Potentiation of Platinum Antitumor Effects in Human Lung Tumor Xenografts by the Angiogenesis Inhibitor Squalamine: Effects on Tumor Neovascularization[1]

Joan H. Schiller[2] and Gerard Bittner

William S. Middleton Veterans Administration Hospital, Madison, Wis., 53705 and University of Wisconsin Department of Medicine, Medical Oncology Section, Madison, Wisconsin 53792

ABSTRACT

Squalamine is a novel anti-angiogenic aminosterol that is postulated to inhibit neovascularization by selectively inhibiting the sodium-hydrogen antiporter exchanger. To determine how to most effectively use this agent in patients with cancer, we examined the antitumor effects of squalamine with or without cytotoxic agents in human lung cancer xenografts and correlated these observations with the degree of tumor neovascularization. No direct cytotoxic effects of squalamine against tumor cells were observed *in vitro* with or without cisplatin. Squalamine was effective in inhibiting the establishment of H460 human tumors in BALBc nude mice but was ineffective in inhibiting the growth of H460, CALU-6, or NL20T-A human tumor xenografts when administered i.p. to mice bearing established tumors. However, when combined with cisplatin or carboplatin, squalamine increased tumor growth delay by ≥1.5-fold in the three human lung carcinoma cell lines compared with cisplatin or carboplatin alone. No enhancement of antitumor activity was observed when squalamine was combined with paclitaxel, vinorelbine, gemcitabine, or docetaxel. Repeated cycles of squalamine plus cisplatin administration delayed H460 tumor growth >8.6-fold. Squalamine plus cisplatin reduced CD31 vessel formation by 25% compared with controls, squalamine alone, or cisplatin alone; however, no inhibition in CD31 vessel formation was observed when squalamine was combined with vinorelbine. These data demonstrate that the combination of squalamine and a platinum analog has significant preclinical antitumor activity against human lung cancer that is related to the anti-angiogenic effects of squalamine.

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[2] To whom requests for reprints should be addressed, at University of Wisconsin, Department of Medicine, Medical Oncology Section, K4/666 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792. Phone: (608) 263-8600; Fax: (608) 263-8613; E-mail: jhschill@facstaff.wisc.edu.

INTRODUCTION

Lung cancer is responsible for approximately one-third of all cancer-related deaths in the United States each year, killing more Americans than breast cancer, colon cancer, and prostate cancer combined (1). Systemic treatment with cytotoxic agents has been the mainstay of treatment in the advanced disease setting. New cytotoxic agents have recently been developed that yield response rates of 35–40%. Despite these advances, chemotherapy has been largely ineffective in producing complete responses or cures in the advanced disease setting, and over 85% of patients with lung cancer eventually succumb to the disease (2). Therefore, the investigation of new paradigms and therapeutic approaches for lung cancer has become an urgent priority for the oncology community.

Several new therapeutic strategies currently under investigation involve modulation of aspects of cellular homeostasis, including the inhibition of tumor neovascularization. Angiogenesis, the sprouting of capillaries from pre-existing vessels, is an essential event in many physiologic processes such as reproduction, development, and wound healing. Neovascularization is also a key component of many pathologic processes such as inflammation, diabetic and other retinopathies, and tumor formation. Angiogenesis inhibition has become a potential antitumor treatment strategy because vascular tumors would be expected to be incapable of growth and have little metastatic potential. Strategies to prevent the development of new blood vessels in tumors and metastases have been effective in suppressing the growth of these tumors in preclinical models, and a number of new angiogenesis inhibitors are currently being explored in clinical trials (3–7).

Squalamine is a newly identified, selective, noncytotoxic inhibitor of new blood vessel formation (Fig. 1; Ref. (8)). Originally developed in a screen for antimicrobial agents, it is an aminosterol that is postulated to inhibit new blood vessel growth by selectively inhibiting the sodium-hydrogen antiporter sodium-proton (Na+/H+) exchangers (NHE3) and inhibiting hydrogen efflux out of the endothelial cell, with a consequent inhibition of intracellular alkalinization and of cellular proliferation (9, 10). Squalamine has further been demonstrated to be anti-angiogenic (11). Preclinical studies have demonstrated no intrinsic cytotoxic activity of squalamine against tumor cells *in vitro* at clinically relevant concentrations and only modest antitumor activity *in vivo* as a single agent (12). However, more pronounced antitumor activity has been observed when given in conjunction with or after cytotoxic agents (12, 13). To determine the optimal conditions for the administration of squalamine plus chemotherapy for lung cancer patients, we examined the antitumor effects of squalamine with or without cytotoxic agents in human lung cancer xenografts. We also measured tumor neovascularization in an effort to correlate the

