) and radiation (10 × 2 Gy on days 1–5 and 8–12). Under this schedule, addition of AQ4N gave rise to an additional 11-day growth delay compared with 10 × 2 Gy (Fig. 1C). The combination of AQ4N, cisplatin, and radiation further improved response and yielded a highly significant 18-day increase in growth delay compared with radiotherapy alone (P = 0.003). In addition to the enhanced mean growth delay afforded by AQ4N-containing schedules, there was a notable increase in the uniformity of tumor response (Fig. 1C). Tumor regression was also apparent in both AQ4N-containing treatment groups, in particular, in combination with cisplatin and radiation, where the lowest average tumor volume was 43 ± 8 mm³ at day 26, a significantly reduced comparison with that upon treatment initiation (256 ± 8 mm³; P < 0.000001; Fig. 1C).

**AQ4N Activation Ex Vivo**

Having shown that the addition of 60 mg/kg AQ4N to radiation and cisplatin was effective in both Calu-6 and RT112 xenograft tumors, we determined whether or not these xenograft tumors were capable of activating AQ4N to AQ4 under anoxic conditions ex vivo. Lysates were prepared from Calu-6 and RT112-xenografted tumors and their ability to metabolize AQ4N ex vivo under anoxia was determined. Rates of formation of AQ4 and the two-electron intermediate AQ4M were analyzed by HPLC (Table 1). Both AQ4M and AQ4 were detected in all samples, demonstrating that both xenografts possessed the appropriate enzyme complement to facilitate bioreductive activation under anoxic conditions.

**Analysis of AQ4N Activation In Vivo**

We then determined whether AQ4N was metabolically activated to its cytotoxic product AQ4 in tumors in vivo. RT112 and Calu-6 tumors that had been treated 24 hours earlier with AQ4N (60 mg/kg) were excised and split into three pieces: two were used to assess AQ4 accumulation and the third to assess hypoxia using the endogenous marker glucose transporter-1 (Glut1). This allowed correlation between data from different analyses of the same tumor (Table 2).

**Analysis of AQ4N and Metabolites by HPLC/Mass Spectrometry**

Frozen tumor samples for control and AQ4N-treated animals were analyzed for the presence of AQ4N and its metabolites, AQ4M and AQ4, by HPLC/mass spectrometry (MS). No AQ4N was detected in any tumor samples, consistent with the rapid excretion of AQ4N observed in pharmacokinetic studies (23). The intermediate mono-N-oxide, AQ4M, was also below detectable limits for all samples, also as predicted from the plasma pharmacokinetics. In contrast, all treated tumors contained substantial levels of the activated cytotoxic agent AQ4 (Fig. 2A). Tumor concentrations of AQ4 were calculated for the samples (Table 2). The mean concentration of AQ4 in RT112 tumors was 0.23 μg/g (n = 3; SD, 0.06), and in Calu-6 tumors, the mean concentration was higher at 1.07 μg/g (n = 3; SD, 0.15). This clearly shows that AQ4N can be activated to give its potentially cytotoxic product in tumors in vivo.

**Detection of AQ4 in Tumors by Laser-Scanning Confocal Microscopy**

Cryosections of frozen xenograft tumor tissue slices were examined for AQ4 fluorescence and distribution by confocal microscopy. Because no AQ4N, AQ4M, or other metabolites were detected by LC/MS, we assumed that any fluorescence we observed was likely to be due to AQ4. The high DNA affinity of AQ4 (14) is expected to result in a predominantly nuclear localization and tight binding to cells and, thus, is likely to maintain any heterogeneous distribution. Clear nuclear fluorescence was observed in regions of both RT112 and Calu-6 tumors, providing direct evidence of cellular accumulation of AQ4 (Fig. 2B). The heterogeneous distribution of this fluorescence was expected to be from the selective activation of AQ4N in the hypoxic regions of tumors. Some degree of faint fluorescence was observed in the untreated RT112, but not Calu-6, control tumors (data not shown). However, this background fluorescence was readily distinguishable from the strong nuclear fluorescence associated with tumors from AQ4N-treated mice. The distribution of fluorescence was scored for a section of each tumor sample (Table 2). The mean fluorescence score was very similar for both tumor types.
Quantification of Glut1 as an Endogenous Marker of Hypoxia in Tumors. Using immunofluorescent staining, we examined the distribution of Glut1 as a marker for tumor hypoxia (24) in sections of formalin-fixed, paraffin-embedded samples of RT112 and Calu-6 tumors. We found clear evidence of Glut1 expression in all xenograft tumors (Fig. 3A). A semiquantitative scoring system for Glut1 staining revealed that an increased fraction of the Calu-6 tumors was Glut1-positive compared with the RT112 tumors (mean scores, 2.6 versus 1.7, respectively; Table 2). In addition, there was a clear difference in distribution of Glut1 staining between the tumor types. RT112 tumors display a classic perinecrotic expression around a few large regions of necrosis (Fig. 3A). In contrast, Calu-6 tumors show more extensive regions of Glut1 spread throughout the section, with Glut1 expression bordering many small regions of necrosis (Fig. 3A).

