Evaluation of the Antiangiogenic Potential of AQ4N

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Abstract

Purpose: A number of cytotoxic chemotherapy agents tested at low concentrations show antiangiogenic properties with limited cytotoxicity, e.g., cyclophosphamide, tirapazamine, and mitoxantrone. AQ4N is a bioreductive alkylaminoanthraquinone that is cytotoxic when reduced to AQ4; hence, it can be used to target hypoxic tumor cells. AQ4N is structurally similar to mitoxantrone and was evaluated for antiangiogenic properties without the need for bioreduction.

Experimental Design: The effect of AQ4N and fumagillin on human microvascular endothelial cells (HMEC-1) was measured using a variety of in vitro assays, i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, wound scrape, tubule formation, rat aortic ring, and invasion assays. Low-dose AQ4N (20 mg/kg) was also given in vivo to mice bearing a tumor in a dorsal skin flap.

Results: AQ4N (10^{-11} to 10^{-5} mol/L) had no effect on HMEC-1 viability. AQ4N (10^{-9} to 10^{-5} mol/L) caused a sigmoidal dose-dependent inhibition of endothelial cell migration in the wound scrape model. Fumagillin showed a similar response over a lower dose range (10^{-13} to 10^{-9} mol/L); however, the maximal inhibition was less (25% versus 43% for AQ4N). AQ4N inhibited HMEC-1 cell contacts on Matrigel (10^{-8} to 10^{-5} mol/L), HMEC-1 cell invasion, and sprouting in rat aorta explants. Immunofluorescence staining with tubulin, vimentin, dynein, and phalloidin revealed that AQ4N caused disruption to the cell cytoskeleton. When AQ4N (20 mg/kg) was given in vivo for 5 days, microvessels disappeared in LNCaP tumors grown in a dorsal skin flap.

Conclusions: This combination of assays has shown that AQ4N possesses antiangiogenic effects in normoxic conditions, which could potentially contribute to antitumor activity.

It is now well established that angiogenesis is a major requirement in the process of tumorigenesis (1). When tumor cells sense a decrease in the concentration of oxygen, they release a range of potent angiogenic factors that encourages the development of new capillary-like vessels, allowing the tumor to continue growing (2). In recent years, the concept of antiangiogenic therapies for cancer has become increasingly attractive, because it involves the specific targeting of activated endothelial cells, which are quiescent in normal tissues; this provides a specificity of action directed at the tumor-derived endothelial cells. Current antiangiogenic-targeted strategies include (a) interference with angiogenic ligands, their receptors, or downstream signaling, (b) up-regulation or delivery of endogenous inhibitors, and (c) directly targeting the tumor vasculature (3).

Antiangiogenic compounds are derived from a range of different sources, including plant materials (4), fruit extracts (5), animal extracts (6), and snake venoms (7). In addition, a new category of antiangiogenic drugs has been identified, known as the “accidental” inhibitors, which include a range of cytotoxic chemotherapeutic drugs. For example, the alkaloid 10-hydroxycamptothecin, which inhibits DNA topoisomerase II, has recently been identified as a potent antiangiogenic compound in vitro and in vivo (8).

An important category of cancer chemotherapeutic agents is the hypoxic cytotoxins or bioreductive drugs, designed specifically to be activated in hypoxic conditions commonly found in tumors. Bioreductive drugs are therapeutically important due to their specific activation and targeting of cells in hypoxic areas of tumors (9, 10). The 1,2,4 benzotriazine 1,4-di-N-oxides, including the bioreductive drug tirapazamine, have been identified as antiangiogenic compounds in vitro and are currently being investigated in vivo (11).

