

An Antiangiogenic Urokinase-derived Peptide Combined with Tamoxifen Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer¹

YongJing Guo, Andrew P. Mazar,² Jean-Jacques Lebrun, and Shafaat A. Rabbani³

Department of Medicine, McGill University Health Centre, Montreal, Quebec, H3A 1A1 Canada [Y. J. G., J. J. L., S. A. R.] and Ångstrom Pharmaceuticals Inc., San Diego, California 92121 [A. P. M.]

ABSTRACT

Expression of urokinase (uPA) and its receptor (uPAR) is associated with increased tumor-cell invasion and metastasis in several malignancies including breast cancer. An 8-mer peptide derived from the nonreceptor-binding domain of urokinase (Å6) has been shown to have antiangiogenic and proapoptotic effects to block the progression of breast cancer *in vivo*. In the present study, we evaluated the effects of Å6 and the antiestrogen tamoxifen (TAM) alone and in combination on estrogen-receptor-positive Mat B-III rat breast cancer cells *in vitro* and *in vivo*. Treatment of Mat B-III cells with Å6 and TAM resulted in a dose-dependent decrease in tumor-cell invasion through Matrigel; these effects were more marked when Å6 and TAM were tested in combination. In addition, treatment of Mat B-III cells with either Å6 or TAM resulted in a significant reduction of vascular endothelial growth factor receptor (flk-1) expression and in transforming growth factor β activity, effects that were significantly higher after combined treatment with Å6 and TAM. For *in vivo* studies, female Fischer rats were inoculated with Mat B-III cells (1×10^6) into the mammary fat pad. These orthotopic tumors were staged to 30–40 mm³ in volume and then treatment was initiated with Å6 (75 mg/kg/day) and TAM (3 mg/kg/day) alone or in combination. Both Å6 and TAM caused a significant reduction in tumor volume; however, these antitumor effects were significantly greater in animals receiving both Å6 and TAM, which demonstrated a 75% reduction in tumor growth as compared with control animals. The number of macroscopic tumor foci was significantly reduced in Å6-treated animals, whereas TAM failed to exhibit any antimetastatic effects. Histological analysis of primary tumors from different groups showed a decrease in new blood-vessel density and increased tumor-cell death in Å6- and TAM-treated animals, and these effects were greater in experimental animals receiving Å6 and TAM in combination. Collectively, these studies demonstrate that the addition of novel antiangiogenic/antimetastatic agents like Å6 to hormone therapy can enhance the anti-tumor effects of hormone therapy through increased inhibition of angiogenesis and induction of tumor-cell death.

INTRODUCTION

uPA⁴ plays a key role in degrading the extracellular matrix and basement membrane in various cancers (such as breast and prostate cancers) and therefore promotes metastasis and angiogenesis (1–3). uPA is secreted as a single chain zymogen (scuPA) that exhibits very low or no intrinsic enzyme activity (4). After enzymatic cleavage by plasmin, the scuPA is converted into an active, disulfide bond-linked, two-chain HMW-uPA. This HMW-uPA, comprised of an A-chain (a.a. 1–158) and a low molecular-weight uPA (a.a. 159–411), con-

tains the catalytic activity for extracellular matrix degradation (5). Two subdomains are located within the A-chain; the growth factor domain is the site that mediates binding of uPA to its cell-surface receptor, uPAR, and a single kringle domain (6, 7). In addition to the uPAR, uPA binds with low affinity to an unidentified membrane-associated protein to trigger uPA-induced signal transduction (8). In addition to the growth factor domain and kringle, the A-chain contains a connecting peptide (a.a. 136–158). Phosphorylation of uPA at Ser138 within this region has been demonstrated to abolish the adhesion of chemotaxis of myelomonocytic cells (9). An 8-mer capped peptide Å6 (a.a. 136–143), derived from this region, was demonstrated previously to inhibit cell motility and contractility including endothelial-cell migration and tumor invasion (Fig. 1; Ref. 10).

Breast cancer is one of the leading malignancies affecting women and results in a high incidence of morbidity and mortality (11). In hormone-dependent breast cancer, the steroid hormone estrogen stimulates tumor-cell proliferation (12, 13). The use of antiestrogen agents such as TAM is thus a standard therapeutic regimen for patients with ER-positive tumors (13, 14). In addition to steroid hormones, the expression of proteolytic enzymes, such as uPA, promotes the progression of breast cancer by enhancing angiogenesis and tumor-cell invasion (10, 15).

A series of basic and clinical studies have demonstrated a correlation between uPA production and tumor invasiveness and disease stage in several malignancies including breast cancer (16–18). Analysis of uPA production in a variety of human breast cancer cell lines has revealed high levels of uPA expression in the highly invasive human breast cancer cells MDA-MB-231. In contrast, uPA mRNA was undetectable in the low-invasive MCF-7 cells. This lack of uPA expression in MCF-7 cells was attributable to hypomethylation of the uPA promoter region, resulting in silencing of uPA gene transcription (19). Previous reports by us and others have demonstrated the species specificity of uPA actions where human uPA fails to interact with rat uPAR (20, 21). These studies underscore the significance of the evaluation of the effect uPA/uPAR interaction in an allogeneic or syngeneic system that can allow complete interaction between uPA and uPAR produced by tumor cells with host proteins produced by a tumor surrounding stromal cells to fully elicit effects of the interaction on tumor angiogenesis and apoptosis (10).

In the present study, we used the ER-positive rat breast cancer cell line Mat B-III, which produces high levels of uPA and serves as a useful model for the study of breast cancer progression. Using this syngeneic *in vivo* model of breast cancer, we examined the ability of Å6 alone and in combination with TAM to decrease Mat B-III tumor invasion and metastasis *in vivo* and explored the underlying mechanisms of the inhibitory effects.

