Squalamine Inhibits Angiogenesis and Solid Tumor Growth in Vivo and Perturbs Embryonic Vasculature


ABSTRACT

The novel aminosterol, squalamine, inhibits angiogenesis and tumor growth in multiple animal models. This effect is mediated, at least in part, by blocking mitogen-induced proliferation and migration of endothelial cells, thus preventing neovascularization of the tumor. Squalamine has no observable effect on unstimulated endothelial cells, is not directly cytotoxic to tumor cells, does not alter mitogen production by tumor cells, and has no obvious effects on the growth of newborn vertebrates. Squalamine was also found to have remarkable effects on the primitive vascular bed of the chick chorioallantoic membrane, which has striking similarities to vascular beds in response to an angiogenic stimulus. A number of cytokines and growth factors that activate endothelial cells have been identified, and the development of new vessels in pathological neovascularization can be directly correlated with their expression. These mitogens induce an alteration of endothelial gene expression with initiation of proliferation and production of proteolytic enzymes leading to matrix degradation, cell migration, and subsequent tissue invasion. For tumor-induced angiogenesis, endothelial activation is mediated by a variety of soluble mitogens produced by the tumor cells that act in a paracrine manner via specific receptors on the endothelial cell (5). Angiogenesis inhibition has become a well-described antitumor treatment strategy, because avascular tumors are incapable of growth and have little metastatic potential. Antagonists of receptors of specific mitogens have been used to inhibit neovascularization in experimental models, but a specific receptor antagonist does not necessarily offer protection against tumors that are known to secrete a variety of angiogenic factors.

In these studies, we examined the effects of the novel aminosterol, squalamine, on both mitogen and tumor-induced angiogenesis as well as on the vascular bed of the chick embryo. Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to a spermidine molecule to a steroid derived from stigmasterol. This compound was originally identified for its broad spectrum antimicrobial activity, its steroid characteristics led us to investigate its activity and possible mechanism(s) as an inhibitor of angiogenesis and tumor growth in multiple in vitro and in vivo models.

MATERIALS AND METHODS

Endothelial Cell Proliferation Assays. The effects of squalamine on endothelial cell proliferation were assayed with and without mitogen stimulation. Rat brain endothelial cells of the RBE-4 clone, immortalized as described previously by La1 et al. (12), were maintained at 37°C in 5% CO2 in fibronectin-coated plates in DMEM containing 10% fetal bovine serum, 1% l-glutamine, 10 mM HEPES, and geneticin (complete media) with basic fibroblastic growth factor at 1 ng/ml. At the time of experimentation, RBE-4 cells (10,000 cells/well) were plated in fibronectin-coated plates and allowed to attach overnight in complete media without growth factors. The medium was then replaced with either fresh complete medium or medium supplemented with the mitogen to be studied. Mitogens used were VEGF (20 ng/ml), bFGF (10 ng/ml), PDGF (10 ng/ml), scatter factor/hepatocyte growth factor (5 ng/ml), tumor conditioned medium from the 9L rat glioma (9LCM), and human hemangioblastoma cyst fluid. 9LCM was generated by growing 9L cells in supplemented MEM as described previously by Cahan et al. (13) for 3 days, followed by centrifugation and resuspension of the pellet in the RBE-4 complete media (50% dilution) without additional mitogens. Human hemangioblastoma cyst fluid was obtained from an intraoperative specimen of a cyst associated with an intracranial hemangioblastoma. This fluid was diluted (1% dilution) into complete RBE-4 media (no additional mitogen) for proliferation studies. Squalamine to create a final concentration of 10, 30, or 50 µg/ml or buffer alone was added simultaneously with mitogen. All assays were performed in triplicate, and data were analyzed using an ANOVA method for multiple comparisons.

Endothelial Cell Migration Assays. The effect of squalamine on the mitogen-stimulated movement of RBE-4 endothelial cells across "wounded" monolayers was quantified. RBE-4 cells were maintained and stimulated with basic fibroblastic growth factor at 1 ng/ml. At the time of experimentation, RBE-4 cells (10,000 cells/well) were plated in fibronectin-coated plates and allowed to attach overnight in complete media without growth factors. After overnight attachment in complete media without added growth factors, the confluent monolayer was wounded with a sterile straight edge, and all adherent cells to one side of the wound were removed by mechanical stripping under microscopic guidance. The remaining adherent cells were rinsed and replenished with complete media containing purified mitogens with or without squalamine at 10 or 50 µg/ml. Cultures were incubated at 37°C to allow cells to migrate out from the wounded monolayer. At 24 h, cultures were fixed and stained with 3,3-diaminobenzidine and washed with distilled water. Stained areas were quantified using a blinded observer using a digital analysis program with a blinded observer using a digital analysis program with a 24-bit color system.
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Table 1 Squalamine inhibits growth and vascularity of 9L glioma implanted in rat flank