The novel alkylaminoanthraquinone N-oxide (AQ4N) is a nontoxic prodrug, which is bioreductively activated in hypoxia to a cytotoxic product, AQ4 (12). AQ4N shows a considerable antitumor effect when used in combination with oxic cell killers like radiation (13, 14) or chemotherapeutic drugs (15, 16) and is currently in phase I/phase II clinical trial. Mitoxantrone, an analogue of AQ4 and AQ4N, shows antiangiogenic activity in the chorioallantoic membrane assay (17), as well as exhibiting potent anticancer properties. Research on natural anthraquinones isolated from Watersipora

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subtorquata has shown them to possess potent antiangiogenic properties in bovine aortic endothelial cells (18), suggesting that antiangiogenic activity may be a feature of anthraquinones. Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) has been identified as a potent antiangiogenic molecule in vitro and ex vivo (19); this effect is caused by induction of cell cycle arrest in G2-M phase through inhibition of microtubule arrangement. A by-product of the brewing process 1,2,5,7-tetrahydroxy-anthaquinone was recently identified as a potent inhibitor of human microvascular endothelial cell-1 (HMEC-1) migration (20), again suggesting that anthraquinones may have a functional role in the inhibition of angiogenesis.

In this paper, we describe the antiangiogenic effects of AQ4N in aerobic conditions in a range of well-established in vitro, ex vivo, and in vivo assays compared with the well-characterized, potent antiangiogenic agent, fumagillin.

Materials and Methods

Cell culture. HMEC-1 were obtained from the Ophthalmology Department at Queens University of Belfast and cultured in Nuclo Labware. The cells were maintained in MCDB 131 medium (Life Technologies-Bethesda Research Laboratories) supplemented with 10% FCS (PAA Laboratories), 2.5 mmol/L L-glutamine (Life Technologies-Bethesda Research Laboratories), and 10 ng/mL epidermal growth factor (Roche). The cells were routinely split at a ratio of 1:3, harvested with trypsin (Life Technologies-Bethesda Research Laboratories), and used in experiments between passages 14 and 21. HT29 cells were obtained from Cancer Research UK and were grown in MEM media (Life Technologies-Bethesda Research Laboratories) with 10% FCS. All experiments were carried out at 37°C in 95% air/5% CO2 in humidified incubators.

Cell proliferation assay. A proliferation assay was carried out, using the colon adenocarcinoma cell line HT29, to determine the toxicity of AQ4N and fumagillin. Briefly, 2 × 10³ cells were seeded in 35-mm diameter Petri dishes and incubated overnight. The medium was removed, and fresh medium containing either AQ4N (10⁻⁸, 10⁻⁹, 10⁻¹⁰ mol/L, KuDOS Pharmaceuticals) or fumagillin (10⁻⁷ mol/L, Sigma) was added. The dishes were incubated for 48, 72, and 96 h, before trypsinization and counting using trypan blue (Life Technologies-Bethesda Research Laboratories) and a hemocytometer.

Cell migration assay. HT29 (5 × 10⁴) or HMEC-1 cells (2.5 × 10⁵) were seeded in 96-well plates and allowed to attach for 5 h. The cells were treated with a range of concentrations of AQ4N or fumagillin and incubated for 24, 48, and 72 h. After incubation, the cells were exposed to a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) for 4 h. The media was aspirated, and 200 μL of DMSO (Sigma) was added to reduce the salt and induce a color change. Samples were analyzed at 550 nm, and the results were compared with untreated control cells. The experiment was repeated thrice.

In vitro “wound” scrape assay. HMEC-1 migration was measured using a wound scrape assay as described previously (21). Briefly, HMEC-1 cells were grown to confluence on oven-baked (180°C) microscope slides (BDH Laboratory Supplies). The monolayer was wounded with a 200-μL pipette tip in a length-wise stripe and supplemented with fresh medium containing AQ4N (1 × 10⁻⁷ to 1 × 10⁻³ mol/L) or fumagillin (1 × 10⁻⁷ to 1 × 10⁻⁶ mol/L). The slides were incubated at 37°C for 7 h, i.e., the time for an untreated wound to decrease in size by 50%. They were then fixed in 4% PBS buffered paraformaldehyde (Sigma), and the wound width was measured at 200× magnification (Olympus BX 50) using a calibrated eyepiece graticule (1 mm/100 μm graduation); the observer was blinded to the treatment. Twenty measurements were taken at random intervals along the length of the wound. This experiment was repeated in triplicate, and data were expressed as a percentage of the migration in untreated endothelial cells. The experiment was then modified so that wounded monolayers were exposed to 10⁻⁵ mol/L AQ4N and slides were removed regularly until wound closure had occurred. Slides were fixed and analyzed as previously described. The experiment was repeated in triplicate.