MATERIALS AND METHODS

Cells and Cell Culture. The rat mammary adenocarcinoma cell line Mat B-III was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in culture *in vitro* in McCoy's 5A modified medium (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 100 units/ml of penicillin-

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² Present Address: Attenuon, LLC, San Diego, California 92121.

³ To whom requests for reprints should be addressed, at McGill University Health Centre, 687 Pine Avenue West, Room H4.67, Montreal, Quebec H3A 1A1 Canada. Phone: (514) 843-1632; Fax: (514) 843-1712, shafaat.rabbani@mcgill.ca.

⁴ The abbreviations used are: uPA, urokinase; HMW, high molecular weight; a.a., amino acid(s); uPAR, uPA receptor; TAM, tamoxifen; ER, estrogen receptor; RT, room temperature; AB, Alamar Blue; TGF, transforming growth factor; PAI, plasminogen activator inhibitor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; VEGF, vascular endothelial growth factor.

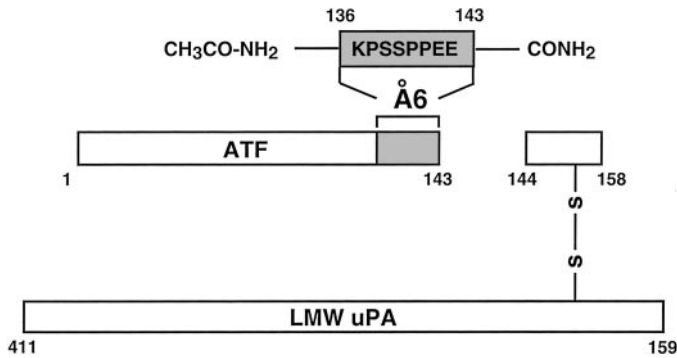


Fig. 1. Structure of uPA and $\hat{A}6$. After its proteolytic processing, HMW uPA is converted to an amino terminal fragment (ATF) and proteolytically active low molecular-weight uPA. From the COOH terminus of ATF, an 8-mer peptide corresponding to a.a. 136–143 was selected and termed $\hat{A}6$, which was used throughout the course of this study.

streptomycin sulfate (Life Technologies, Inc.) and 0.2% gentamycin (Sigma, St. Louis, MO).

In Vitro Assays. The effect of $\hat{A}6$ and TAM on Mat B-III cells invasion was determined by two compartment Boyden chambers (Transwell, Costar, Cambridge, MA) and basement membrane Matrigel invasion assay as described previously (10, 22). The 8- μ m-pore polycarbonate filters were coated with basement membrane Matrigel (50 μ g/filter). Matrigel was then reconstructed by adding 0.1 ml of serum-free culture medium to the upper chamber and incubated for 90 min. After removal of medium, cells (5×10^4) in 0.1 ml of medium with or without $\hat{A}6$ or TAM were added to the upper chamber and placed in a lower chamber prefilled with 0.8 ml of serum-free medium supplemented with 25 μ g/ml fibronectin (Sigma, Oakville, Ontario, Canada) and incubated at 37°C for 24 h. At the end of incubation, medium was removed and filters were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4) at RT for 30 min. After washing with PBS, all filters were stained with 1.5% toluidine blue and filters were mounted onto glass slides. Cells were examined under a light microscope. Ten fields under $\times 400$ magnification were randomly selected and the mean cell number was calculated. Additionally, the degree of cell invasion was determined by counting the number of cells having migrated through the membrane into the lower chamber. Results were presented as the percentage of change in invasion where the number of cells invading in the presence of vehicle alone was considered as 100%.

In the dose-response studies, the effect of $\hat{A}6$ and TAM on Mat B-III cell proliferation was determined by measuring the reduction of AB as described by the manufacturer (Serotec, Kidlington, United Kingdom) with modifications. The assay is based on metabolic reduction of the AB dye into a fluorescent species, which can be detected easily after excitation of the reduced AB dye at 560 nm and subsequent emission at 580 nm. The assay was adapted to 96-well fluorescent plates with clear-bottomed wells, which allowed direct reading of the plates without sample transfer. AB reduction was measured every second day by removal of the growth medium and substituting it with AB-containing medium. Reduction of AB was determined at 2 h after the addition of the AB substrate using a total volume of 300 μ L of a 1:20 diluted AB stock solution per well. Fluorescence was measured at a slit width of 5 nm for both excitation and emission using a Perkin-Elmer luminescence spectrophotometer LS 50B, equipped with a microtiter plate reader (23). After reading the plates, the AB-containing medium was removed, the cells were rinsed twice with PBS, and fresh medium containing the test compounds was added to the plates. The proliferative activity after 4 days of treatment was presented as absorbance at 580 nm.

To evaluate the effect of $\hat{A}6$ and TAM on TGF- β activity, Mat B-III cells were seeded at a density of 2×10^5 /6-well plate. Cells were transfected 24 h after with 5 μ g of the reporter plasmid p3TP-Lux, together with pSV- β -Galactosidase Control Vector (1 μ g; Promega, Madison, WI) using Lipofectamine (24, 25). Fifteen hours later, the medium was changed to McCoy's 5A modified medium containing 4% FCS, and the cells were incubated for an additional 9 h followed by treatment with $\hat{A}6$ (5.0 μ M), TAM (100.0 nM), or $\hat{A}6$ and TAM for 48 h. Cells were lysed, and the luciferase activity was measured and normalized to the relative β -galactosidase activity as described (25).