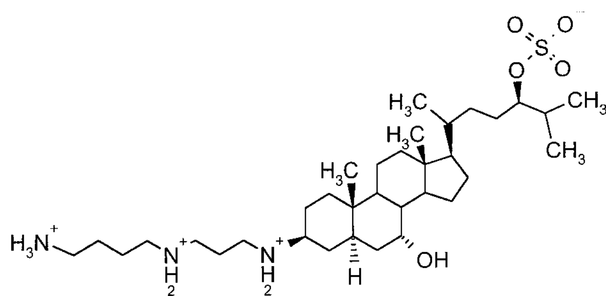

Fig. 1 The structure of squalamine.

antitumor effects of squalamine in combination chemotherapy with its anti-angiogenic property.

MATERIALS AND METHODS

Squalamine was obtained from Magainin Pharmaceuticals, Inc. (Plymouth Meeting, PA). The rat antimouse CD31 and biotinylated goat antirat antibodies were obtained from Pharmingen (San Diego, CA). H460, Calu-6, and Lewis lung tumor cell lines were all obtained from the American Type Culture Collection (Rockville, MD). NL2OT-A cells were derived in our laboratory from an origin of replication-deficient SV40 Large T immortalized normal human bronchial epithelial cell line that spontaneously became tumorigenic after 180 passages *in vitro* (14).

H460 cells were cultured in RPMI-1640 plus 8% FBS[3] and subcultured 1:1600 once a week. Calu-6 and Lewis lung cells were cultured in MEM with Earls salts plus 8% FBS and subcultured 1:200 once a week. NL2OT-A cells were cultured in F12 plus 8% FBS and 10 μg/ml insulin, 1 μg/ml hycrocortisone, 5 μg/ml transferrin, 2.7 mg/ml dextrose and 10 ng/ml epidermal growth factor. NL2OT-A cells were subcultured 1:500 once a week.

Cisplatin and carboplatin were obtained from Sigma (St. Louis, MO) and dissolved in D5W. Paclitaxel was obtained from Sigma and dissolved in cremephore. Vinorelbine was obtained from Burroughs Wellcome (Research Triangle Park, NC) and dissolved in PBS. Docetaxel was obtained from Rhone-Poulenc Rorer Pharmaceuticals Inc. (Collegeville, PA) and dissolved in supplied diluent (13% ethanol in H_2O) and diluted in PBS. Gemcitabine was obtained from Eli Lilly (Indianapolis, IN) and dissolved in PBS.

***In Vivo* Studies.** Three different human lung cancer xenografts were used for this study, each of which varied in their growth characteristics and malignant potential. They included (*a*) the Calu-6 cell line, a human adenocarcinoma cell line; (*b*) the H460 cell line, a very rapidly proliferating large-cell carcinoma cell line; and (*c*) the NL20T-A cell line, a slowly growing tumorigenic cell line derived in our laboratory from an immortalized human bronchial epithelial cell line that underwent spontaneous transformation to the tumorigenic phenotype (14). The Lewis lung murine tumor cell line was also utilized.

Three-to-four-week-old female BALBc nu/nu nude mice obtained from Harlan Sprague-Dawley (Madison, WI) were inoculated s.c. behind the right foreleg with 5×10^6 tumor cells in 0.2 ml of antibiotic-free, serum-free tissue culture media. Once s.c. tumors were visible (3–5 days) with a mean volume of approximately 50–80 mm^3, the mice were treated i.p. with squalamine and/or one of the cytotoxic drugs (generally 0.1–0.2 ml in D5W, saline, or other appropriate vehicle). Squalamine (20 mg/kg) was dissolved in H_2O and diluted 1:10 in Intralipid. Intralipid was used to buffer the irritation caused by squalamine. Control mice were treated i.p. with saline. Tumors were measured two or three times per week (depending on growth rate) with a calipers, and tumor volume was calculated using the following formula.

$$\text{Volume (mm}^3) = \frac{\Pi \times \text{ longest diameter} \times (\text{perpendicular diameter})^{2-}}{6}$$

Tumor growth delay was calculated by graphing the volume of each treatment group (eight mice/treatment group) and calculating the number of additional days it took to reach 500 mm^3 compared with control (12).