Tube formation on Matrigel. HMEC-1 tube formation was carried out using a modification of the method of Ashton et al. (22). BD BioCoat Matrigel Matrix Thin layer 24-well plates (BD Discovery Labware) were hydrated with 500 μL of MCDB 131 medium and incubated at 37°C for 30 min. Excess medium was aspirated, and HMEC-1 cells (1 × 10⁵ per well) were plated onto the gel surface in 1 mL of growth medium and incubated for 1 h. The wells were further supplemented with 1 mL of medium and AQ4N (10⁻¹⁵ to 10⁻⁵ mol/L) or fumagillin (10⁻¹² to 10⁻⁶ mol/L), in triplicate. After 18 h, excess medium was aspirated from the gel surface, and the tube-like structures were fixed in 4% PBS buffered paraformaldehyde. These were analyzed at 100× magnification, and the number of tube structures formed between adjacent endothelial cells was determined in five different fields of view per well. For example, two endothelial cells sharing a common branching point would be scored as 1; contact between three endothelial cells as 2 and so on. An independent investigator assessed each well blindly. Data was expressed as a percentage of tube formation compared with control. Experiments were repeated four times.

Invasion assay. Endothelial cell invasion was measured using the BD BioCoat Angiogenesis System (BD Biosciences) according to manufacturer’s instructions. Briefly, 1.5 × 10⁵ cells in serum-free media were added to the chamber inserts, with FCS-containing media inside the chamber to act as a chemoattractant. AQ4N (10⁻¹⁵ to 10⁻⁶ mol/L) was added to the endothelial cells and incubated for 24 h at 37°C. The insert plate was removed and incubated for 90 min in fluorescein (5 μg/mL) at 37°C. Fluorescence was read in a fluorescent plate reader (Tecan, Bio-Rad, United Kingdom) at excitation/emission wavelengths of 485 of 530 nm as a measure of invaded cells. Results were plotted as percentage of cell invasion compared with control. Experiments were repeated thrice.

Rat aortic ring assay. This assay was carried out as described by Suchting et al. (23) with some modifications. In brief, aortas were aseptically removed from 250 to 350 g male Wistar rats and immediately placed in complete MCDB 131 medium supplemented with 100 units/mL penicillin and 100 μg streptomycin. The connective tissue was removed, and the aorta was sectioned into 5-mm rings. Twenty-four well plates coated with Matrigel were hydrated as previously described. The rings were gently placed onto the hydrated gel surface and covered with a thin layer of Matrigel. AQ4N (10⁻⁶ mol/L) was added to the wells in a final volume of 250 μL of medium. The plates were incubated for 7 days. Endothelial cell sprouting from the aortic ring was assessed at 4× using a graduated eye piece graticule. Each ring was scored by four independent observers on a scale of 0 to 5, depending on the extent of outgrowth, wherein 0 equals no growth and 5 represents profuse growth. The experiment was repeated in triplicate.

Evaluation of cytoskeletal changes in endothelial cells on exposure to AQ4N. HMEC-1 cells were seeded onto four-well chamber slides and incubated overnight until confluent monolayers had formed. Medium was removed from each well, and the monolayer was wounded as previously described. The cells were supplemented with medium and AQ4N (10⁻⁶ mol/L) and incubated for 5 h. After four washes in PBS, cells were fixed in 4% PBS buffered paraformaldehyde and treated with 0.1% Triton X for 20 min. The cells were washed (3×) in PBS, incubated for 20 min in 2% bovine serum albumin containing 0.1% Triton X and washed (1×) in PBS. They were then incubated for 90 min with a primary monoclonal antibody to α-tubulin (1:500), vimentin (1:200), dynein (1:200), or the FITC conjugate phalloidin (1:20; Sigma). The cells were washed in PBS followed by 1 h incubation with FITC-conjugated antimouse secondary antibody (Sigma 1:30) at room temperature. The cells were mounted with Vectashield mounting medium.

