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13. ABSTRACT (Maximum 200 Words)
With 1,000,000 new cases worldwide annually, breast cancer is the most common malignancy in women. Genesis of these cancers depends, in part, on formation of a good blood supply, a process termed angiogenesis. Vascular endothelial growth factor (VEGF) stimulates growth of endothelial cells, and this critical protein is produced by breast cancers. High levels of VEGF secretion occur in tumors expressing EGF and HER-2 growth factor receptors. Antibodies to HER-2 receptor elicit direct antitumor effects but also reduce VEGF secretion from tumors and, thereby, decrease angiogenesis. Optimal suppression of tumor growth may be achieved by combining anti-growth factor receptor therapies with agents that disrupt tumor angiogenesis, such as squalamine, a steroid that blocks VEGF-induced activity. This study probes the notion that treating both the tumor and the tumor-associated vasculature may be more effective than treating cancer cells alone. We are assessing specific binding and biologic activities of squalamine using vascular endothelial cells, with evidence for localization of squalamine in caveolae signaling domains. Moreover, we are evaluating the efficacy of squalamine alone and combined with other biologic agents, such as Herceptin and 2C4, in blocking growth and progression of human breast cancer xenografts *in vivo*.

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INTRODUCTION

With 1,000,000 new cases in the world annually, breast cancer is the most common malignancy in women (1). Recent advances in surgery, radiation and chemotherapy have improved overall survival, but improved treatment options for patients afflicted with breast cancer are urgently needed. Although the cause of most breast cancers is unknown, epidermal growth factor receptor (EGFR) family members, HER-2 and EGFR, are often implicated in the pathogenesis of this cancer (2,3). HER-2 receptors are overexpressed in 25-30% of newly diagnosed breast cancers, and HER-2 amplification correlates with poor clinical course (4-7). Moreover, antibodies specific for HER-2 receptor (8-10) elicit *in vivo* a marked cytostatic growth inhibition of breast cancers that overexpress the HER-2 gene product (see Fig. 1).

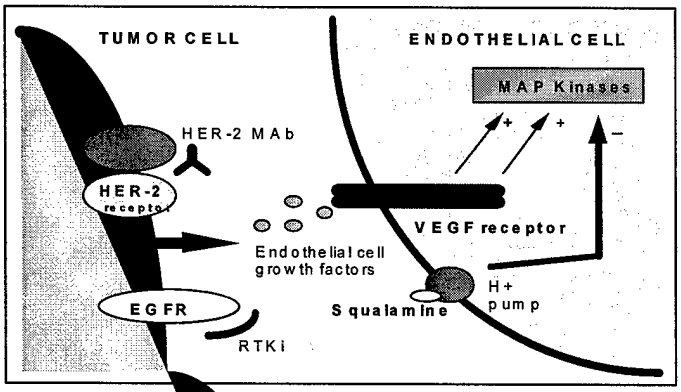

FIG. 1. Breast tumor-associated angiogenesis. Cancers with HER-2 and/or EGF receptors secrete endothelial growth factors. VEGF binds it's receptor (11) and growth factor regulation of vessel growth converges with signaling by MAP kinases (12,13). Squalamine, possibly by binding surface proton pumps (14,15) or unknown membrane sites, may disrupt MAPK signaling in endothelail cells to block growth. Receptor tyrosine kinase inhibitor (RTKi); HER-2 monoclonal antibody (MAb); epidermal growth factor receptor (EGFR).

Growth of breast cancer depends, in part, on formation of an adequate blood supply. Tumor angiogenesis has prognostic significance in breast cancer (16), and vascular endothelial growth factor (VEGF) may play a critical role in breast cancer progression (17). HER-2 and EGF receptor signaling are reported to up-regulate secretion of VEGF in solid tumors (18), including breast cancer (18,19), and treatments targeted to block VEGF or other angiogenic molecules may stop tumor progression (20). Therapy directed to the vasculature of solid tumors is now being pursued as an important new direction in cancer treatment, because avascular tumors exhibit limited growth (4,5) and tumor aggressiveness and metastatic potential commonly correlate with tumor vascularity (6).