Antiproliferative Studies. Cells were dissociated, counted on a hemacytameter, and plated at 10^4 cells/well in 6-well culture plates. Twenty-four h later, the cells were exposed to the cytotoxic agent for 1 h, rinsed, and refed with media or media containing squalamine. After 5 more days, the cells were dissociated, and total cell number was determined by hemacytometer counts. All of the data points were done in triplicate.

Immunohistochemistry. Tumors were collected at various times after chemotherapy treatment; 6-μm frozen sections cut, fixed in cold acetone, dried, and stored frozen until stained. Sections were stained with 5 μg/ml rat antimouse CD31 (Pharmingen) at 37° for 1 h, rinsed, and subsequently stained with 2.5 μg/ml biotinylated goat antirat antibody (Pharmingen) at 37° for 30 min. Sections were rinsed and incubated at room temperature for 30 min with streptavidin-alkaline phosphatase conjugate (Vector Labs, Burlingame, CA) at a 1:500 dilution, then were rinsed and developed with Vector Labs Alkaline Phosphatase Kit III for 15 min at room temperature in the dark. Slides were then rinsed, counterstained for 20 min with nuclear Fast Red (Vector Labs), rinsed, dried, and mounted.

Quantification of angiogenesis was performed as described by Vermeulen *et al.* (15). The most highly vascularized area of each tumor was identified on low power and five high-powered (×400) fields were counted in this area of greatest vessel density. Results are presented as the percent vessel number compared with untreated controls (four tumors/group). The mean SD of all of the experiments was 11.5% (range, 4–21%).

RESULTS

In Vitro Antiproliferative Effects of Squalamine

The *in vitro* effects of squalamine plus cisplatin were tested in H460 cells (Fig. 2). Cells were exposed to cisplatin for 1 h

[3] The abbreviations used are: FBS, fetal bovine serum; VEGF, vascular epithelial (cell) growth factor; NHE, normal human epithelial.

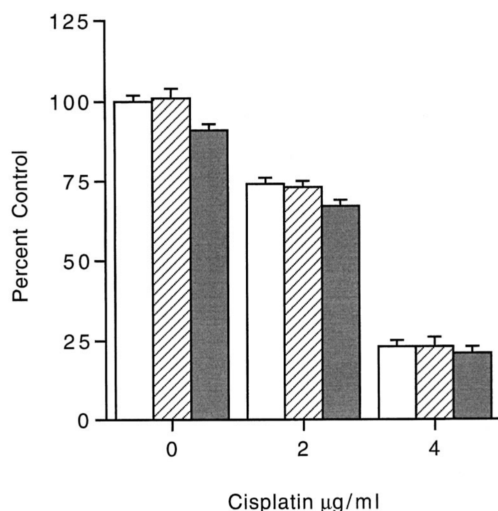

Fig. 2 *In vitro* effects of cisplatin 2 and 4 µg/ml (6.65 and 13.3 µM, respectively) alone (*open columns*) or 10 µg/ml (15.9 µM) (*hatched columns*) or 20 µg/ml (31.8 µM) (*solid columns*) squalamine plus cisplatin in H460 cells. Squalamine had no direct antitumor effects as a single agent and did not potentiate the antiproliferative effects of cisplatin.

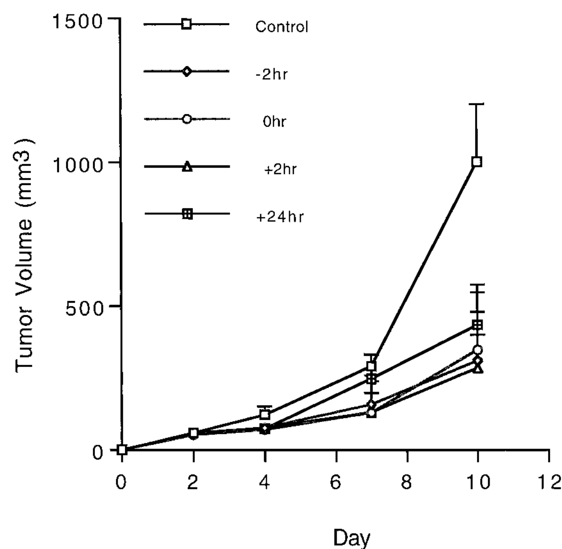

Fig. 3 Effects of squalamine on H460 tumor formation. Squalamine (20 mg/kg) was administered i.p. either 2 h before (*−2 hr*), concurrent with (*0 hr*), 2 h after (*+2 hr*), or 24 h after (*+24 hr*) the innoculation of mice with H460 cells. The mean tumor volume of eight mice per group is depicted. Squalamine inhibited the formation of H460 xenografts when administered 2 h before or up to 24 h after tumor innoculation.

and/or squalamine for 5 days. Cisplatin decreased the number of viable tumor cells in a concentration-dependent fashion. Squalamine had no cytotoxic effects *in vitro;* the addition of squalamine to cisplatin did not result in any enhanced tumor cell kill, nor did squalamine alone lead to a reduction in tumor cell number.