To gain direct evidence of colocalization of AQ4 and hypoxic regions, we then analyzed the relationship between Glut1 expression and the distribution of AQ4 fluorescence in cryostat sections of Calu-6 tumor xenografts (Fig. 3B).
We found nuclear AQ4 accumulation to be coincident with membranous Glut1 expression in these tumors (Fig. 3B). This is consistent with the notion that AQ4N is metabolically activated to give AQ4 in these Glut1-positive, putative hypoxic regions.

**Significance of Intratumor AQ4 Concentrations**

To equate the intratumor concentrations of AQ4 to cytotoxicity, in vitro experiments were carried out where RT112 cells were grown in culture and exposed to a range of AQ4 concentrations (1–500 ng/mL) for 1 hour. Cells were washed and centrifuged to give cell pellets and then the pellets were weighed before examination by HPLC/MS using identical conditions to the tumor analysis. A linear relationship between intracellular concentration (in μg/g) and concentration of drug in the media (in μg/mL) was observed (R² = 0.99; Fig. 4). For example, an exposure of cells to 40 ng/mL for 1 hour, gave an intracellular concentrations of 1 μg/g. This is consistent with a sequestration of AQ4 from the media as a result of the high nuclear affinity of AQ4. No saturation of intracellular concentrations was observed at the doses used (up to 8,000 ng/g), indicating a large capacity for incorporation of AQ4 into the cells. To determine the significance of this level of exposure, we used a clonogenic assay to determine the AQ4 concentration required to kill 90% of cells (IC₉₀) following 1 hour of exposure. RT112 cells were found to have an IC₉₀ value of 25 ng/mL, which is equivalent to an intracellular concentration of AQ4 of 40 to 50 ng/g (Fig. 4). When mice were given 60 mg/kg AQ4N and RT112 tumors harvested were 24 hours later, they were found to contain 230 ng/g AQ4. From Fig. 4, this is equivalent to 10 ng/mL (extracellular), which far exceeds the IC₉₀ for these cells. When considering the in vivo measurements were made from the whole tumor and how Fig. 3 shows clearly that AQ4 localization is focal, this suggests that local AQ4 concentrations should be sufficiently high to elicit toxicity toward hypoxic tumor cells in vivo. Therefore, this uptake and predicted toxicity would be expected to make a significant contribution to tumor growth delay. A similar analysis can be made using Calu-6 cells and tumors where directly measured intratumor concentrations of AQ4 are ~1,000 ng/g. If the relationship between drug exposure and uptake is similar to that given in Fig. 4, then this equates to an in vitro dose of 40 ng/mL, which exceeds the IC₉₀ value (25 ng/mL).

**Discussion**

The hypoxia-activated prodrug AQ4N is currently in phase I/II clinical development. Preclinical evaluation of AQ4N has previously focused upon murine tumor models, with AQ4N administered as an adjunct to either radiotherapy or chemotherapy. One aim of the present study was to establish the efficacy of AQ4N in a cisplatin-based chemoradiation schedule in human xenografts using clinically relevant fractionation protocols. Lung and bladder xenografts were seen as relevant models for these evaluations as radiotherapy with platinum chemotherapy is commonly applied in the clinical setting, and hypoxia has been identified as a potential cause of therapy failure in both diseases (25).

We show here, for the first time, that the addition of AQ4N to chemoradiotherapy yields antitumor benefit when used to treat RT112 and Calu-6 xenografts, with no apparent increase in host toxicity. The mean tumor growth delays of this treatment are comparable for both models (Fig. 1B). However, the response of individual tumors varies; Calu-6 tumors show a notably increased uniformity of response in combinations including AQ4N. In contrast, RT112 tumors show a considerable variation in response to the addition of AQ4N, with certain tumors showing a dramatic and prolonged regression, but others appearing to show no improvement over chemoradiation alone (Fig. 1A). This variation could have its basis in the relatively low level of hypoxia in the RT112 tumors suggested by the Glut1 staining of the peri-necrotic regions of these tumors.