The rat 9L glioma was implanted in the flanks of rats, followed for 25 days, and measured and examined following necropsy. Animals received either a single maximum tolerated dose of the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, n = 6), daily injections of squalamine (n = 6), or saline (n = 4), 5 days after implantation as described in the text. Results are expressed as the mean ± SE. The squalamine group exhibited a significant effect for each parameter (P < 0.002).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor weight (g)</th>
<th>Tumor volume (mm³)</th>
<th>Vessel density (no. per ×400 field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.41 ± 1.34</td>
<td>7121 ± 1675</td>
<td>10.67 ± 1.52</td>
</tr>
<tr>
<td>Squalamine</td>
<td>1.67 ± 0.60</td>
<td>1466 ± 388</td>
<td>5.54 ± 1.82</td>
</tr>
<tr>
<td>BCNU</td>
<td>2.30 ± 0.76</td>
<td>2494 ± 877</td>
<td>11.09 ± 5.09</td>
</tr>
</tbody>
</table>

Macintosh computer (NIH Image Analysis program). The total area of RBE-4 migration across the wound was calculated and expressed in mm² for each plate. Four assays were run for each squalamine concentration using each mitogen, and comparisons were made using ANOVA.

Effects of Squalamine on Endothelial Cell Proton Secretion. Studies were conducted to assess the effects of squalamine on the cellular metabolism of both resting and mitogen-stimulated endothelial cells. Effects of squalamine and related substances on in vitro endothelial cell proton secretion after VEGF stimulation were measured using a Cytosensor microphysiometer (Molecular Devices Corp., Sunnyvale, CA) as described previously by McConnell et al. (14). Human microvascular endothelial cells (Cell Systems, Kirkland, WA) were maintained in supplemented EBM media. Cells were used at a density of 200,000–300,000 cells per Capsule Cap and were serum starved for 24 h prior to the assay to deplete any growth factors. Cells were maintained at 37°C on the Cytosensor system in low-buffered balanced salt solution (138 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.81 mM KH₂PO₄, 0.11 mM KH₂PO₄, and 10 mM glucose) with a flow rate of 120 ml/min. After equilibration on the system for 30 min, cells were exposed to squalamine, 1436 (another natural aminosterol isolated from the shark that differs from squalamine in that the polyamine moiety is spermine rather than spermidine, which exhibits no effect on endothelial cell proliferation), or methylisobutyl amiloride (a potent inhibitor of the NHE isoform, NHE-1; Ref. 15), each at a

Fig. 1. Inhibition of mitogen-stimulated angiogenesis in the mouse Matrigel assay. Masson trichrome-stained sections of ventral abdominal wall showing bFGF-containing Matrigel plug (top), fibroblast layer with vessels, and abdominal muscle tissue. A and B, squalamine-treated animal with few vessels in fibroblast layer. C and D, control animal showing much thicker fibroblast layer with numerous microvessels. A and C, ×200; B and D, ×630.
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Days after tumor implantation

Fig. 2. Summary of inhibition of angiogenesis by squalamine in one trial of this assay (five eyes per experimental group). ○, squalamine treated; □, controls. Bars, SE.

Concentration of 10 mM for 1 h, followed immediately by exposure to 20 ng/ml of VEGF. Extracellular acidification rates were measured every 2 min throughout the duration of the experiment.

Angiogenesis and solid tumor growth assays in the rabbit cornea.
To determine whether squalamine might have therapeutic value in vivo in the treatment of solid tumor-induced angiogenesis, it was incorporated into a sustained release polymer for testing in the rabbit cornea model. Squalamine was incorporated at a 20% (w/w) loading into ethylene vinyl acetate copolymer (Elvax, Dupont, Wilmington, DE), which provides local sustained release (16).