In Vivo Effects of Squalamine

Single Agent Effects of Squalamine. The effects of squalamine on preventing the establishment of H460 tumors was studied *in vivo.* Nine sequential days of squalamine was effective in reducing tumor formation when administered starting immediately before, simultaneously with, or up to 24 h after the inoculation of H460 tumor cells (Fig. 3). However, when administered in mice bearing established tumors starting on day 3, squalamine was ineffective in inhibiting the growth of H460 or Calu-6 human tumors (Fig. 4) as well as the NL20T-A human tumor xenograft (data not shown).

***In Vivo* Effects of Squalamine plus Chemotherapy.** Experiments were conducted in athymic nude mice bearing established tumors, in which squalamine was administered i.p. for 5–9 days starting approximately 24 h after i.p. administration of a cytotoxic agent. Doses of the cytotoxic agent that resulted in approximately 50% inhibition of tumor growth were chosen for these combination experiments. These doses were chosen in order to ensure the use of an active agent at a dose that would not totally suppress tumor growth, thus making it difficult to observe a squalamine-drug interaction.

The effects of combining squalamine and cisplatin were studied in four tumor lines (H460, Calu-6, NL20T-A, and Lewis lung; Table 1). The effects of squalamine and the alternative platinum agent carboplatin were separately studied in H460 and Calu-6 tumor xenografts. In all of the cases, administration of multiple doses of squalamine in combination with the platinum analogue resulted in at least a 1.5 to 2-fold growth delay of the tumor compared with the platinum agent alone, without squalamine having any demonstrable effect as a single agent.

Interestingly, no enhancement of antitumor activity was seen when squalamine was administered for 5–7 days after treatment with paclitaxel, vinorelbine, gemcitabine, or docetaxel (Table 1).

A potentiation of cisplatin and carboplatin antitumor activity was observed in the H460 and Calu-6 cell lines when only one injection of squalamine was administered 24 h after treatment with the platinum analogue (Fig. 4). For example, whereas one injection of 20 mg/kg squalamine had no antitumor activity as a single agent, the combination of squalamine and cisplatin increased tumor growth delay by >2-fold compared with cisplatin alone.

A single administration of cisplatin and squalamine was compared with the combination of cisplatin and squalamine administered three times over a 12 day period of time in H460 xenografts (Fig. 5). Administration of three doses of squalamine plus cisplatin increased tumor growth delays by >2.3-fold compared with three injections of cisplatin. Three injections of the combination resulted in a 4.1-fold increased delay in tumor growth compared to one administration of cisplatin and squalamine, and a >8.6-fold increased delay compared with no treatment.

Effects of Squalamine on Tumor Neovascularization. The effects of squalamine on tumor vascularization were studied with the H460 tumor xenograft (Fig. 6). Squalamine had no effect as a single agent on H460 tumor growth (Table 1) and, similarly, had no effect on the number of CD31-staining vessels in established H460 tumors (Fig. 6). However, the combination