For Western blot analysis to determine the level of flk-1 production, Mat B-III cells treated with vehicle, $\hat{A}6$ (5.0 μ M), TAM (100.0 nM), or $\hat{A}6$ + TAM were homogenized in radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.2), 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 8 μ M aprotinin, 2 mM phenylmethylsulfonyl-fluoride, 10 mM Leupeptin, and 2 mM sodium orthovanadate]. The supernatant was removed and total protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of total protein from various groups of Mat B-III cells were separated by SDS-PAGE in 7.5% gels under reducing conditions. The proteins in gel were electrophoretically transferred to nitrocellulose membranes and reacted with antibodies used at a dilution of 1:50 with 0.5% skimmed milk in this buffer saline (pH 7.5). All membranes were soaked in buffer containing flk-1 or peroxidase conjugated antimouse rabbit immunoglobulin for flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA). All membranes were stripped and probed with β -tubulin monoclonal antibody (Sigma) to assure equal protein loading in each lane. Reaction bands were analyzed by developing in film with an enhanced chemiluminescence kit (Amersham). The level of flk-1 production was quantified by determining the ratio of flk-1/ β -tubulin levels (24).

For PAI-1 ELISA, Mat B-III cells were treated with vehicle alone, $\hat{A}6$ (5.0 μ M), TAM (100.0 nM), $\hat{A}6$, and TAM for 72 h. Cell-conditioned medium with cells from control and experimental cells was collected and tested at different concentrations for the PAI-1 production using a rat PAI-1 ELISA kit (American Diagnostica Inc., Greenwich, CT) according to manufacturer's instructions.

Animal Protocols. Inbred female Fischer 344 rats weighing 200–220 g were obtained from Charles River Inc. (St. Constant, Quebec, Canada). Before inoculation, Mat B-III tumor cells grown in serum-containing medium were washed with Hank's balanced buffer and trypsinized for 5 min. Cells were then collected in Hank's balanced buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were resuspended in 0.2 ml of saline and injected using 1-ml insulin syringes into the mammary fat pad of rats anesthetized with ethanol/Somnotal (MTC Pharmaceuticals, Cambridge, Ontario, Canada). Control and experimental animals were injected i.p. with PBS, $\hat{A}6$ (75 mg/kg/day), TAM (3 mg/kg/day), or $\hat{A}6$ and TAM in combination twice a day for 17 days. All animals were numbered, kept separate, and examined for the development of tumors daily for up to 18 days. The tumor mass of control and experimental animals was measured in two dimensions by calipers and the tumor volume was calculated. Control and experimental animals were sacrificed at the end of this study (day 18), examined, and scored for the development of macroscopic tumor metastases in various tissues (10, 21, 22). Primary tumor tissues were also removed from control and experimental animals for histological examination.

Histology and Immunohistochemistry. Primary tumors were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin (Fisher Scientific, Montreal, Quebec, Canada) the next day. Tumor sections (4 μ m) were deparaffinized and then rehydrated and stained with hematoxylin/eosin (H&E). For TUNEL assay, paraffin-embedded tissue sections were soaked in Toluene for deparaffinization, then rehydrated in graded alcohol series (100 to 70%). For enzyme predigestion of formalin-fixed tissue, the sections were incubated for 30 min at RT in 15 μ g/ml of proteinase K/10 mM Tris/HCl. The terminal deoxynucleotidyl transferase (Boehringer Mannheim, Quebec, Canada) diluted in TUNEL reaction mixture, was added to the slides and incubated at 37°C for 60 min. After rinsing with PBS, the sections were coated with 50 μ L of anti fluorescein antibody conjugated with converter-POD (horseradish peroxidase) at 37°C for 30 min. For factor VIII staining, enzyme predigestion of formalin-fixed tumors was performed by incubation at 37°C in 0.1 g of Pronase type 14/100 ml of PBS, followed by washing in 10 mM PBS (pH 7.6). Antihuman endothelial-cell antibody against factor VIII-related antigen (Von Willebrand factor; DAKO Diagnostica Inc., Mississauga, Ontario, Canada) was used as the primary antibody diluted in serum/PBS (1:600). Tumor sections were incubated for 60 min at RT followed by further incubation for 30 min with biotinylated antirabbit link antibody (Zymed Laboratories Inc., San Francisco, CA). Sections were rinsed with PBS, followed by coating with streptavidin conjugated to horseradish peroxidase for 10 min. The substrate 3,3'-diaminobenzidine was then incubated with factor VIII-related antigen sections for 10 min at RT (Sigma, Oakville, Ontario, Canada). Finally, the sections were counterstained with hematoxylin and mounted. All histological examinations were carried out by light and fluorescence microscopy using a