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medium containing propidium iodide (Vector Laboratories) and sealed to prevent dehydration. The slides were covered in tinfoil and stored at 4°C for analysis using fluorescence microscopy (Bio-Rad Micro-Radiance confocal scanning laser microscope).

**Dorsal skin flap model.** Twelve-week-old male BALB-c SCID mice were anesthetized using i.p. injection of fentanyl (10 mg/kg; Hypnorm, Vetapharma Ltd.) and midazolam (10 mg/kg; Hypnovel, Roche). Animals were kept warm using heated pads throughout the surgery. A viewing chamber, consisting of two aluminum frames holding two parallel glass windows 200 μm apart, was attached to a raised skin flap on the dorsal surface of the mouse. The skin on one side of the viewing region was removed, exposing the dermis on the opposite skin layer. A fragment (~ 0.5 mm) of LNCaP prostate tumor (excised from a donor mouse) was placed on the microvascular bed, and the glass slide was replaced. After surgery, animals were maintained at 32°C to 34°C.

The vasculature was imaged 12 days postsurgery, when the tumors measured ~2 mm in diameter, by injection of 50 μL of 150 kDa FITC-labeled dextran (50 mg/mL; Sigma) into the tail vein under light anesthesia. The viewing chamber was illuminated with a mercury lamp, and the FITC signal was detected with an ORCA-ER camera system (Hamamatsu Photonics). Images were recorded using IPLab software (BD Biosciences). Mice then received AQ4N (20 mg/kg) i.p. daily for 5 days; control animals received daily i.p. injections of PBS. Imaging was repeated at days 13 and 17. Experiments were carried out in accordance with the Animal (Scientific Procedures) Act 1986 and conformed to the current United Kingdom Coordinating Committee on Cancer Research guidelines.

**Results**

**Effect of AQ4N on the proliferation of HT29 colon cancer cells.** AQ4N (10^-6, 10^-8, 10^-10 mol/L) had no significant effect on the proliferation of HT29 cells (Fig. 1A), whereas fumagillin (10^-6 mol/L) inhibited the proliferation of HT29 cells over the 72-h incubation. AQ4N (10^-10 to 10^-5 mol/L) had no effect on HT29 cell viability (Fig. 1B). Fumagillin (10^-5 to 10^-8 mol/L) caused a decrease in cell viability compared with control (Fig. 1C). However, it had no effect on viability below 10^-9 mol/L.

**Effect of AQ4N on the proliferation of HMEC-1 cells.** AQ4N (10^-6 mol/L) had no significant effect on the proliferation of HMEC-1 cells over 72 h (Fig. 1D). At 72 h, there was a small, nonsignificant inhibition (P = 0.13). A similar lack of effect was shown at all other doses tested (10^-6 to 10^-11 mol/L). When an equimolar concentration of fumagillin (10^-6 mol/L) was tested, a significant inhibition of HMEC-1 proliferation was found compared with both the control and AQ4N-treated cells at 24 and 48 h (P < 0.05; Fig. 1E).

**Effect of AQ4N on migration of HMEC-1 cells in the wound scrape assay.** This assay was used to evaluate the effect of the drugs on the chemotactic migration of endothelial cells (Fig. 2A). Untreated HMEC-1 cells closed the wound by 50% in 7 h (Fig. 2B). AQ4N (10^-10 to 10^-5 mol/L) induced a dose-dependent inhibition of migration (Fig. 2C). At 10^-6 mol/L, AQ4N induced a maximal decrease in wound closure of 43% compared with untreated control. In contrast, the maximal decrease caused by fumagillin was 25%, although this occurred at a lower concentration (10^-9 mol/L). This difference in maximal effect and concentration was reflected in the EC50 values of each compound: 7.4 x 10^-11 mol/L (R^2 = 0.78) for fumagillin and 2.9 x 10^-8 mol/L for AQ4N (R^2 = 0.93).