Final polymer shape was a disc with a diameter and height of 0.5 mm. Sustained first-order drug release for 14 days was quantified in vitro by placing a squalamine polymer into a vial containing 5% dextrose in water at 37°C and then replacing the solution daily for analysis of the amount of drug present. The VX2 carcinoma, a tumor syngeneic to the New Zealand White rabbit was propagated by serial transplantation in the flank of a carrier animal. To stimulate angiogenesis, a 1-mm³ solid piece of the VX2 tumor was inserted into a corneal micropocket of an anesthetized rabbit 3 mm from the limbus (17).

For efficacy testing, either a squalamine loaded or blank polymer (no drug) was inserted into the pocket just distal to the tumor piece between the limbus (the source of new vessels) and the tumor (the angiogenic stimulus). For each animal, one eye received a squalamine polymer, whereas the contralateral eye received a blank polymer so that each animal served as its own control. Because the cornea is normally avascular, the ingrowth of new vessels from the limbus toward an angiogenic stimulus was easily quantified by slit lamp stereomicroscopic examination at 7, 14, and 21 days after implantation by two observers blinded to the treatment. An angiogenesis index (16) was calculated for each cornea at each time point. For biocompatibility testing, only a squalamine-containing polymer was inserted into the corneal pocket (no tumor), and the corneal reaction was assessed. Three separate experiments were performed with a total of 50 corneas studied. Statistical analysis was performed by ANOVA with ranked data and a nonparametric method. With cell culture methodology identical to that used for 9L glioma cells (13), squalamine (up to 30 µg/ml) was tested for cytotoxicity against VX2 cells in vitro.

Capillary ingrowth into Matrigel plugs in the mouse flank. To determine the efficacy of systemic administration of squalamine as an angiogenesis inhibitor in vivo, bFGF-impregnated Matrigel (Collaborative Biomedical Products, Bedford, MA) pellets were implanted s.c. in the ventral abdominal wall of mice, and vessel ingrowth was quantified. Human recombinant bFGF (R&D
**Squalamine is a novel angiogenesis inhibitor**

Squalamine is a novel angiogenesis inhibitor. A VEGF, bFGF, 1E+06, 1E+05, 1E+04, 1E+03, 1E+02, 1E+01, 1E+00, 1E-01, 1E-02, 1E-03, 1E-04, 1E-05, 1E-06. Days 0 1 2 3 4 5 6

**Fig. 4.** Squalamine inhibition of mitogen-induced endothelial cell proliferation. Growth curves for rat brain capillary endothelial (RBE-4) cells stimulated by various mitogens with and without addition of squalamine. • no drug added; ▲ 10 μg/ml squalamine added; △ 30 μg/ml of squalamine added; ○ 50 μg/ml of squalamine added. RBE-4 growth stimulated by VEGF (20 ng/ml; A), bFGF (10 ng/ml; B), 9L glioma conditioned media (C), and human hemangioblastoma cyst fluid (D) is shown. All proliferative values are averages of triplicate determinations with comparisons made using an ANOVA method of multiple comparisons (P < 0.0001 for all mitogens with squalamine at 50 μg/ml).

**Systems, Minneapolis, MN** was incorporated at 150 ng/ml into liquid Matrigel. Plugs of 0.5 ml were injected into the ventral abdominal wall of C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). The bFGF released from the plug served as an angiogenic stimulus for ingrowth of vessels (18). Animals were treated with twice-daily injections of either sterile water or squalamine (given s.c. at a distal site at 50 mg/kg/day) for 7 consecutive days beginning on the day of plug injection. All animals were sacrificed 7 days after plug placement.

The plug and adjacent peritoneal wall were removed en bloc and fixed in 10% formalin. Sections (4 μm) were cut and stained with either Masson's trichrome for standard histology or with an antibody directed against the endothelial cell integrin CD34 for immunohistochemistry. Vessel density was quantified by a blinded observer examining the sections at high power (X630) and counting the number of vessels in 10 consecutive fields in the zone of tissue adjacent to the Matrigel plug.