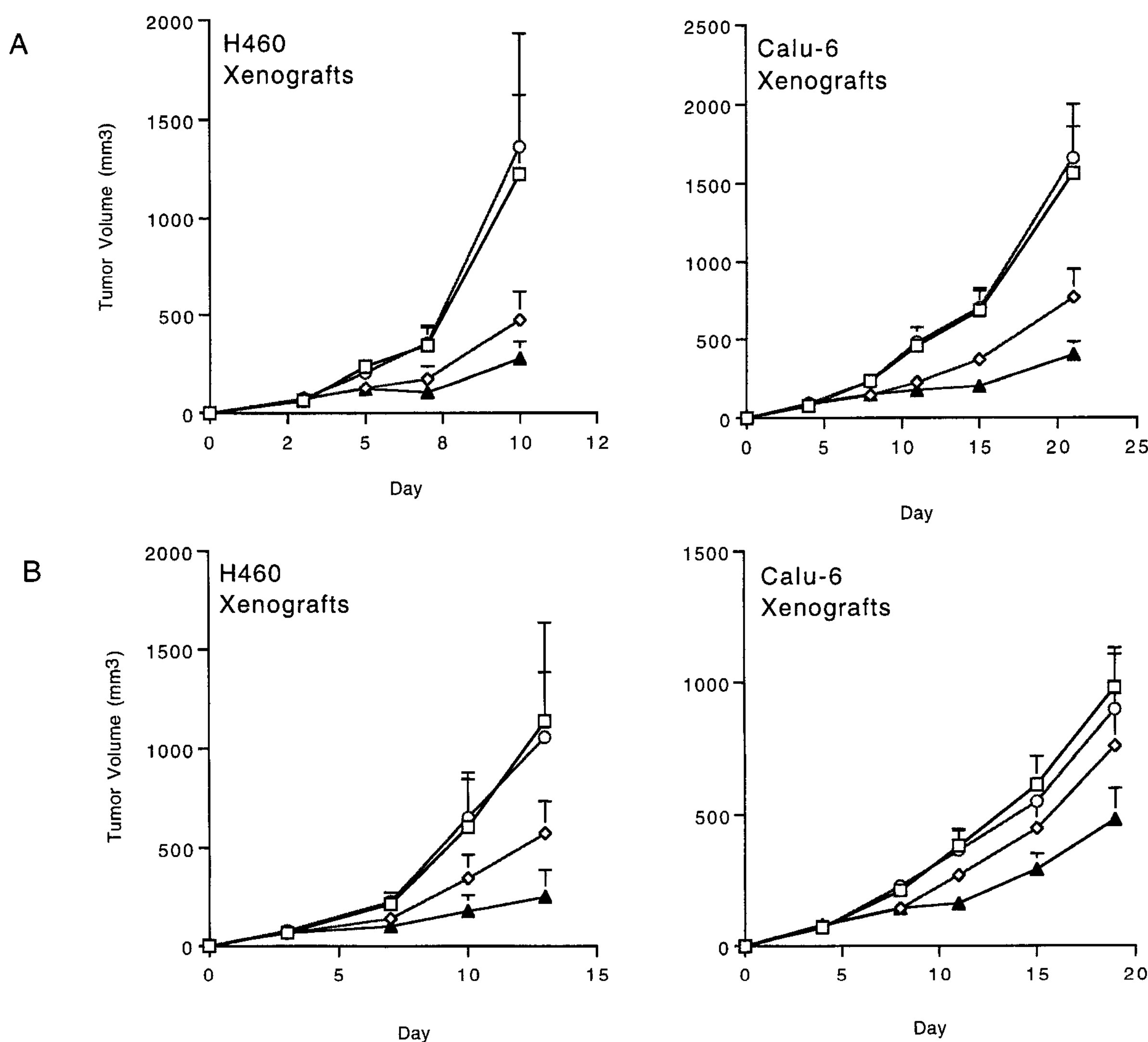

Fig. 4 *In vivo* effects of a single administration of squalamine in established H460 and Calu-6 tumor xenografts. Squalamine was administered alone or in combination with cisplatin (3 mg/kg; *A*) or carboplatin (60 mg/kg; *B*) on day 4, when the tumors were 50–80 mm^3 in size. □, control tumors; ◇, platinum-treated tumors; ○, squalamine; ▲, platinum plus squalamine. The mean tumor volume of eight mice per group is depicted.

of squalamine and cisplatin reduced the number of vessels approximately 25% (Figs. 6 and 7). This reduction was observed as early as 2 h after squalamine-plus-cisplatin treatment, lasted for 24 h, and had dissipated by 48 h (Fig. 6). By contrast, no difference in the number of CD31 staining vessels was observed with squalamine and vinorelbine at any time after chemotherapy treatment (data not shown). At 4 h, tumors treated with vinorelbine had 111% of control CD31-staining vessels, whereas vinorelbine plus squalamine had 108%.