Squalamine, a natural steroidal molecule, has antiangiogenic activity in models of breast, ovary, brain and lung cancer (21-23). Angiostatic steroids were first described 20 years ago (20,24), but squalamine, a 7,24 dihydroxylated 24-sulfated cholestane steroid conjugated to a spermidine at C-3 (25,26), differs in structure from the earlier agents. Squalamine is unique among most angiostatic agents currently in development because it blocks endothelial growth induced by a wide range of growth factors including bFGF and VEGF (15,26). This inhibition may result from interaction with endothelial cell surface proton pumps, thus altering intracellular pH and impeding growth factor signaling (15,25,26), or with other membrane signaling components (23). In vascular endothelial cells, squalamine blocks growth factor-induced activation of MAP kinase signaling, an enzyme at a crucial point of convergence for several growth factor signaling cascades (23). Administered as a single agent, squalamine is relatively non-toxic and well tolerated in early clinical trials (27-29).

BODY: RESEARCH PROGRESS

AIM 1. *Assessment of the specific binding, subcellular distribution and biologic activities of squalamine, a newly-synthesized antiangiogenic steroid, using human vascular endothelial cells in vitro.*

1.1. Squalamine blocks endothelial growth factor-stimulated proliferation of endothelial cells *in vitro*. To assess biologic mechanisms for antitumor effects of squalamine, human umbilical vein endothelial cells (HUVEC) were grown *in vitro*. VEGF is a critical endothelial growth factor secreted by breast cancers, and the peptide elicits growth of HUVEC cells by 72 h (Fig. 2). In the absence of VEGF, squalamine has no significant effect on proliferation of endothelial cells. However, squalamine elicits a marked reduction in VEGF-induced endothelial growth ($P < 0.001$). This growth-inhibitory effect of squalamine appears restricted to endothelial cells since it had no direct effect on growth of breast cells.

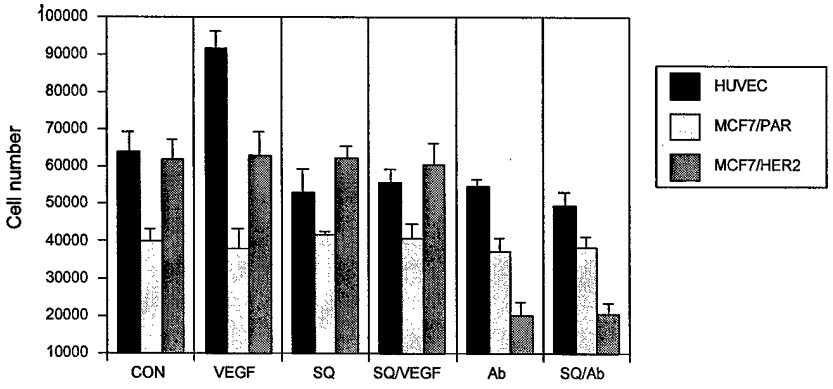

FIG. 2. Squalamine (SQ) disrupts vascular endothelial cell growth. Human vascular endothelial cells (HUVEC), MCF-7/PAR or MCF-7/HER2 cells were treated with control vehicle (CON), VEGF (20 ng/ml), squalamine (SQ;16μM), Herceptin (Ab) or a combination of squalamine and Herceptin (SQ/Ab).

1.2. Squalamine does not affect VEGF secretion *in vitro* for breast cancer cells with or without HER-2 gene overexpression. HER-2 is thought to elicit tumor progression via its effects on promoting cancer cell growth, but recent data show that HER-2 may also regulate angiogenesis by promoting tumor VEGF production (18,19). To evaluate HER-2 effects on VEGF, MCF-7 parent and HER-2 cells were plated for 72 h *in vitro*, and secretion of VEGF into the media was determined by ELISA (11,23,29) (FIG. 3).