A single i.p. dose of 60 mg/kg AQ4N was used for this study as it has been shown to be effective and well tolerated

**Table 1. AQ4N metabolism by S9 fractions derived from Calu-6 and RT112 cells taken from xenograft tumors**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of metabolite formation (μmol/L/min/mg protein)</th>
<th>AQ4</th>
<th>AQ4M</th>
<th>Ratio AQ4M/AQ4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor 1</td>
<td>1.0</td>
<td>13.4</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>Tumor 2</td>
<td>0.9</td>
<td>12.4</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>Tumor 3</td>
<td>1.3</td>
<td>21.2</td>
<td>16.3</td>
<td></td>
</tr>
<tr>
<td>RT112</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor 1</td>
<td>4.4</td>
<td>56.2</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>Tumor 2</td>
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</tr>
<tr>
<td>Tumor 3</td>
<td>14.1</td>
<td>59.0</td>
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<td></td>
</tr>
<tr>
<td>Tumor 4</td>
<td>13.3</td>
<td>47.3</td>
<td>3.6</td>
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</tr>
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**Table 2. Analysis by confocal microscopy of levels of AQ4 and the putative hypoxia marker Glut-1 in Calu-6 and RT112 tumors taken from mice treated 24 h previously with 60 mg/kg AQ4N**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Treatment</th>
<th>Scoring following confocal analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AQ4N</td>
<td>AQ4</td>
</tr>
<tr>
<td>Calu-6</td>
<td>AQ4N</td>
<td>1.17</td>
</tr>
<tr>
<td>Calu-6</td>
<td>AQ4N</td>
<td>0.89</td>
</tr>
<tr>
<td>Calu-6</td>
<td>AQ4N</td>
<td>1.14</td>
</tr>
<tr>
<td>Calu-6</td>
<td>Control</td>
<td>BLQ*</td>
</tr>
<tr>
<td>Calu-6</td>
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<td>BLQ</td>
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<tr>
<td>Calu-6</td>
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<tr>
<td>RT112</td>
<td>AQ4N</td>
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<tr>
<td>RT112</td>
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<td>Control</td>
<td>BLQ</td>
</tr>
<tr>
<td>RT112</td>
<td>Control</td>
<td>BLQ</td>
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</table>

NOTE: Absolute levels of AQ4 were also quantified using HPLC/MS.
*BLQ, below limit of quantitation.
†Non-nuclear background fluorescence was observed in RT112 control tumors.
in combination with other therapies in murine tumor models (6, 9). Similar pharmacokinetics have been obtained for i.p. versus i.v. dosing in mice, and clinically, the drug has been given as a 30-minute infusion. The dose of AQ4N used here is equivalent to 180 mg/m² in man, which has previously been shown to be well tolerated (10, 11). A comparable dose of 200 mg/m² was selected for clinical evaluation in a phase I pharmacodynamic trial (12), which was modeled on the studies described here to allow potentially meaningful comparisons between the pharmacodynamic parameters in mouse and man. A single dose was used to simplify most of the studies detailed here, although we anticipate that administration of repeated doses of AQ4N will be used clinically, and is likely to yield improved efficacy, as shown for Calu-6 2-weekly protocol compared with the single administration (Fig. 1C).

AQ4N was designed to be activated in hypoxic regions of tumors. Although the clinical importance of hypoxia is increasingly widely recognized, there remains some controversy in the choice of method for quantifying hypoxia (26). Glut1 is well recognized to be regulated by hypoxia, and there are numerous studies demonstrating its utility as a marker of endogenous hypoxia and its association with tumor aggressiveness and poor prognosis in numerous diseases (24–27). The use of an endogenous marker of hypoxia such as Glut1 does not require exogenous administration of

Figure 2. The active cytotoxic agent AQ4 is present in tumors from AQ4N-treated animals. A, typical chromatograms of extracts from (a) Calu-6 tumors and (b) RT112 tumors following treatment of mice with 60 mg/kg of AQ4N, followed by tumor excision 24 h later. c, standards of AQ4 (6.8 min) and AQ4N (9.1 min) and (d) an extract of tumor from a nondrug-treated control. B, representative confocal laser scanning microscopy images of sections of Calu-6 and RT112 tumors at 633 nm, demonstrating nuclear staining with AQ4 in AQ4N-treated tumors. Right, a control untreated Calu-6 tumor, demonstrating the absence of background fluorescence.
an agent such as pimonidazole and, thus, facilitates the translation of any preclinical findings into a clinical context. In the present study, we were unable to observe any staining using the alternative endogenous marker of hypoxia, carbonic anhydrase IX. However, we have previously observed pimonidazole staining in both tumor types (data not shown), suggesting that the Glut1 staining we observe is due to hypoxia. Both RT112 and Calu-6 tumors were found to have a typically heterogeneous distribution of hypoxia, as indicated by Glut1 expression (Fig. 3). AQ4 was directly visualized in tumor sections by confocal microscopy and also observed to be heterogeneously distributed, consistent with activation in these hypoxic regions. Simultaneous analysis of AQ4 and Glut1 in Calu-6 tumors revealed their colocalization and provided convincing evidence of activation of AQ4N exclusively in hypoxic regions in vivo. This is also supported indirectly by the observation that Calu-6 tumors accumulated higher levels of AQ4 than RT112 tumors, and have a higher mean staining for Glut1 (Table 2). They also indicate that Glut1 may be a useful biomarker for AQ4N activation in future preclinical or clinical studies.