DISCUSSION

Squalamine is a natural aminosterol originally purified and characterized from several tissues of the dogfish shark. Identified as an antimicrobial substance, it was subsequently confirmed as an anti-angiogenic compound, partly because of its structural similarities to the angiostatic steroids. However, squalamine differs in structure from previously described angiostatic steroids, and does not interact with glucocorticoid or mineralcorticoid receptors (9, 11). Squalamine inhibits endothelial cell growth in three dimensional matrices of collagen in tissue culture and inhibits angiogenesis in a dose-dependent manner in a chick embryo chorioallantoic membrane assay *in vitro* and in a rabbit corneal micropocket assay in which the allogeneic VX2 carcinoma was implanted (11, 16). Squalamine specifically inhibits VEGF-stimulated bovine retinal endothelial cell proliferation but not the growth of VEGF-stimulated tumor cell lines (17).

Although the mechanism of steroid hormone function on gene transcription has been well studied, the mechanism by which angiostatic steroids exhibit multiple diverse effects on cells and tissues have been less well characterized. They have been implicated in the direct blocking of *in vitro* and *in vivo*

Table 1 Tumor growth delay in established tumors[a]

	H460	Calu-6	NL20T-A	Lewis lung
Squalamine	<0.2 (0.02)	<0.8 (0.1)	<0.1 (0.01)	<0.02 (0.01)
Cisplatin	2.5 (0.9)	5 (0.8)	12 (3.9)	2.1 (0.3)
Cisplatin + squalamine	>5 (0.9)	>10.7 (1.7)	>29 (8.3)	3.6 (0.5)
Carboplatin	2.9 (0.9)	2.5 (0.6)		3.1 (0.5)
Carboplatin + squalamine	>6.7 (2.0)	>6.3 (1.6)		5.7 (1.7)
Paclitaxel	1.7 (0.4)	7.4 (1.9)		
Paclitaxel + squalamine	1.8 (0.6)	7.4 (1.5)		
Vinorelbine	1.8 (0.5)	4.3 (1.1)		
Vinorelbine + squalamine	2 (0.7)	4.6 (0.8)		
Docetaxel	2.2 (0.5)			1.7 (0.2)
Docetaxel + squalamine	2.9 (0.8)			2.1 (0.4)
Gemcitabine	2.7 (0.5)			
Gemcitabine + squalamine	2.7 (0.6)			

[a] In days (SD).

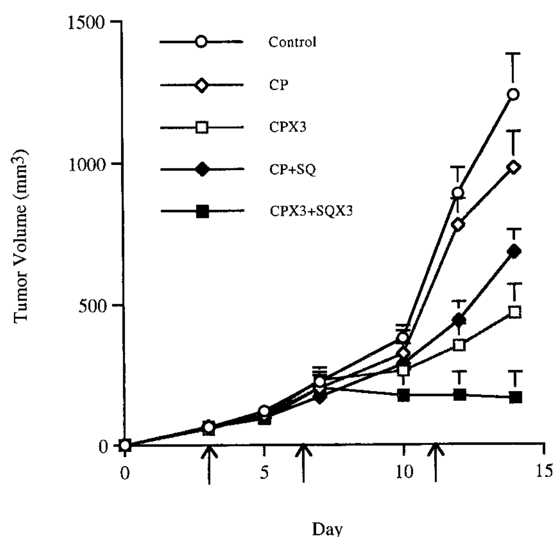

Fig. 5 Antitumor effects of multiple injections of cisplatin plus squalamine on established H460 tumor xenografts. Mice were treated with a single dose of 3 mg/kg of cisplatin on day 3 with squalamine (*CP+SQ*) or without squalamine (*CP*); or with three doses of cisplatin on days 3, 7, and 12 (*CPX3*); or with three doses of cisplatin plus squalamine on days 3, 7, and 12 (*CPX3+SQX3*). *Arrows*, days of treatment.

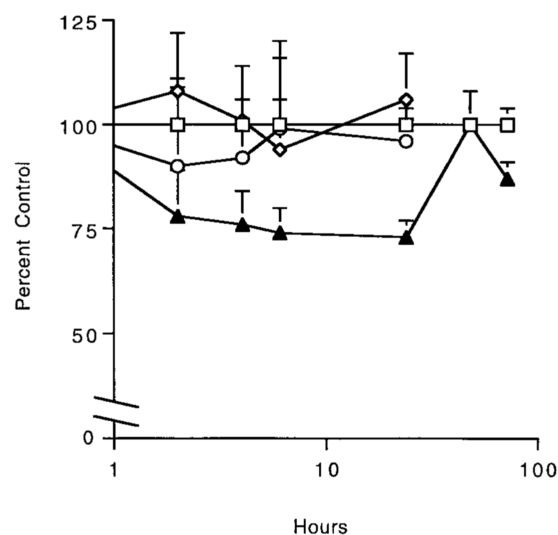

Fig. 6 Inhibition of CD31 staining vessels in H460 tumors. Mice were treated with cisplatin with or without squalamine. Results are expressed as percentage of CD31 staining vessels in control tumors. □, control tumors; ◇, cisplatin; ○, squalamine; ▲, cisplatin plus squalamine.