Using the optimal concentration for inhibition of migration, the rate of HMEC-1 cell migration was evaluated in the presence of AQ4N (10^-6 mol/L; Fig. 2B). In AQ4N-treated monolayers, the wound closed in 41 h compared with the untreated controls, which took 18 h.

**Effect of AQ4N on HMEC-1 microvessel tubule formation on Matrigel matrix.** AQ4N induced a dose-dependent inhibition

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**Fig. 1.** The effect of AQ4N and fumagillin on HT29 cell proliferation and HMEC-1 viability. **A,** the effect of AQ4N (10^-6, 10^-8, and 10^-10 mol/L) or fumagillin (10^-6 mol/L) on the proliferation of HT29 cells. Cells (2 x 10^5) were seeded in 35-mm dishes and incubated overnight. Cells were resupplemented with medium containing AQ4N (10^-6, 10^-8, and 10^-10 mol/L) or fumagillin (10^-6 mol/L) at 0, 24, 48, and 72 h, cell counts were determined. **B,** the effect of AQ4N (10^-10 to 10^-5 mol/L) and C, fumagillin (10^-10 to 10^-5 mol/L) on HT29 cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **D,** the effect of AQ4N (10^-10 to 10^-5 mol/L) on HMEC-1 cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **E,** comparison of the effect of fumagillin (10^-6 mol/L) and AQ4N (10^-8 mol/L) on HMEC-1 cell viability measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results are the mean of three independent experiments. *P* < 0.05.
of HMEC-1 microvessel tube formation on Matrigel in the concentration range 10^{-12} to 10^{-5} mol/L (Fig. 3A and B). The dose-response curve was sigmoidal and yielded an EC_{50} value of 1.31 \times 10^{-7} \text{mol/L} (R^2 = 0.91). The maximal effect of AQ4N was observed at 10^{-5} \text{mol/L} when a 34\% inhibition of tube formation was observed compared with untreated control cells. Fumagillin showed a greater inhibition of tube formation (64\%) at 10^{-6} \text{mol/L} and exhibited a lower EC_{50} value (2.79 \times 10^{-10} \text{mol/L}; R^2 = 0.88).

**Effect of AQ4N on HMEC-1 cell invasion.** The effect of AQ4N on HMEC-1 invasion was evaluated in the endothelial cell invasion assay. AQ4N showed a sigmoidal dose-dependent inhibition of HMEC-1 invasion over the dose range 10^{-11} to 10^{-5} \text{mol/L} (Fig. 3C).

**Rat aortic ring assay.** In control conditions, the ring exhibited significant growth, with sprouts tending to be long, forming intricate high-density networks around the ring (Fig. 4A). In contrast, the addition of 10^{-6} \text{mol/L} AQ4N induced a significant inhibition of endothelial sprouting from the explanted aortic ring (P = 0.001). On the sprouting index scale, scored by four independent investigators, the mean score for spraying in control conditions was 3.5 whereas AQ4N-treated rings exhibited a score of 0.38 (Fig. 4B).

**Effect of AQ4N on the cytoskeletal elements of the human microvascular endothelial cell during chemotactic migration.** To determine whether the observed antimigratory effect of AQ4N may be a consequence of cytoskeletal changes in the endothelial cell, the morphology of the tubulin networks was investigated by immunofluorescence using the \alpha\text{-tubulin antibody.} Figure 5A shows a quantitation of the reorientation of microtubules in the presence or absence of AQ4N. In control conditions, 60\% of the HMEC-1 in the first two rows of cells adjacent to the wound edge show complete realignment of microtubules into the wound, with a further 34\% of the cells showing partial realignment of their structural cytoskeleton. In contrast, the response of cells in the presence of AQ4N show a significantly different microtubule orientation, with 64\% of the cells showing no realignment of tubules and 36\% of the cells showing partial realignment.