**In Vivo Assay of Tumor Growth and Vessel Density.** The effect of systematically administered squalamine on the growth of solid tumors in vivo was evaluated in the rat flank 9L glioma model. 9L rat glioma was propagated in the flank of a carrier animal. At the time of tumor implantation, the tumor was excised, and solid tumor pieces measuring 1 mm³ were cut using the operating microscope. A single piece was then implanted s.c. into the flank of Fischer 344 rats. Treatment was begun 5 days later with a twice-daily dose of either i.p. saline or squalamine (20 mg/kg/day). A separate group of animals received a single dose of 1,3-bis(2-chloroethyl)-1-nitrosourea (14 mg/kg) given 5 days after tumor implantation, a regimen previously shown to inhibit tumor growth in this model (19). Animals were sacrificed 25 days after tumor implantation, and the tumor mass was exposed and measured with calipers. Tumor volume was estimated, and the tumor was weighed and processed for staining with H&E. Immunohistochemistry against the endothelial cell integrin, CD34, was also done. Microvessel density was then quantified by two blinded observers using a double-headed microscope to review the most vascularized areas from the middle of the tumor in the CD34-stained sections at ×400 magnification as described previously by Weidner et al. (20). Results were expressed as number of microvessels per ×400 field (mean ± SD) for each sample, and comparisons were made between treatment groups by an ANOVA.

Using the methodology described previously for culture of 9L glioma cells (13), squalamine (30 mg/ml) was tested in vitro for toxic and antiproliferative effects on 9L cells. The effect of squalamine on VEGF production by 9L cells was measured by ELISA.

**Effects of Squalamine on Chick Embryo Vasculature.** Effects of squalamine on the yolk sac vessels of the 4-day chick embryo were investigated. Two-day-old chick embryos were purchased from a local hatchery and maintained at 37°C. Shell caps were removed at day 4, exposing the embryos and their extraembryonic vasculature. Dulbecco's PBS (without magnesium and calcium; 0.3 ml) containing 30% (w/w) Ficoll 400 (Pharmacia) with or
without 0.1 μg/ml squalamine was applied directly over the entire vascular embryonic structure. The vasculature was studied under a Zeiss stereomicroscope and photographed at various times after initial treatment. One group of yolk sac membranes exposed to 0.1 μg/ml squalamine for 1 h was fixed overnight in situ by direct application of 10% phosphate-buffered formalin onto the exposed surface. The egg contents were emptied into a dish, and the yolk sac was carefully removed, embedded in paraffin, sectioned, and stained with H&E for microscopic examination of vessel morphology.

RESULTS

Squalamine Inhibits Tumor Growth and Reduces Vessel Density and Ingrowth in Vivo without Affecting Tumor Cell Growth or VEGF Secretion in Vitro. Systemic treatment with squalamine effectively inhibited rat flank 9L glioma tumor growth as estimated by direct tumor measurement. In fact, monotherapy with squalamine was as effective in this model as the chemotherapeutic agent carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea] in retarding tumor growth. Immunohistochemical analysis of the tumor specimens and surrounding flank tissue for the endothelial cell integrin CD34 demonstrated significant reduction in vessel density in the tumors harvested from the squalamine-treated rats when compared with controls (Table 1).

Squalamine at 30 μg/ml had no effect on the proliferation of 9L glioma cells in vitro. In fact, inhibition of the proliferation of nonendothelial cells was only observed at concentrations of squalamine that caused almost immediate cellular lysis. Furthermore, squalamine did not inhibit the secretion of VEGF by 9L glioma cells into tissue culture medium as measured by ELISA assays.

Squalamine-treated mice showed an 85% decrease (P < 0.002) in growth of new vessels into bFGF-impregnated Matrigel plugs and adjacent peritumoral tissues when compared histologically to untreated controls (Fig. 1).

Squalamine Inhibits Angiogenesis and Tumor Growth in the Rabbit Cornea. Squalamine polymers (20%) were found to be biocompatible in all cases. Squalamine produced a significant reduction in angiogenesis index at all time points (P < 0.0001) as measured by slit lamp stereomicroscopy (Figs. 2 and 3, A–D). Inhibition of tumor growth was seen in the squalamine-treated corneas as evidenced by the presence of small, poorly vascularized tumors in contrast to the large, vascularized control tumors. Histological analyses of the corneas demonstrated the continued presence of tumor cells but few vessels in squalamine-treated eyes when compared with controls (Fig. 3, E and F). These experiments have been repeated with consistent results in over 50 animals. This result was not due to cytotoxicity because in vitro proliferation assays showed that squalamine at concentrations up to 30 μg/ml had no direct toxic or antiproliferative effects on VX2 cells.

Squalamine Inhibits Mitogen-induced Endothelial Cell Proliferation and Migration. In the absence of mitogen(s), squalamine at concentrations up to 50 μg/ml had no effect on the survival or proliferation of endothelial cells. However, squalamine did inhibit endothelial cell proliferation in a dose-dependent fashion in all mitogen stimulation assays. The inhibitory effect was similar for all of the studied mitogens. Squalamine at a concentration of 50 μg/ml reduced rat brain endothelial (RBE-4) cell proliferation by 50% after 24 h (Fig. 4). Squalamine concentration of 10 μg/ml resulted in about 10% inhibition at day 6 for all mitogens tested (Fig. 4).