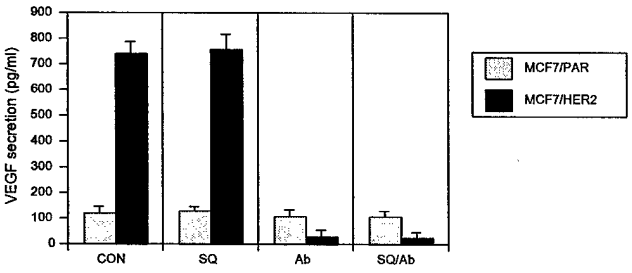

FIG. 3. HER-2 gene overexpression promotes enhanced secretion of VEGF in breast tumor cells *in vitro*. Parental MCF-7 cells (PAR) and HER2-overexpressing (HER-2) cells were incubated without (CON) or with squalamine (SQ), Herceptin (Ab) or squalamine-Herceptin (SQ/Ab) for 72 hr, with VEGF in extracellular media assayed by ELISA. Data given as mean ±SEM. See text for details (23,29).

Parent cells exhibit significant secretion of VEGF, and, after HER-2 transfection, a further increase in VEGF secretion was found. In parallel studies, squalamine elicited no effect on VEGF secretion (FIG. 3). Thus, HER-2 may contribute to angiogenesis via up-regulation of VEGF secretion in breast cancer, but squalamine is not antiangiogenic at this step in angiogenesis since it does not appear to directly affect secretion of VEGF by breast tumor cells. However, as reported previously (23,32), Herceptin elicits a significant reduction in VEGF secretion in the presence or absence of squalamine ($P<0.01$).

1.3. Squalamine blocks VEGF-induced activation of MAP kinases *in vitro*. VEGF exerts its effects by binding with receptor tyrosine kinases, Flt-1 and Flk-1/KDR, at endothelial cell surfaces (12,30). Post-receptor signaling regulates the proliferative action of VEGF that is associated, in turn, with tyrosine phosphorylation and stimulation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase ERK-1 ($p44^{MAPK}$) and ERK-2 ($p42^{MAPK}$)(12,31). We reported that blockade of endothelial cell growth by squalamine may occur, in part, by suppression of MAPK signaling induced by growth factors, such as VEGF. VEGF, given alone to vascular endothelial cells, promotes tyrosine phosphorylation of p42/p44 MAPK isoforms, but, after co-administration with squalamine, VEGF-induced phosphorylation of MAPK is significantly suppressed, thus suggesting inhibitory effects of squalamine (23,32). Thus, these and independent studies suggest that MAPK's may be a major point of convergence for many angiogenic cascades, and assay for MAPK activation may be a useful screen for effects of squalamine.

Importantly, the SAPK2/p38 MAP kinase pathway is a stress-activated kinase cascade that is also stimulated by VEGF in human vascular endothelial cells. To assess potential inhibitory effects of squalamine on this downstream signaling pathway, we conducted experiments to investigate VEGF-induced effects on p38 MAPK. To detect phosphorylated forms of p38 MAP Kinase, human vascular endothelial cells were plated on glass slides in complete media. When cells reached about 80% confluency, they were serum-starved for 16-18 h and washed twice with PBS before treatment. We then treated cells with control, 160 nM of squalamine control for 1 h, 50 ng/mL of VEGF for 10 min, or a combination of a pre-treatment with squalamine for 1 h followed by the addition of 50 ng/mL of VEGF the last 10 min. After treatment, cells were immediately rinsed with cold PBS and prepared for viewing by confocal microscopy, using antibodies against phospho-p38 MAP kinase

(Thr180/Tyr182)(Cell Signaling)(32). As expected, treatment of vascular endothelial cells with VEGF *in vitro* elicited significant phosphorylation of p38 MAP kinase by 10 min. However, after addition of squalamine, the VEGF-stimulated phosphorylation of p38 MAP kinase was significantly reduced and far less predominant. Squalamine-mediated changes in VEGF-induced activation of SAPK2/p38 MAP kinase may have mechanistic significance, since activated p38 MAP kinase is associated with F-actin formation and focal adhesion assembly, important functions in the migration and proliferation of vascular endothelial cells (30-34).