Untreated cells showed a highly structured microtubule arrangement, generally aligned toward the direction of cell migration. The tubules originated from one central point the microtubule organization center, which was identified as a dense, clearly defined area of staining situated in front of the nucleus. In AQ4N-treated cells, at the optimum concentration for inhibition of migration (10^{-6} \text{mol/L}), the microtubule
organization center was poorly defined. In addition, the microtubules present were not orientated in the expected direction of migration (i.e., into the wound) and instead showed a more random orientation with a significant number of the cells exhibiting microtubules with curled or twisted terminal endings (Fig. 5B and C).

To determine the effect of AQ4N on other cytoskeletal elements, immunofluorescence staining of the intermediate filaments, actin fibers, and dynein motor protein with vimentin, phalloidin, and dynein, respectively, was carried out. The structure of the intermediate filaments in control conditions showed dense fibrous structures elongating into the wound, indicating migration. In contrast, in cells treated with AQ4N, the vimentin staining was significantly altered, with the majority of the staining showing a partial encircling of the nucleus or condensation behind the nucleus at the farthest point in the cell from the wound edge. Any filaments present in the cytoplasm were laterally aligned to the wound edge and showed no signs of reorientation toward the wound (Fig. 5D and E).

In control cells, dynein seemed as dense staining throughout the cytoplasm of migrating cells. In AQ4N-treated cells, the dynein staining was significantly different from the untreated cells with a dense region of staining behind the nucleus at the farthest point from the wound edge and the remaining fibers orientating laterally to the wound edge (Fig. 5F and G).

In control cells, the actin fibers spread out over the complete cell cytoplasmic area and generally orientated toward the wound. In AQ4N-treated cells, the actin fibers remained intact and did not orientate in the direction of migration (Fig. 5H and I).

The effect of AQ4N on tumor vessels growing in LNCaP tumors in a dorsal skin flap model. A subtherapeutic dose of AQ4N (20 mg/kg) was given for 5 days to mice bearing a LNCaP tumor implanted in a dorsal skin flap. When examined 24 h after treatment was started, no effect was observed (data not
shown). After 5 days of treatment, a clear reduction in tumor vessels was observed especially in the center of the tumor; vessels around the tumor edge were less affected (Fig. 6). No effect was seen in the controls.

**Discussion**

Angiogenesis is a key component in the development of tumors and ultimately in the process of metastasis. For this reason, the search for efficient antiangiogenic agents has been considerable; currently, there are a large number of these compounds in different stages of clinical trial (24, 25). Significant benefit has been shown with several agents, and one, Avastin, has recently been given Food and Drug Administration approval.

The present study reports on the ability of the alkylaminoanthraquinone N-oxide AQ4N to inhibit endothelial cell function in vitro, ex vivo, and in vivo. Recent studies looking at the antiangiogenic properties of alkylating agents have identified proliferating endothelial cells as highly sensitive to their apoptotic actions (26). The main problem associated with antiangiogenic drugs that target endothelial proliferation is their frequently reported toxicity, e.g., TNP470 (27), often due to the drugs ability to inhibit the proliferation of other cell.

**Fig. 5.** The effect of AQ4N on cytoskeletal proteins. A, the effect of AQ4N (10^{-6} mol/L) on the directional rearrangement of the cellular microtubules in the in vitro wound scrape model of endothelial migration compared with time-matched controls. Cells were examined using fluorescent microscopy for alignment in the direction of the wound. When AQ4N was present, no cells showed full realignment. The results represent the data from three independent experiments and 10 different fields of views in each experiment. B-I, the effect of AQ4N (10^{-6} mol/L) on actively migrating HMEC-1 cells showing cytoskeletal elements. Immunofluorescence photomicrographs of HMEC-1 cells stained for α-tubulin (B and C), vimentin (D and E), dynein (F and G), and phalloidin (H and I). B, D, F, and H, control cells; C, E, G, and I, AQ4N-treated cells.