Squalamine at 50 μg/ml reduced the migrated area of stimulated cells compared with controls by 94.9% for VEGF, 93.1% for bFGF, 84.3% for PDGF, and 98.9% for 9L conditioned medium (P < 0.0001 for all mitogens; Fig. 5). The effect on migration was not due simply to reduction in cell number, because the cell density adjacent to the line of migration was not altered by squalamine.

Squalamine Inhibits Proton Secretion by Mitogen-stimulated Endothelial Cells but to a Lesser Degree than MIA. Under conditions established in vitro (i.e., bicarbonate-free medium), acid secretion is dependent on both the rate of proton generation due to cellular metabolic activity and the rate of secretion through the action of the sodium-hydrogen exchanger (NHE; Ref. 17). MIA fully inhibited the rise in rate of acid secretion by VEGF to a value below that observed for all mitogens; Fig. 5). The effect on migration was not due simply to reduction in cell number, because the cell density adjacent to the line of migration was not altered by squalamine.

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Squalamine Exhibits Profound Effects on the Vasculature of 4-Day Chick Embryos. The direct application of squalamine had a rapid effect on the chick embryo vasculature. Prior to squalamine exposure, the vascular network displayed a uniformly well-perfused system comprised of several large vessels interconnected by secondary and tertiary branches (Fig. 7, panel 0'). Following application of squalamine solution to the embryo, vascular structure and flow characteristics remained unaffected for about 20 min. Soon thereafter, constriction of the smallest capillaries occurred throughout the yolk sac, trapping red cells. These capillaries were reduced to near-invisible remnants of the original vessels (Fig. 7, panels 30', 35', and 40'). Continuous blood flow through even the largest vessels ceased, replaced by a pulsatile expansion and contraction of vessels as blood appeared to be forced into the occluded large vessels with each heartbeat. About 1 h after exposure of the embryo to squalamine, some of the previously occluded capillaries opened, as evidenced by resumption of blood flow, whereas others remained totally or partially constricted (Fig. 7, panels 60', 75', and 100'). The integrity of the vascular bed during this remodeling process did not appear to be compromised, because hemorrhage was not observed. The net effect appeared to be a reduction in the flow of blood coursing through the capillary network.

This effect on the vasculature of the chick embryo was highly dependent on the specific chemical structure of squalamine. Minor structural changes in the polyamine or in the stereochemistry of the ring or side chain yield analogues that no longer induced rapid microvessel changes at concentrations of aminosterol as high as 100 μg/ml. In addition, embryos treated with a Ficoll solution lacking squalamine did not show significant change over the time interval presented.

Histological examination of the treated and untreated yolk sac vascular beds revealed that squalamine induced the appearance of narrowed vascular segments that effectively blocked erythrocyte movement (Fig. 8). Such structures were not seen in untreated embryos. The fate of the constricted vessels was not determined in this study.

Fig. 6. Effects of squalamine, 1436, and MIA on endothelial cell proton secretion after VEGF stimulation. The data plotted represent the instantaneous rate of acid secretion measured at the indicated time point. Each plot is averaged from several (n) independent experiments; bars, SD.

Fig. 7. Effects of squalamine on the yolk sac vessels of the 4-day chick embryo. Panel numbers refer to the time interval between treatment and photography. ×50.
DISCUSSION

The current model of tumor angiogenesis suggests that the growth of solid tumors is supported by angiogenesis, a process involving the ingrowth of vessels that have sprouted from existing capillary beds (22, 23). The treatment of solid tumors with antiangiogenic agents has received experimental support in preclinical studies and continues to elicit optimism over its eventual application in the treatment of human cancer (22). Inhibitors of angiogenesis block any of several steps in the angiogenic cascade, including proliferation of endothelial cells, attachment of endothelial cells to the substratum, and migration and invasion through the tissue space that is required for the extension of capillary sprouts into new territory (3, 24–30). Our experiments have reproducibly demonstrated the strong inhibitory effects of squalamine on this pathway. We have shown that squalamine inhibits in vitro endothelial cell proliferation and migration induced by multiple mitogens, including those produced in combination by very angiogenic tumors.