1.4. Squalamine suppresses VEGF-stimulated endothelial cell tube-like formation *in vitro* To assess the role of squalamine inhibition of VEGF signaling early in the process of angiogenesis, human vascular endothelial cells were plated on growth factor-depleted Matrigel (32,33). At doses as low as 160 nM, squalamine was found to block VEGF-induced capillary tube formation. However, VEGF-stimulated capillary tube formation was not significantly disrupted by 1.6 nM squalamine. The inhibitory effect of squalamine on capillary tube formation by endothelial cells appears to occur in a dose-dependent manner. Cells in plates in which networked capillary tubes were inhibited by squalamine displayed a profound alteration in shape and size (i.e., round and small) as compared with more characteristic spindle-shaped, elongate cells that form tubes in the absence of squalamine. These experiments are continuing in the current period.

1.5. Squalamine Interacts with Caveolae Signaling Domains of Human Vascular Endothelial Cells *in vitro*. To further assess potential biologic mechanisms for antiangiogenic and antitumor effects of squalamine noted above, human umbilical vein endothelial cells (HUVEC) were grown *in vitro*. We have hypothesized that antiangiogenic effects of squalamine may be due, in part, to binding of squalamine with caveolae localized in plasma membranes of human vascular cells. Caveolae are plasma membrane domains that act as signaling platforms to intercept a variety of agonists, both protein and lipid, in endothelial cells. To evaluate these membrane subfractions as a primary site of squalamine interaction with endothelial cells, we prepared caveolae membranes from HUVEC cells using a detergent-free method and measured specific binding of [7β-^{3}H]-squalamine (Perkin Elmer-New England Nuclear) using established methods (35-37). An example of purification of a caveolae membrane fraction from human vascular endothelial cells is shown in Fig. 4. The endothelial cells exhibit enrichment of caveolin-1 in caveolae-related domains. In addition, we find localization of specific [^{3}H]-squalamine binding-sites in caveolin-enriched membrane fractions.

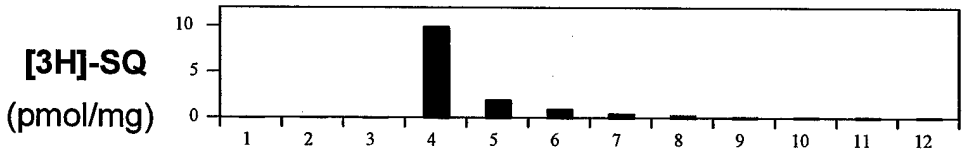

Fig. 4. Purification of caveolae membrane sub-fractions from human vascular endothelial cells. The cells show significant enrichment of caveolin-1 in caveolae domains, gradient fractions 4-6, isolated from vascular endothelial cells by use of established detergent-free methods. In addition, specific binding of [3H]-squalamine localizes to the same gradient fractions that contain caveolin (gradient fractions 4-6, see figure above). Specific binding of [3H]-squalamine was assessed by use of established methods. Results of one representative experiment are shown.

The nature of squalamine binding with caveolae was investigated further in a set of equilibrium binding studies. Data obtained from these experiments indicate that *in vitro* binding of [^{3}H]-squalamine at concentrations ranging from 10-400 nM to caveolae membranes is saturable (data not shown). The squalamine-binding properties of the caveolae were analyzed further to obtain an estimate of both the concentration of specific binding sites for squalamine and the equilibrium constant. Preliminary analysis of the data by the method of Scatchard indicate that the dissociation constant (Kd) for the binding process is 7.5×10^{-8} M. Total binding sites for squalamine at saturation correspond to a maximal binding capacity (Bmax) of 10 pmol/mg protein. The ligand specificity of [^{3}H]-squalamine binding to caveolae was analyzed by competitive binding of a 100-fold molar excess of unlabelled squalamine or other common steroidal compounds. The extent of [^{3}H]-squalamine binding by caveolae was largely unaffected by such excess of estradiol-17β, progesterone, or cortisol. In contrast, unlabelled squalamine elicited nearly complete reduction of specific [^{3}H]-squalamine binding to caveolae

membranes. The data suggest that caveolae at the surface membrane of endothelial cells may offer a primary interaction site for squalamine.