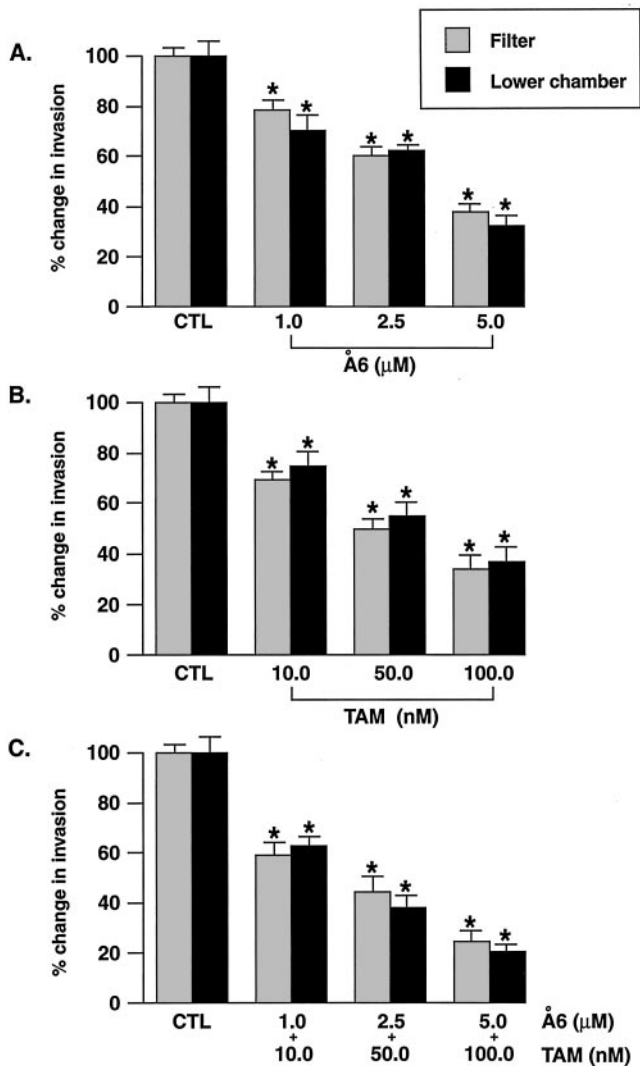


Fig. 2. Effect of Å6 and TAM alone or in combination on Mat B-III cell invasion. Mat B-III rat breast cancer cells were grown in culture and the number of tumor cells migrating to the lower aspect of Boyden chamber filter and into the lower chamber after treatment with different doses of Å6 (A), TAM (B), or a combination of Å6 and TAM (C) were counted. A comparison was made among the number of cells invading after treatment with vehicle alone (CTL). Results represent \pm SE of three different experiments. Significant difference in tumor-cell invasion as compared with control groups are represented by * ($P < 0.001$).

Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, VT). For quantitation of TUNEL-positive cells, three sections from each tumor were analyzed using NIH Image Version 1.61 and expressed as integrated density per field of examination. In control and experimental tumors, microvessels were counted and expressed as angiogenesis density representing the mean of at least 10 random fields of observation in three different sections from each tumor. All slides were interpreted by two independent investigators (10).

Statistical Analysis. Results were expressed as the mean \pm SE of at least triplicate determinations and statistical comparisons were based on Student's *t* test or ANOVA using sigmaplot software (Rockware Inc., Golden, CO). Significance was considered as $P < 0.05$.

RESULTS

Effect of Å6 and TAM on Mat B-III Cell Invasion and Proliferation *in Vitro*. The effect of different concentrations of Å6 (1.0–5.0 μ M) and TAM (10.0–100.0 nM) on the invasive capacity of Mat B-III was determined using a modified two compartment Boyden

chamber Matrigel invasion assay. Treatment with both Å6 and TAM caused a significant dose-dependent decrease in the number of cells invading through Matrigel as compared with vehicle-treated control. Both Å6 and TAM demonstrated similar anti-invasive effects as stand-alone agents (30–50% for Å6 and 40–60% for TAM over the range of concentrations tested (Fig. 2, A and B). An additive inhibitory effect (45–80%) was observed when cells were treated with the combination of Å6 and TAM (Fig. 2C).

The effect of Å6 (5 μ M) or TAM (100.0 nM) alone and in combination on Mat B-III cell proliferation was evaluated using the AB assay. After 4 days of treatment of Mat B-III cells, Å6 had no significant effect on cell-doubling time, whereas treatment with TAM caused a small but statistically significant decrease in cell proliferation. Combination treatment with Å6 and TAM exhibited a similar effect on cell growth as observed by TAM alone (Fig. 3). Throughout the course of these studies, no noticeable effect on cell morphology or viability as determined by trypan blue assay underscoring the noncytotoxic nature of these agents (data not shown).

Effect of Å6 and TAM on Tumor Growth *in Vivo*. Mat B-III cells were inoculated into the mammary fat pads of female Fischer rats. Tumors were staged to 30–40 mm³ and then animals were randomized and injected daily with vehicle alone, Å6 (75 mg/kg/day), TAM (3 mg/kg/day), and the combination of Å6 and TAM for 17 days. Tumor volume was determined every other day beginning on day 16 posttumor cell inoculation. Animals receiving either Å6 or TAM exhibited a significant decrease in the tumor-growth rate by ~50%. This decrease in tumor volume was 50–60% at the end of this study on day 18 as compared with control animals. The decrease in tumor growth was significantly greater (75%) in animals receiving both Å6 and TAM (Fig. 4). Animal weight did not change throughout the study (data not shown), indicating no overt toxicities and good tolerance of this regimen by the animals.