Other factors undoubtedly contribute to AQ4N activation in tissues, such as the levels of CYP450s and other haem-containing reductases, and the distribution and the level of hypoxia present in different regions of tumors. Ex vivo lysates from both tumors were shown to activate AQ4N under anoxia, demonstrating that both cell types express appropriate bioreductive enzymes. It was interesting to note that the RT112 S9 fractions were able to metabolize AQ4N ex vivo more effectively than the Calu-6 S9 fractions, whereas Calu-6 was found to accumulate more AQ4 in vivo. However, the S9 fractions were incubated in complete anoxia.

Figure 3. Both Calu-6 and RT112 tumors exhibit Glut1 staining that is coincident with AQ4 fluorescence. A, immunofluorescence images of Calu-6 and RT112 tumors stained for Glut1 (green) and 4',6-diamidino-2-phenylindole (blue). The heterogeneous perinecrotic localization of Glut1 membrane staining can be seen. B, images of Glut1 (left) and AQ4 fluorescence (right) in Calu-6 tumors. Inset, an overlay of Glut1 and AQ4 in a positive region, highlighting nuclear localization of AQ4.
with generation and accumulation in hypoxic areas. As such, reveals the distribution of AQ4 to be very heterogeneous, tion throughout the tumor. In fact, confocal microscopy These concentrations are generated from a homogenization regions of three dimensional multicell spheroids.5 with other recently published work (3) and with effect to these regions, which is consistent these regions to become activated. The finding of AQ4 in hypoxia-activated prodrug must also be able to penetrate regions, contributing to their refractory nature (2). Thus, a penetration and delivery of cytotoxic agents into hypoxic oxygen and nutrients to these areas. This also limits the absolute level of pO2 in the hypoxic regions of the tumor, which is necessary for activation and toxicity of AQ4N.

Hypoxic regions of tumors arise due to inadequate vascular and high interstitial pressures that limit access of oxygen and nutrients to these areas. This also limits the penetration and delivery of cytotoxic agents into hypoxic regions, contributing to their refractory nature (2). Thus, a hypoxia-activated prodrug must also be able to penetrate these regions to become activated. The finding of AQ4 in internal regions of tumors suggests that AQ4N can indeed effectively penetrate to these regions, which is consistent with other recently published work (3) and with in vitro data demonstrating rapid penetration of AQ4N into internal regions of three dimensional multicell spheroids.5

We have shown that the prodrug AQ4N is activated to the cytotoxic agent AQ4 in tumors of two xenograft models. A single dose of 60 mg/kg led to the intratumoral accumu-

Figure 4. Intracellular accumulation of AQ4. RT112 Cells were treated with AQ4 for 1 h, washed, and intracellular levels of AQ4 quantified by HPLC/MS. Intracellular concentrations of AQ4 determined by HPLC/MS are plotted against the administered doses of AQ4 and show a clear linear relationship (broken line, R² = 0.9936). From Table 2, AQ4 levels in RT112 xenografts are 230 ng/g; in vitro, this is equivalent to treatment with 10 ng/mL (solid line), which far exceeds the IC₉₀ of the compound. Dotted line, a similar analysis for the levels of AQ4 in Calu6 cells and tumors.

whereas in tumors, there will not only be regions of anoxia but also gradients of oxygen concentration. These will differ in the two tumor types. Further, we have preliminary data, using nitric oxide synthase as the activating system,4 to suggest it is the proportion of cells in the tumor that exist below physiologically normal aerobic condition, rather than the absolute level of pO₂ in the hypoxic regions of the tumor, which is necessary for activation and toxicity of AQ4N.

Hypoxic regions of tumors due to inadequate vascula-

expected to be much higher, and considerably in excess of in vitro IC₉₀ values.

This study was designed to parallel a pharmacodynamic investigation of AQ4N activation in man in a phase I clinical study. In this clinical study (12), 200 mg/m² AQ4N was administered 24 hours before elective surgery, and biopsy samples were taken for analysis. This dose is roughly equivalent, by standard allometric scaling, to the 60 mg/kg dose in mice used here. The clinical study identified tumor-spe-
cific activation of AQ4N in a range of human cancers, and found mean tumor AQ4 concentrations of >0.2 μg/g in 22 of 32 patients (12). In xenografts, we have shown here that an equivalent dose of AQ4N leads to similar intratumoral levels of AQ4 of 0.2 to 1 μg/g, and that these levels are sufficient for therapeutic benefit in combination therapy in these models. This provides evidence that therapeutic doses of AQ4 can be delivered into human tumors and that this might potentially lead to improved patient response to com-

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In vivo activation of the hypoxia-targeted cytotoxin AQ4N in human tumor xenografts

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