AIM 2. *Investigation of the efficacy of squalamine alone and combined with other antitumor agents in blocking growth and progression of human breast cancer xenografts in nude mice.*

2.1. Squalamine and Herceptin block growth of breast tumors with HER-2 overexpression in vivo Antitumor activity of the antiangiogenic steroid, squalamine, was evaluated using human MCF-7 breast tumors with or without overexpression of HER-2. Tumors were grown as subcutaneous xenografts and treated with control, Herceptin, squalamine or squalamine in combination with Herceptin (Fig. 5).

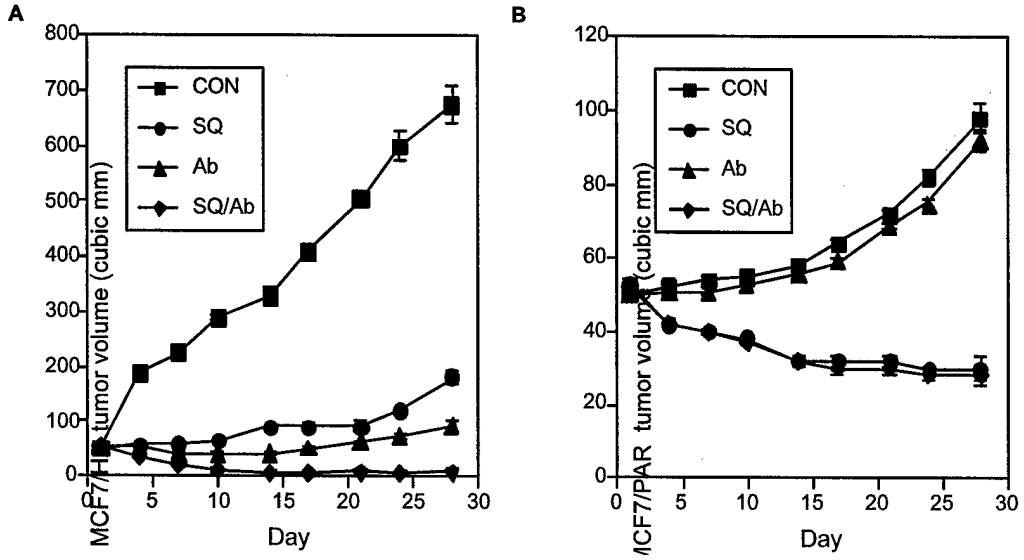

FIG 5. Squalamine and Herceptin stop tumor growth *in vivo*. MCF-7 tumors with (MCF7/H2)(**A**) or without (MCF-7/PAR)(**B**) HER-2 overexpression were grown as SC xenografts in nude mice to 50-75 mm^3(see *y*-axis). Mice were then randomized and treated with control (CON), Herceptin (Ab; 8 mg/kg loading dose, 4 mg/kg maintenance dose 1 wk later), squalamine (SQ; 2 mg/kg on days 1-10) or squalamine plus Herceptin.

Squalamine alone retards growth of MCF-7 cells with or without HER-2 overexpression ($P<0.001$), while Herceptin alone blocks growth of MCF-7/HER-2 but not MCF-7/PAR cells ($P<0.001$). More profound regression of HER-2-overexpressing tumors was found with squalamine plus Herceptin. In parallel studies, MCF-7/PAR and HER-2 tumors were harvested after 7 days treatment (Fig. 5) and prepared for IHC staining to detect blood vessels (23). On scoring tumor microvessel density, HER-2 tumors exhibited more angiogenic activity than parent cancers ($P<0.001$). Squalamine alone elicited a reduction of vessel density for either PAR or HER-2 tumors ($P<0.001$), and IHC analyses revealed a further reduction of angiogenesis in mice with MCF-7/HER-2 tumors treated with a combination of Herceptin with squalamine ($P<0.01$) (data not shown).