Effect of Å6 and TAM on Tumor Metastases. All control and experimental animals were euthanized on day 18 and evaluated for the presence of macroscopic tumor metastases. Control animals inoculated with Mat B-III tumor cells routinely showed the presence of macroscopic tumor metastases to the lungs, liver, and axillary and retroperitoneal lymph nodes. A significant decrease in the number of macroscopic tumor metastases foci was seen in animals receiving Å6, whereas no change in tumor metastases was observed after treatment

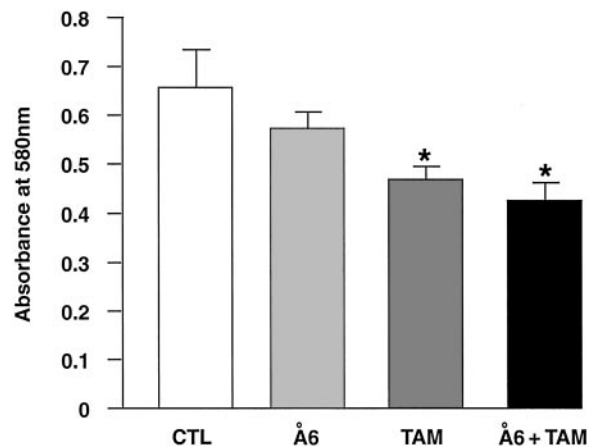


Fig. 3. Effect of Å6 and TAM on Mat B-III cell growth. Mat B-III cells were grown in culture alone and in the presence of Å6 (5.0 μ M) or TAM (100.0 nM) alone and Å6 and TAM in combination for 4 days. Cells for triplicate Petri dishes were trypsinized and counted daily by AB assay as described in "Materials and Methods." Total absorbency at 580 nm after 4 days of treatment with these agents is shown. Results represent \pm SE of three different experiments. The significant difference in the number of cell growth as compared with control groups are represented by * [$P < 0.05$ (TAM) and $P < 0.01$ (Å6 and TAM)].

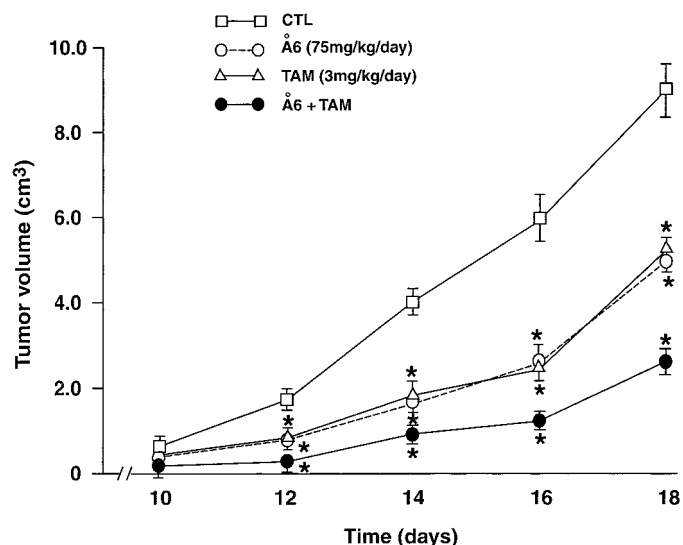


Fig. 4. Effect of Å6 and TAM on tumor volume. The tumor volume of animals inoculated with Mat B-III tumor cells was determined after treatment with vehicle alone (CTL), Å6, TAM, and a combination of Å6 and TAM at timed intervals, as described in "Materials and Methods." Results represent \pm SE of six starting animals in each group in three different experiments. The significant difference from control is shown by * [$P < 0.05$ for Å6 and TAM alone and $P < 0.01$ for the Å6 and TAM].

with TAM (Table 1). Treatment with both Å6 and TAM exhibited a decrease in tumor metastases that was similar to that observed after the infusion of Å6 alone. Collectively, these results showed that although both Å6 and TAM were able to reduce the growth of primary tumor, only Å6 decreased the incidence of tumor metastases in this syngeneic *in vivo* model of breast cancer (Table 1).

Effect of Å6 and TAM on Tumor Angiogenesis and on Tumor-Cell Death. Rapidly growing tumors are highly dependent on the formation of neovessels that fuel tumor growth. To evaluate the effect of Å6 and TAM on tumor neovascularization, control and experimental tumors were examined for new blood-vessel formation by immunohistochemical analysis using antifactor VIII-related antigen. Sections of primary tumors from both Å6- and TAM-treated animals exhibited a decrease in the number of factor VIII hot spots as compared with control tumors (Fig. 5). This decrease in hot spots was more pronounced in tumor sections from animals receiving both Å6 and TAM (Fig. 5).

In previous studies, we have routinely observed that Mat B-III tumors exhibit a significant necrotic area, which is a direct result of tumor-cell death via several mechanisms. In our studies, significantly greater areas of tumor necrosis was observed in primary tumors from all experimental tumors as evaluated by H&E Staining of histological sections (data not shown). To define the mechanism of this tumor-cell death, histological sections of control and experimental tumors were evaluated for apoptotic effects of these agents by TUNEL assay.

Control sections exhibited a limited number of TUNEL-positive cells, the number of which was significantly greater in tumors from Å6- and TAM-treated animals. These effects were found to be further increased in animals receiving Å6 and TAM in combination, as determined by integrated density per field of examination of TUNEL positive cells (Fig. 6).

Molecular Mechanism of Å6 and TAM Mediated Effects *in Vitro* and *in Vivo*. To understand this mechanism of Å6- and TAM-mediated effects in Mat B-III invasion, growth and metastases, we