**Fig. 6.** The effect of AQ4N on blood vessels in LNCaP tumors grown in a dorsal skin flap. LNCaP prostate tumors were implanted in a dorsal skin flap on the backs of SCID mice. FITC-dextran was injected i.v. 10 min before imaging. A, before treatment. B, after treatment with AQ4N (20 mg/kg) given i.p. for 5 d. Arrows show orientation mark.
types. Encouragingly in our study, AQ4N (10^{-12} to 10^{-5} mol/L) showed no significant antiproliferative effects on HMEC-1 or the colon cancer cell line HT29 over a 72-h exposure period. Fumagillin, the precursor of TNP-470, exhibited an antiproliferative effect in both cell lines at concentrations of >10^{-8} mol/L, which was similar to those previously described (28, 29); however, it should be noted that the antiangiogenic activity of fumagillin was evident at lower doses.

A vital component of the initial stages of angiogenesis is the migration of endothelial cells from the existing tumor vasculature to the site of new vessel development. Using the wound scrape assay, AQ4N was seen to be an efficient inhibitor of migration inducing an optimal inhibition of 43% at 10^{-6} mol/L, a dose that causes no measurable cytotoxicity. Fumagillin showed an effect on migration at a lower dose; however, the magnitude of the maximal inhibition of migration was 25%. When the time for wound closure was evaluated, it took over twice as long for the wound to close in the presence of AQ4N (10^{-7} mol/L) compared with control.

The ability of HMEC-1 to form tube-like structures on the basement membrane-rich Matrigel matrix (30) provides a second method of evaluating angiogenic promoters or inhibitors. Angiogenic events will require directional migration, proliferation, and the rearrangement of endothelial cell position that leads to the formation of tube-like structures. AQ4N was found to be a potent inhibitor of HMEC-1 tube formation. AQ4N was ~1,000-fold less potent than fumagillin, although the efficacies of inhibitions were similar. The ability of AQ4N to prevent the differentiation of the HMEC-1 into tube structures indicates its possible effectiveness as an antiangiogenic therapy and an antitumor treatment. The important consideration for clinical application, however, is not simply the ED_{50} for antiangiogenic end points but the relative ED_{50}s for therapeutic and toxic effects. In this respect, our experiments support previous reports that the drug has a low toxicity under oxic conditions in vitro and in vivo (13, 31). In our experiments, no significant inhibition of HMEC-1 proliferation was observed at concentrations that effectively inhibited migration and tube formation. The invasion assay confirmed the ability of AQ4N to reduce the migration of HMEC-1 cells across a membrane.

The ex vivo aortic ring assay was first described in 1990 (32). The usefulness of the assay is that it provides an environment of multiple cell types, allowing the investigation of cells and soluble mediators implicated in angiogenesis and novel inhibitors of these processes, e.g., endostatin (33). It has recently been used to confirm the antiangiogenic effects of the pan vascular endothelial growth factor receptor tyrosine kinase inhibitor CEP7055. The reported efficacy mirrored antiangiogenic responses in vivo (34). In these investigations, 0.4 μmol/L CEP7055 induced maximal inhibition of tube formation at a concentration comparable with that reported in this paper.

As the most significant antiangiogenic behavior was observed in the migration assay, we investigated the effect of AQ4N on the cytoskeletal morphology of endothelial cells. Microtubules have been identified as the major element responsible for orientation of most cell types in response to a migratory stimulus (35) and maintenance of cell shape (36). The structure of the microtubules in AQ4N-treated cells is clearly abnormal. In control cells, the microtubules were long, thin, and fiber-like, attached to the leading edge of the cell or pseudopodia. In contrast, the microtubules in AQ4N-treated cells were shortened and tortuous, with excessive twisting of the tubules. This would suggest an almost complete destabilization of the microtubule network. The twisting of the microtubules is consistent with previous reports in which a range of compounds, including the Vinca alkaloids (37) and nocodazole (38), was shown to interfere with microtubule organization.