Studies with 2C4 antibody to HER-2 receptors are underway. Preliminary findings suggest that 2C4 antibody (38,39), unlike Herceptin, has antitumor activity in both MCF-7 parent and HER2-overexpressing breast tumors. Combination of 2C4 and receptor tyrosine kinase inhibitors (40,41) with squalamine is also planned in the coming funding period.

AIM 3. *Investigation of the efficacy of squalamine administered by oral and intravenous routes in blocking in vivo growth and progression of human breast cancer xenografts in nude mice.*

Experiments to assess the potential oral bioactivity of squalamine have begun. MCF-7 breast tumor cells are being implanted subcutaneously in estrogen-primed nude mice by established methods. After tumors achieve a size of 50-100 mm^3, mice will be treated with squalamine at 5, 20 or 50 mg/kg by oral gavage. Antitumor activity and post-treatment assays of tumor microvessel density and apoptosis are planned, as well as assessments of animal weight and any notable treatment-related physiologic or behavioral effects.

KEY RESEARCH ACCOMPLISHMENTS

- Significant growth inhibition was elicited by squalamine alone for both parental and HER-2-overexpressing human breast tumor xenografts *in vivo*.

- Immunohistochemical evaluation of tumors revealed decreased microvessel density and increased tumor cell apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types appear to be similarly reduced by treatment with squalamine.
- In HER-2-overexpressing breast tumors, combination of squalamine with Herceptin antibody to HER-2 receptors provides a more profound antitumor effect.
- In *in vitro* studies, we find that squalamine does not directly affect proliferation of breast tumor cells. However, squalamine significantly blocks VEGF-induced activation of p42/p44 and p38 MAP kinases and cell proliferation in human vascular endothelial cells.
- Squalamine binds with specificity and significant affinity to caveolae domains isolated from human vascular endothelial cells. This membrane region is known to function as a signaling platform for the regulation of vascular endothelial cell growth.

REPORTABLE OUTCOMES

Presentations

1. Pietras, R.J. "Squalamine blocks tumor-associated angiogenesis in human breast cancers". Presented at Jonsson Cancer Center Seminar Series, UCLA (June, 2004).
2. Pietras, R.J. "Steroidal agents and tumor-associated angiogenesis". Presented at FASEB Summer Research Conference, Tucson, Arizona (August, 2004).

Publications

1. Pietras, RJ and Weinberg, O (2005). Antiangiogenic steroids in human cancer therapy. *Evidence-Based Complementary and Alternative Medicine* (submitted for publication).

Additional Research Opportunities

Dr. Pietras, the Principal Investigator, was the Chairman of a FASEB Summer Research Conference on "Steroid Hormone Receptors" that was held July 31-August 5, 2004 in Tucson, Arizona, and work derived from the funded research was discussed at the Conference (see presentations above).

No patents, development of cell lines, informatics or additional funding opportunities to be reported at this time.

PERSONNEL

Richard J. Pietras, PhD, MD, Principal Investigator
Diana Marquez, MD, Assistant Researcher
Olga Weinberg, BS, Research Assistant

CONCLUSIONS

This study is intended to evaluate the notion that treating both breast cancer cells and the tumor-associated vasculature may be more effective than treating breast cancer cells alone. Studies in the first year of this award show that the antiangiogenic steroid, squalamine, directly inhibits VEGF-induced growth of vascular endothelial cells and, thereby, elicits a potent antitumor effect. In addition, growth factor receptors, such as HER-2, in breast tumors enhance VEGF secretion, and therapies targeted to this signaling axis appear to elicit beneficial indirect effects on the process of angiogenesis. This project will continue to evaluate antitumor effects of squalamine alone and combined with other therapies targeted to tumor cell growth factor receptors,

such as Herceptin, 2C4 antibody and receptor tyrosine kinase inhibitors, and, thereby, test the potential value of direct and indirect blockade of angiogenesis as an antitumor strategy in breast cancer. In conclusion, research goals from the 'statement of work' have been achieved for year 01 of this project.

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