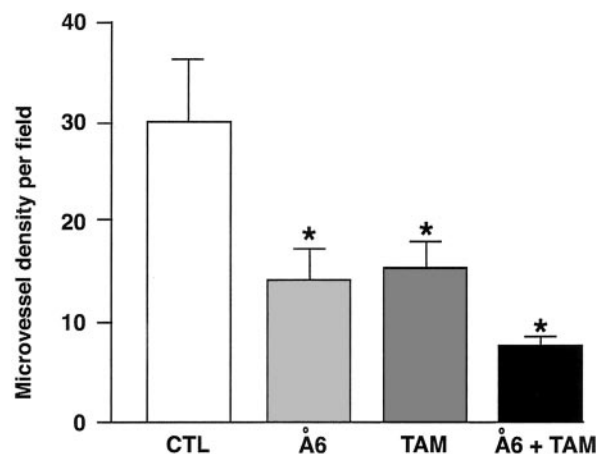
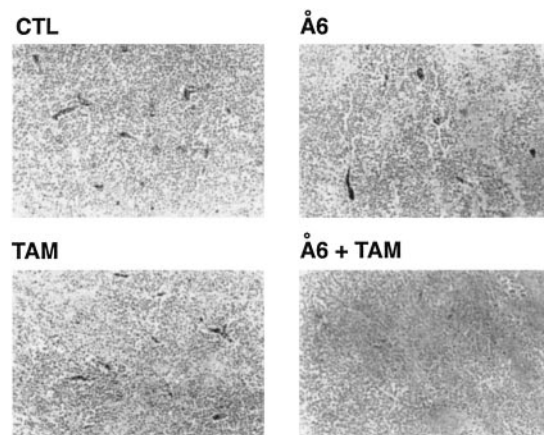


Fig. 5. Immunohistochemical analysis of tumors from animals treated with Å6 and TAM. Primary tumors from all control and experimental animals were removed, formalin fixed, and paraffin embedded. Four-micrometer sections were prepared and stained with antifactor-VIII-related antibody. Representative photomicrographs from vehicle-treated control (CTL), Å6, TAM, and Å6 and TAM in combination are shown (top; $\times 100$ magnification). Areas of high vascularization in at least 10 random fields of observation in all groups were counted for microvessel density as described in "Materials and Methods" and plotted (bottom). Results represent \pm SE of three determinations in each section from all groups. The significant difference in microvessel density per field of examination from CTL is shown by * ($P < 0.01$).

Table 1 Effect of Å6 and TAM on Tumor Metastases

Female Fischer rats were inoculated with Mat B-III breast cancer cells and treated with vehicle (CTL), Å6 (75 mg/kg/day), TAM (3 mg/kg/day) or Å6 and TAM in combination as described in "Materials and Methods." All control and experimental animals were sacrificed at day 18 posttumor cell inoculation and evaluated for the presence of macroscopic tumor metastases. The total number of metastatic foci in various organs were counted and compared. Results represent at least four animals in each group in three different experiments. *n* represents the number of animals in each group. The significant difference from control groups was evaluated by ANOVA with student-Newman-Keuls test.

| | CTL <i>n</i> = 12 | Å6 <i>n</i> = 12 | TAM <i>n</i> = 12 | Å6 + TAM <i>n</i> = 12 |
|-----------------------------|----------------------|------------------------------|----------------------|----------------------------|
| Lung | 5.0 \pm 1.90 | 2.0 \pm 0.94 ^a | 5.0 \pm 1.18 | 2.1 \pm 0.7 ^a |
| Liver | 3.0 \pm 1.00 | 0.8 \pm 0.4 ^a | 2.8 \pm 1.7 | 1.0 \pm 0.4 ^a |
| AX lymph nodes ^b | 4.0 \pm 1.55 | 1.10 \pm 0.70 ^a | 4.0 \pm 1.0 | 0.9 \pm 0.7 ^a |
| RP lymph nodes | 3.3 \pm 1.00 | 1.0 \pm 0.8 ^a | 3.0 \pm 1.4 | 1.0 \pm 0.6 ^a |

^a Significant difference from control groups ($P < 0.001$).

^b AX, axillary; RP, retroperitoneal.

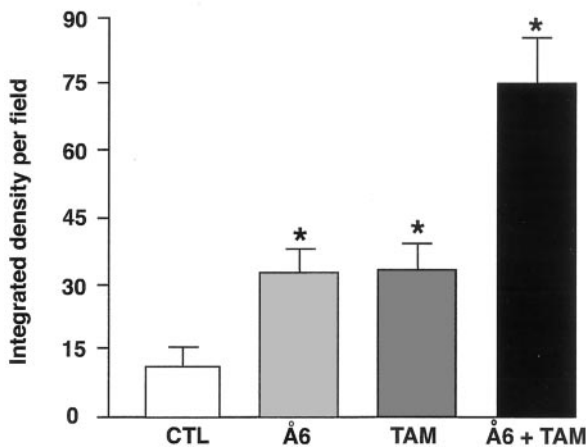
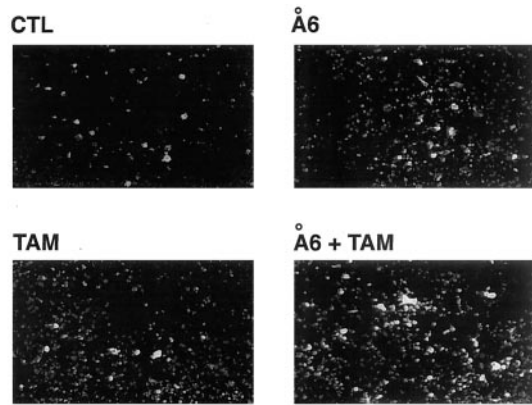


Fig. 6. Histological analysis of Å6- and TAM-treated Mat B-III tumors. Animals inoculated with Mat B-III tumors and receiving vehicle alone (CTL), Å6, TAM, and Å6 and TAM in combination were sacrificed on day 18 posttumor cell inoculation. Primary tumors from all animals were removed, formalin fixed, and paraffin embedded. Four-micrometer sections of tumors were cut and subjected to TUNEL assay and quantified, as described in "Materials and Methods." A representative photomicrograph from each group is shown ($\times 400$ magnification). Results represent \pm SE of three different determinations in each tumor. The Significant difference from CTL is represented by * ($P < 0.01$).