The profound in vitro antiangiogenic effects of squalamine held true in a variety of standard in vivo models in several species. Squalamine dramatically reduced angiogenesis and subsequently VX2 tumor growth in the well-established rabbit cornea model. In the mouse, squalamine inhibited the ingrowth of vessels into bFGF-impregnated Matrigel plugs, and in the rat, it inhibited the growth of solid tumors with an associated reduction of tumor vessel density. These results are consistent with a mechanism by which squalamine inhibits tumor growth in vivo by interfering with tumor vascularization. Direct tumor cytotoxicity seems mechanistically unlikely because squalamine had no direct toxic or antiproliferative effects on any tumor cell lines that we used.

Although the effects of squalamine clearly appear to be directed toward the endothelial cell, no direct cytotoxicity was observed against endothelial cells either. The observed inhibitory effects on proliferation and migration were appreciated only in the presence of endothelial cell mitogens. Blockade of action of specific growth factor receptors is also unlikely as a significant mechanism because squalamine acts in the face of a wide variety of mitogens, including those found in combination in highly angiogenic tumors. Inhibition of mitogen production further seems a poor candidate because squalamine caused no reduction in VEGF production by cultured 9L glioma cells.

The precise intracellular impact of the presence of squalamine on the physiology of the endothelial cell is yet to be clearly elucidated. Although the sodium-hydrogen exchanger (NHE) has long been thought to play a role in the regulation of cellular processes (31) such as growth, volume regulation, and communication between a cell and its external attachment sites, drugs such as amiloride and its analogues have been found to have minimal effect on endothelial cell proliferation in vitro at concentrations where they completely inhibit one...
form of NHE, NHE-1 (32). In contrast, squalamine inhibits endothelial cell proliferation while exerting a lesser effect on proton secretion. Thus, although we conclude that squalamine inhibits certain growth factor-stimulated cellular events within endothelial cells that involve the disposition of protons, we believe that disturbance of cellular pH regulation alone is not the basis of the effects of squalamine.

Perhaps the most interesting effects of squalamine were those observed in the 4-day CAM assay. The primitive capillary networks of the CAM arise through the embryonic process of vasculogenesis in which mesoderm-derived angioblasts differentiate into endothelial cells, which in turn organize to form vascular networks de novo; in contrast, tumor angiogenesis involves the growth of capillary beds by the branching of existing vascular beds (22). Despite differing in origin, capillaries present within tumors strikingly resemble the squalamine-responsive capillaries of the early embryonic yolk sac of the chick (33). Like the chick embryonic vessels, they are frequently described as morphologically primitive in that they can exhibit very rapid growth, can lack certain associated cells such as pericytes, and can be associated with sparse deposition of basement membrane. Furthermore, anti-VEGF antibodies have been shown recently to induce narrowing of certain tumor-associated capillaries over the course of several days (34), suggesting that certain tumor capillary beds are unstable and require continued growth factor support. Because the vessels of the 4-day CAM are composed solely of endothelial cells and lack supporting cells such as pericytes or smooth muscle cells (33), the luminal narrowing observed with squalamine treatment must arise as a consequence of a change in shape or volume of the endothelial cell rather than as a consequence of the constriction of associated contractile cells. Insight into the intracellular mechanism of the activity of squalamine on CAM vessels is lacking, and it is even possible that this mechanism is different from that observed among cultured endothelial cells or even tumor-induced angiogenesis.

Despite its activity on the "primitive" vessels of the 4-day CAM, squalamine has been found previously to have no obvious effect on the growth of newborn vertebrates, including both linear growth and the development of internal organs. Strikingly, unweaned neonatal mice treated with concentrations of squalamine that effectively inhibit hypoxic induced extraretinal proliferation do not exhibit impairment of normal retinal vessel growth or total body growth, highlighting a selectivity of squalamine for particular "primitive" neovascularization processes while sparing others (35).

We have elucidated a number of properties intrinsic to the novel aminosterol, squalamine. The activities of squalamine include, among others, reduction in solid tumor vascularization and growth in various animal models without direct tumor cytotoxicity, profound inhibition of the proliferation of stimulated endothelial cells in vitro without an effect on unstimulated cells, and constriction of tumor-like capillaries in the 4-day CAM with sparing of normal developmental neovascularization in other models. Based in part on the data presented in this report, Phase I clinical trials have been initiated to evaluate the feasibility of using squalamine in the treatment of cancer.

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