Prevention of Breast Cancer Growth, Invasion, and Metastasis by Antiestrogen Tamoxifen Alone or in Combination with Urokinase Inhibitor B-428

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ABSTRACT

Urokinase (urokinase plasminogen activator, uPA) and its cell surface receptor (uPAR) play an important role in a variety of physiological and pathological processes requiring cell migration and tissue remodeling. Using our syngeneic model of uPAR overexpression by the rat breast cancer cell line Mat B-III, we have examined the ability of the nonsteroidal antiestrogen, tamoxifen (TAM), and of a selective synthetic inhibitor of uPA, 4-iodo benzo[b]thiophene-2-carboxamidine (B-428), to inhibit expression of uPA and uPAR as well as cell growth, invasion, and metastasis of wild-type Mat B-III cells and of cells overexpressing uPAR (Mat B-III-uPAR). Both TAM and B-428 inhibited uPAR gene transcription, mRNA expression, protein production and also decreased the proliferative and invasive capacity of Mat B-III and Mat B-III-uPAR. The effects of TAM and B-428 were more pronounced when these agents were tested in combination. Both control and experimental cells (1 x 10^7 cells) were inoculated orthotopically into the mammary fat pad of syngeneic female Fisher rats, and animals were infused i.p. with either TAM and B-428 alone or in combination for 2 weeks. Control animals receiving vehicle alone developed large tumors and macroscopic metastases to lungs, liver, and lymph nodes. In contrast to this, experimental animals receiving TAM and B-428 showed a significant decrease in primary tumor volume and metastases. Combination therapy had especially marked effects in blocking progression of the primary tumor in experimental animals inoculated with highly aggressive Mat B-III-uPAR cells. These results underscore the utility of anti-proteolytic agents (B-428) in combination with TAM in breast cancer patients where the uPA/uPAR system plays a key role in tumor progression.

INTRODUCTION

Adenocarcinoma of the breast is one of the leading malignancies among women, resulting in a high incidence of morbidity and mortality (1). In this hormone-dependent cancer, the steroid hormone estrogen stimulates tumor cell proliferation, resulting in the use of antiestrogens as a standard therapeutic regimen for patients with ERα-positive tumors (2). This treatment strategy has had limited success in controlling breast cancer progression, due to the transition of breast cancer cells from an estrogen-sensitive to estrogen-insensitive variety in later stages of disease when the tumor is more aggressive (3). In contrast to estrogens, the role of progestins in breast cancer remains poorly defined (4). The nonsteroidal antiestrogen, TAM still remains the most common form of hormone therapy in patients suffering from hormone-sensitive breast cancer (5). Because of its ability to bind ER, TAM can transactivate various estrogen-responsive genes implicated in tumor cell proliferation and differentiation (6). Additionally, TAM can block cells in the early G1 phase of the cell cycle by inhibiting cyclin-dependent kinase activity (7). In addition to its antiestrogenic effects, TAM can also modulate the expression of a variety of growth factors, e.g., TGF-α and TGF-β, implicated in breast cancer progression (8).

A critical event in cancer cell invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix (9). This is achieved through various proteolytic enzymes located at the plasma membrane, which helps to focus these degradative events on the invading cell surfaces (10, 11). One such proteolytic enzyme, uPA, a serine protease, has been implicated in the progression of many malignancies (12, 13). Increased uPA production by tumor cells and their surrounding stroma is associated with higher invasive and metastatic potential in human malignancies (12, 14). In our previous studies, we have demonstrated that overexpression of uPA by the rat prostate cancer cells Mat LyLu results in increased tumor metastasis to both skeletal and nonskeletal sites (15). This higher invasive and metastatic potential is achieved at least partially via focalization of the proteolytic activity of uPA within the tumor cell environment by uPA binding to its receptor (uPAR) expressed on tumor cells (16, 17). Several clinical studies have provided compelling evidence for the role of uPA/uPAR in breast cancer, where higher plasma levels of uPAR were associated with lower overall survival (18). To directly examine the role of uPAR in tumor progression, we have developed a syngeneic model of uPAR overexpression by the rat breast cancer cell line Mat B-III (19). Experimental cells overexpressing uPAR showed higher invasive capacity in vitro. Inoculation of these cells into the mammary fat pad of syngeneic female Fischer rats resulted in the development of large tumors and metastasis to several sites as compared to animals inoculated with control cells (19).

Because the uPA/uPAR system plays a key role in tumor invasion and metastasis, inhibition of cell surface uPA activity is an attractive therapeutic target for controlling cellular invasiveness in cancer (20). The uPA active site specific inhibitor, 4-iodo benzo[b]thiophene-2-carboxamidine (B-428), was able to efficiently block tumor growth and invasion in a dose-dependent manner when tested in our syngeneic model of prostate cancer overexpressing rat uPA (21). This inhibitor has also been shown to block tumor cell invasion in vitro and in vivo in a mouse mammary adenocarcinoma model (22). Because breast cancer is a complex multistep process in which several growth factors and proteases play key roles, appropriate therapeutic strategies to specifically target these mechanisms are required.

In the present study, we have used our syngeneic in vivo model of breast cancer to evaluate the ability of TAM alone and in combination with B-428 to block invasion and metastasis of wild-type Mat B-III cells and cells overexpressing uPAR.