examined the ability of these agents to activate latent TGF- β , which is known to play an important role in tumor progression. For these studies, we used plasminogen activator inhibitor PAI-1 promoter luciferase construct (3TPLux), which allows active TGF- β to bind to its receptor in transfected cells to induce PAI-1 expression, a process that is monitored as an increase in luciferase activity. Indeed, 3TPLux, which combines portions of PAI-1 promoter with three TPA responsive elements, is highly responsive to active TGF- β (25). Mat B-III cells were transfected with 3TPLux plasmid and treated with Å6 (5.0 μ M) or TAM (100.0 nM) alone or in combination, and TGF- β activity was assessed by luciferase activity assay. In this assay, both Å6 (34%) and TAM (30%) reduced the activity of TGF- β , as seen by the reduction in relative luciferase activity. However, this reduction in TGF- β -mediated luciferase activity was further reduced to 70% after treatment with both Å6 and TAM (Fig. 7).

Mat B-III cells treated with vehicle, Å6, TAM, and a combination of Å6 and TAM for 48 h were isolated; total protein extract was analyzed by Western blot analysis for the level of production of VEGF receptor for flk-1. Both Å6 and TAM decreased the production of flk-1 as compared with control cells; however this decrease in flk-1 levels was found to be more pronounced in cells treated with both Å6 and TAM (Fig. 8).

Mat B-III cell-conditioned culture medium from vehicle-treated, Å6-treated (5.0 μ M), TAM-treated (100.0 nM), or Å6- and TAM-

treated cells for 72 h was removed and evaluated for the level of PAI-1 production by ELISA in different concentrations. These studies showed that although both Å6 and TAM were able to decrease PAI-1 production, these effects were significantly greater in Mat B-III cells treated with a combination of Å6 and TAM (Fig. 9). The ability of Å6 and TAM to decrease PAI-1 production was time dependent with increased reduction after 48 h of treatment with these agents (data not shown).

DISCUSSION

In this study, we explored the inhibitory effects of Å6 on experimental breast cancer progression when combined with TAM, as well

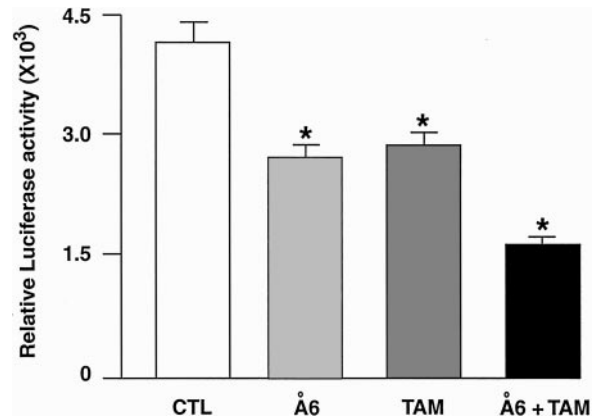


Fig. 7. Effect of Å6 and TAM on TGF- β activity. Mat B-III cells were transfected with PAI-1-luciferase plasmid, and after 20 h of incubation cells were treated with Å6 (5.0 μ M), TAM (100.0 nM), and Å6 and TAM in combination. Induction of PAI-1-mediated relative luciferase activity was determined as described in "Materials and Methods." Results represent \pm SE of three different experiments. The significant difference in luciferase expression from vehicle treated control (CTL) is represented by * ($P < 0.01$).

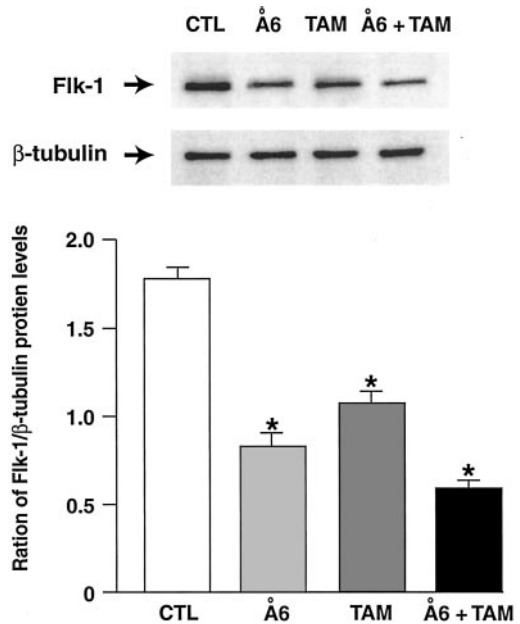


Fig. 8. Effect of Å6 and TAM on flk-1 production. Mat B-III cells were treated with vehicle alone (CTL), Å6 (5.0 μ M), TAM (100.0 nM), and Å6 and TAM in combination for 48 h. Fifty micrograms of total protein were loaded in each Lane, electrophoresed in a SDS-PAGE, and immunoblotted with anti flk-1 and anti β -tubulin antibody (top). Blots were scanned by laser densitometric scanning, and the ratio of flk-1/ β -tubulin production was plotted (bottom) as described in "Material and Methods." Results represent \pm SE of three different experiments. The significant difference from control is represented by * ($P < 0.01$).