In endothelial cells, intermediate filaments are a major constituent of the cytoskeleton (39) and are classed as relatively stable when compared with microtubules (40). The most abundant intermediate filament in endothelial cells is vimentin, which is thought to have many roles, including maintenance of cell shape and targeting of molecules between the nucleus and cytoplasm (41). In control HMEC-1 cells, the filaments were organized in a network originating at the nucleus and extending to the inner surface of the outer membrane, indicating an active role in migration as previously reported (21). In contrast, the AQ4N-treated nonmigrating cells seemed to have no cytoplasmic evidence of vimentin, although dense vimentin staining was observed behind the nucleus. This phenomenon in endothelial cells has previously been reported when the cells were exposed to the microtubule destabilizing agent colchicine (42). In this study, a close correlation between microtubules and intermediate filaments was suggested due to the collapse of both cytoskeletal elements when treated with the microtubule destabilizing agent. The importance of vimentin to the motility of endothelial cells has been reported; vimentin-deficient cells showed impaired migratory ability (43).

As migration of endothelial cells involves reorientation of the microtubule organization center and microtubules, the process will require a cytoplasmic motor protein, which has recently been identified as dynein (44). Dynein is a large multisubunit protein which is thought to bind cellular compounds and move them along microtubules toward the center of the cell in an ATP-dependent fashion (45). In untreated cells, dynein was observed as dense staining throughout the cytoplasm of migrating cells. In contrast, AQ4N-treated, cells showed an accumulation of dynein in the perinuclear region. Dynein has recently been identified as a linking motor protein between microtubules and vimentin movement (46), facilitating their interaction in the maintenance of cytoskeleton integrity.

In endothelial cells, actin fibers are considered vital for conserving cell integrity and are commonly observed as dense bands of stress fibers crossing the cell (35, 47) and terminating at focal contact points. To initiate migration, the actin cytoskeleton must depolymerize its stress fibers into fibers of G-actin, which form the skeletal elements in lamellipodia (48). In our experiments, little disruption of the actin cytoskeleton was observed, the main difference between control and AQ4N-treated cells being the absence of reorientation of the fibers toward the wound.

To confirm that the in vitro effects also occur in vivo, we assessed the influence of AQ4N on the vasculature of a LNCaP tumor growing in a dorsal window flap. A subtherapeutic dose of AQ4N was investigated to reduce the likelihood of antitumor effects influencing the response. There was a marked reduction in the small capillaries after 5 days; this was not apparent when examined at 24 h (data not shown), confirming an antiangiogenic influence. The larger vessels showed some reduction in the center of the tumor, but peripheral vessels were less...
affected. Interestingly, because AQ4N is widely distributed, the reduction in microvessels should not compromise drug delivery but may enhance reduction of the drug to its cytotoxic metabolite AQ4. This would result in a specific enhancement of solid tumors. Immunofluorescence analysis of AQ4N-specifically inhibiting migration, tube formation, aortic ring vessel sprouting, and invasion with no effect on the proliferative effect of the endothelial cells, suggesting no cytotoxic effects at these concentrations. Immunofluorescence analysis of AQ4N-treated HMEC-1 cells revealed significant damage to the cytoskeletal structure of the cell, with complete disruption of microtubule networks and significant damage to vimentin and dynein function. In a preliminary in vitro experiment, we have confirmed the antiangiogenic effect of AQ4N. It is possible that in addition to the well-established bioreductive cytotoxicity of AQ4N, its antiangiogenic properties could be exploited in cancer therapy, most usefully by combining chronic, low-dose (micromolar) exposure (to target angiogenesis) with acute high dose (millimolar) administration (to kill hypoxic cells). Further studies are planned to examine the timing and dose dependency of this approach.

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References

Evaluation of the Antiangiogenic Potential of AQ4N

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