endothelial cell growth (18), inhibition of collagenolysis and of plasminogen activator production (19–24), altered regulation of plasminogen activator inhibitor synthesis (23–25), and induction of basement membrane dissolution and regulation of collagen metabolism (4, 26, 27). The mechanism of action underlying squalamine's anti-angiogenic effects is similarly not well characterized; however, it is known that squalamine modifies the shape and decreases the volume of endothelial cells in embryonic vascular beds, resulting in lumenal narrowing and occlusion of blood flow (11). One possible molecular mechanism regulating the cellular changes is the inhibition of the sodium-proton exchanger (10). This effect seems to be specific for the NHE isoform NHE3, as contrasted to amaloride, which is specific for NHE1.

In addition to inhibiting the growth of new vessels, squalamine alone or with cytotoxic agents has been reported to inhibit tumor cell growth *in vivo* but not *in vitro.* In a rat 9L glioma model, squalamine effectively inhibited tumor growth and vessel density, without any direct inhibition of 9L glioma tumor cells *in vitro* (11). Squalamine has been used successfully in combination with cyclophosphamide in the human MX-1 breast cancer in a mouse xenograft model (28). When used in combination with cyclophosphamide, squalamine increased the median time to progression and survival and increased the number of animals experiencing a complete regression, despite discontinuation of squalamine after 5 weeks. In human prostate cancer xenografts, mice treated with squalamine after castration