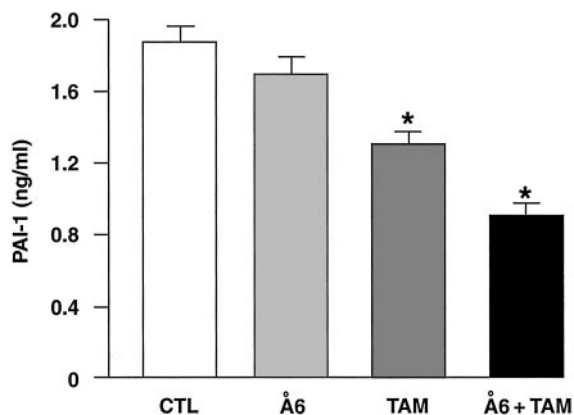


Fig. 9. Effect of Å6 and TAM on PAI-1 production. Mat B-III cells were treated with Å6 (5.0 μ M), TAM (100.0 nM), and Å6 and TAM in combination for 48 h. Cell-conditioned culture medium was removed and tested for the level of PAI-1 production by ELISA as described in "Materials and Methods." Results represent \pm SE of three different experiments. The significant difference in PAI-1 production as compared with vehicle treated control (CTL) Mat B-III cells is represented by * ($P < 0.01$).

as the underlying mechanisms responsible for this inhibition. We, and others, had previously demonstrated that Å6 alone or in combination with cisplatin could significantly inhibit tumor angiogenesis, leading to decreased rates of tumor growth, as well as a survival benefit in a xenograft model of glioma (10, 26, 27). Although Å6 is derived from the human uPA sequence, amino acids KPSS are identical in rat uPA, which may account for its ability to exhibit antitumor effects in both rat syngeneic and human xenograft models of breast cancer (10). Peak plasma levels in rats were consistent with *in vitro* IC₅₀ as described previously (10). Similar effects were observed in the current study in which Å6 and TAM alone each significantly inhibited tumor-cell invasion *in vitro* and angiogenesis and tumor growth *in vivo*. Although both Å6 and TAM were effective in reducing tumor growth, only Å6 was able to reduce the number of tumor metastases. However, the combination of the two agents resulted in super-additive effects, resulting in an additional \sim 50% decrease in microvessel density and a 2.5-fold increase in the number of TUNEL-positive foci observed in tumor sections as compared with either agent alone. Furthermore, we observed a significant decrease in TGF- β activity using a luciferase reporter gene construct. We then identified several other molecules whose regulation could explain the observed decreases in microvessel density and increased TUNEL-positive foci in response to Å6 and TAM treatment through the inhibition of TGF- β activity.

The effect of TGF- β on tumor growth is biphasic: carcinogenesis and early tumor growth are suppressed by TGF- β , whereas this growth factor apparently accelerates tumor progression in more advanced aggressive tumors (28, 29). TGF- β is known to regulate the expression of the serpin inhibitor of uPA catalytic activity, PAI-1. PAI-1 also behaves in a biphasic manner: in some models it has been shown to decrease tumor growth and angiogenesis, whereas in other systems, PAI-1 has been shown to promote angiogenesis and tumor growth (30–32). High PAI-1 levels in cancer patients are almost always associated with poor prognosis, but this paradox may be explained by a concentration effect: although PAI-1 may inhibit angiogenesis at physiological levels by inhibiting uPA-dependent remodeling of basement membrane, as well as adhesion of cells to vitronectin, at higher PAI-1 concentrations, the inhibition of excess proteolytic activity and induction of migration toward fibronectin may act to promote angiogenesis (31–34). In this study, we demonstrated that both TAM and Å6 inhibit the expression of PAI-1 and that the combination of agents inhibits PAI-1 expression by \sim 50% in cell culture *in vitro*. We are currently investigating whether the decrease in

PAI-1 expression by tumor cells treated with TAM and Å6 correlates with the inhibition of angiogenesis *in vivo*.

Recent data have also demonstrated the autocrine regulation of tumor-cell invasion and signaling by VEGF in prostate and breast cancer cells (35, 36). In this study, we also observed the expression of the rodent VEGF receptor, flk-1, in MatB-III cells (homologous to human KDR), and this expression could be attenuated by $>$ 50% in the presence of Å6 alone. This attenuation was further enhanced in the presence of TAM to \sim 33% of control. Recently, a correlation has been demonstrated between VEGF expression and PAI-1 expression in human colorectal tumors (37). The expression of VEGF and PAI-1 correlated with stage, with much higher expression levels being associated with advanced disease. Finally, it has also been demonstrated that blocking PAI-1 expression inhibits the expression of VEGF in smooth muscle cell, raising the possibility that a similar axis of regulation may also exist in tumors (38). The existence of a possible autocrine VEGF loop in tumor cells implies that VEGF receptors, such as flk-1, might also be regulated. Unfortunately, it has been difficult to evaluate flk-1 expression directly in tumor sections because of the lack of a suitable, commercially available antibody against flk-1 that is useful for immunohistochemistry. This necessitates the use of alternative approaches. Experiments are currently ongoing to determine whether a PAI-1-dependent autocrine loop regulated through TGF- β signaling and leading to flk-1 expression is responsible for the invasiveness and tumorigenicity of Mat B-III cells *in vitro* and *in vivo*.

Collectively, this study demonstrates the combination of an antiestrogen (TAM) and an antiangiogenic and anti-invasive peptide derived from uPA (Å6) in reducing the growth rate of rat breast tumors through the inhibition of angiogenesis and invasiveness with a concomitant increase in cell death as measured by TUNEL staining. We present data for the first time describing aspects of the molecular mechanism underlying the antiangiogenic and anti-invasive activity of Å6, which has been demonstrated previously to inhibit angiogenesis, leading to the inhibition of tumor growth and the implication of a VEGF receptor and PAI-1 as part of this mechanism. We also present data suggesting the utility of combining an antiestrogen with noncytotoxic antiangiogenic therapy as a potential therapeutic approach to suppress breast cancer progression, an approach that may improve the efficacy of current first-line therapy.

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