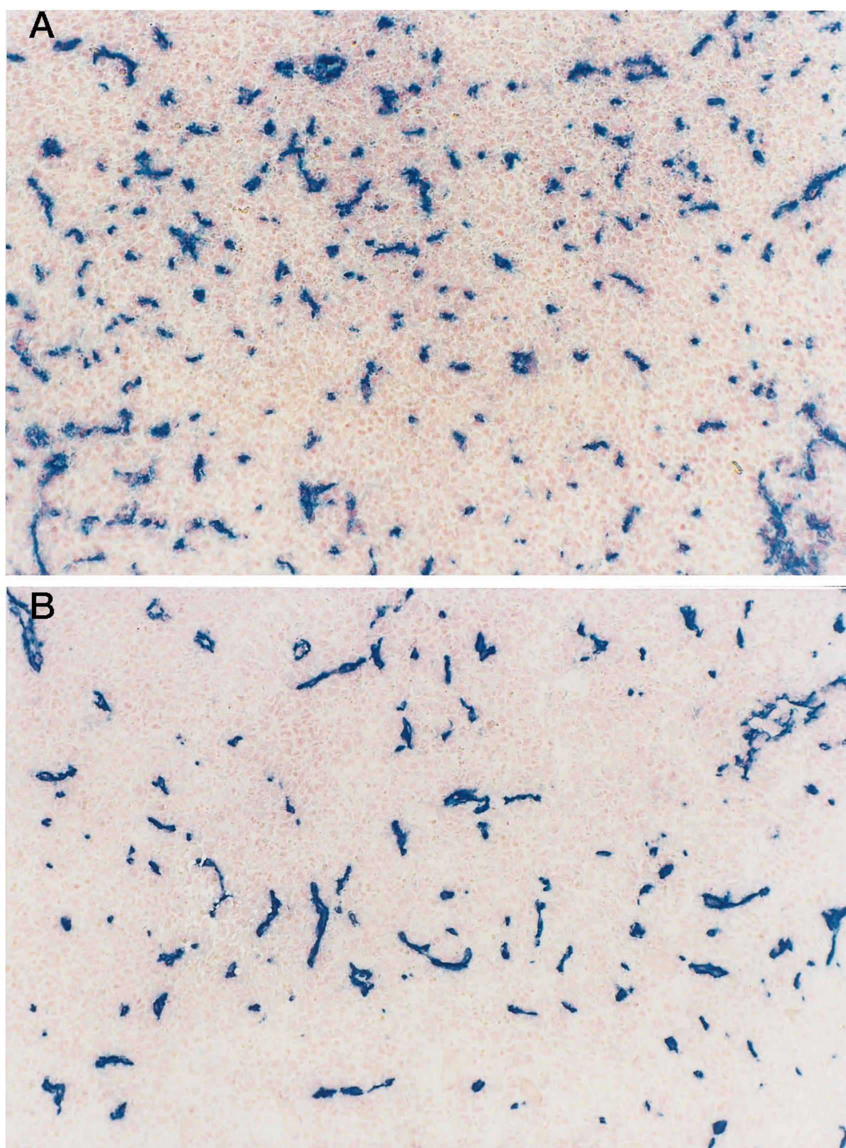

Fig. 7 A representative photomicrograph of CD31 staining vessels in H460 tumors treated with (*A*) saline or with squalamine plus cisplatin (*B*). ×200.

maintained prostate-specific antigen nadir and had eradication of advanced human prostate cancer (29). In the rat 13762 mammary carcinoma and murine Lewis Lung models, squalamine increased tumor growth delays produced by cyclophosphamide, cisplatin, paclitaxel, and 5-fluorouracil by 1.9 to 3.8-fold compared with the anticancer drugs alone (12).

In this report, we investigated the antitumor effects of squalamine when administered alone or in conjunction with cytotoxic agents to human lung tumor xenografts. As anticipated, we found no direct antiproliferative effects *in vitro.* Squalamine was effective in inhibiting tumor establishment but at best only modestly effective in inhibiting the growth of established tumors. Immunohistochemistry studies examining the effects of squalamine on neovascularization also documented that squalamine by itself had no discernable anti-angiogenic effects in established tumors.

Although squalamine had no activity as a single agent, combination therapy with squalamine plus a platinum analogue (cisplatin or carboplatin) was significantly better at inhibiting tumor cell growth than the platinum analogue alone, despite the fact that squalamine had no intrinsic activity when used under the conditions of these experiments. The combination of cisplatin and squalamine did result in a significant decrease in CD31-staining blood vessels. This effect was observed early, within 2–4 h, and had dissipated by 48 h. These findings are consistent with our observations that antitumor effects *in vivo* occur when the squalamine and platinum analogue are administered in close temporal proximity to each other, and that there was no benefit to prolonged squalamine administration. These findings are also consistent with observations in the chick embryo chorioallantoic membrane assay, in which morphological changes consisting of constriction of the smallest yolk-sac capillaries occurred within 40 min and was somewhat reversible by 100 min (11).

Somewhat surprisingly, the combination of squalamine

plus paclitaxel, gemcitabine, docetaxel, or vinorelbine had no enhanced effect on tumor inhibition, whereas such an effect was repeatedly observed when combining squalamine with either cisplatin or carboplatin in three different human lung cancer cell lines. Additionally, no enhanced effect of squalamine plus vinorelbine was seen on reducing the vascularization of H460 tumors. It is possible that the "injury" induced by platinum analogues is different from that produced by other drugs, enabling a squalamine/platinum interaction; additional studies will be necessary to further characterize these differences.

The potentiation of platinum's antitumor activity in human lung tumor xenografts has also been observed by Gonzalez *et al.* (13). However, in the chemoresistant human lung tumor line MV-522, as well as with the rat 13762 mammary carcinoma and murine Lewis lung carcinomas, squalamine also potentiated the effects of paclitaxel (12, 13). This was not observed in our human lung xenografts treated with either docetaxel or paclitaxel.

In conclusion, we have demonstrated that the anti-angiogenic aminosterol squalamine can reduce the formation of human lung tumor xenografts. When combined with platinum analogues, it increases the tumor growth delays produced by cisplatin or carboplatin but not other cytotoxic agents. A transient decrease in tumor vascularization was also observed when squalamine was combined with cisplatin but not with vinorelbine, which suggests that the anti-angiogenic effects of squalamine were important in potentiating the antitumor effect.

These results have important clinical implications. The platinum analogues cisplatin and carboplatin are two of the most commonly used drugs for the treatment of lung cancer in the United States and worldwide. Given the median survival of approximately 8–10 months with platinum combinations in metastatic non-small cell lung cancer, any therapy that could enhance or maintain tumor suppression with minimal toxicity could have major clinical impact on the treatment of this disease. Squalamine has recently completed clinical investigation in the Phase I setting for advanced cancer. The results obtained in our xenograft models support the continued clinical development of squalamine in combination with cisplatin or carboplatin in the treatment of advanced non-small cell lung cancer.

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