MATERIALS AND METHODS

Urokinase Inhibitor B-428 and Antiestrogen Tamoxifen. Urokinase inhibitor B-428 was a kind gift from Dr. B. A. Littlefield of Eisai Research Institute, Andover, MA (23). Tamoxifen citrate salt was purchased from Sigma Chemical Co. (St. Louis, MO).
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Cells and Cell Culture. Mat B-III cells were obtained from American Type Culture Collection (Rockville, MD). The experimental cells overexpressing uPAR (Mat B-III-uPAR) were generated by transfecting the expression vector pRc-uPAR containing the entire coding region of rat uPAR cDNA as described previously (19). Cells were maintained in culture in vitro in McCoy’s 5A modified medium supplemented with 2 mM L-glutamine (Life Technologies, Inc.), 10% fetal bovine serum, 100 units/ml of penicillin-streptomycin sulfate (Life Technologies, Inc.), and 0.2% gentamicin. For transfected Mat B-III and Mat B-III-uPAR cells, the medium was also supplemented with G418 (600 μg/ml).

Animal Protocols. Inbred female Fisher 344 rats weighing 200–220 g were obtained from Charles River, Inc. (St. Constant, Canada). Before inoculation, control and experimental Mat B-III cells were grown in serum-containing medium and washed with Hank’s buffer and trypsinized for 5 min. Cells were then collected in Hank’s 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).

To examine the effect of B-428 on tumor-bearing animals, Alzet osmotic minipumps (models 2 ML4 and 2 MN4; Alza, Palo Alto, CA) were implanted i.p. under light ether anesthesia. Each minipump contained B-428 dissolved at 2.5 mg/ml in 0.9% saline containing 5% DMSO and 5 mg/ml BSA to deliver a constant dose of the compound for up to 2 weeks at a delivery rate of 0.005 ml/h (21). Control animals received vehicle alone (0.9% saline, 5% DMSO, and 5 mg/ml BSA) delivered in an identical manner. To examine the effect of TAM on tumor-bearing animals, TAM was given daily i.p. at a concentration of 3 mg/kg/day for 2 weeks. Combination treatment was given in an identical manner. These doses of B-428 and TAM are sufficient for decreasing tumor growth in rats without any noticeable side effects and represent the pharmacological dose of TAM for humans (21, 24–26). Combination treatment was given in an identical manner.

All animals were numbered and kept separately and were examined for the development of tumors every second day for up to 17 days. The tumor mass at the site of tumor cell inoculation (mammary fat pad) of control and experimental animals was measured in two dimensions by calipers, and the tumor volume was calculated (21, 25). At timed intervals, control and experimental animals were sacrificed, examined, and scored for the development of macroscopic metastases in various tissues by two experts blinded to therapy. Tumor tissues were also removed from the site of inoculation and extracted for RNA analysis.

Cell Proliferation Assay. Control and experimental cells were trypsinized, resuspended into six-well tissue culture dishes (2 × 10^4 cells/well), and allowed to adhere overnight. The cells were treated the next day with either TAM (0.01–1.0 μM), B-428 (0.5–5.0 μM), or a combination of TAM and B-428 (1.0 μM TAM and 5.0 μM B-428). B-428 and TAM were dissolved in DMSO and ethanol, respectively, as 1000× stock solutions and were added directly into the fresh culture medium (2 ml/plate) 24 h after plating the cells. Cell culture medium was replenished every third day, and the total number of control and experimental cells were counted using a Coulter counter (model ZF; Coulter Electronics, Harpenden, Herts, United Kingdom) at 1, 2, 3, and 4 days after the treatments.

Northern Blot Analysis. Total cellular RNA was isolated from tumor tissue and control and treated Mat B-III cells by acid guanidinium thiocyanate-phenol-chloroform extraction. Twenty μg of total cellular RNA were electrophoresed on a 1.1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytian, Amersham, Oakville, Ontario, Canada) by capillary blotting. Filters containing Mat B-III cell RNA were hybridized with a 32P-labeled rat uPAR and rat uPA cDNA (19). All blots were also hybridized with 18S RNA probe labeled with [32P]a-UTP (>600 Ci/mmol; ICN, Costa Mesa, CA) for 60 min at room temperature. After the incubation, DNase I (150 units per reaction) and proteinase K (0.2 mg/ml) were added sequentially and incubated for 30 min at 37°C, respectively (29). Newly synthesized RNAs were isolated by spin column and ethanol precipitation and pelleted by centrifugation. RNAs were hybridized with uPA, uPAR, and 18S cDNAs and with Bluescript vector DNA (Stratagene, La Jolla, CA) previously immobilized on Nitran membranes using a slot blot manifold (Bio-Rad, Richmond, CA). These membranes were incubated in the hybridization solution [6X SSC (pH 7.4), 50% formamide, 1% SDS, and 0.1 mg/ml sonicated salmon sperm DNA] at 42°C for 48 h. After hybridization, membranes were washed in a final wash solution of 0.1X SSC, 0.1% SDS at 42°C and exposed to Kodak XAR film with intensifying screens. The intensity of each band was quantitated using laser densitometry.

Indirect Immunofluorescence. To examine the effect of TAM and B-428 alone or in combination on uPAR expression on Mat B-III cells, 5 × 10^4 cells were plated in Lab Tek tissue culture chambers (Nunc, Naperville, IL) and allowed to grow to 70% confluence. Cells were treated with vehicle, TAM (1.0 μM), B-428 (5.0 μM), and in combination (1.0 μM TAM and 5.0 μM B-428) for 6 days. Indirect immunofluorescence was performed as described previously (19) using anti-rat uPAR IgG (American Diagnostica, Inc., Greenwich, CT).

Statistical Analysis. Results are expressed as the means ± SE of at least triplicate determinations, and statistical comparisons are based on Student’s t test or ANOVA. A probability value of <0.05 was considered to be significant.

RESULTS

Effect of TAM and B-428 on Mat B-III Cell Invasion in Vitro. The effects of TAM (0.01–1.0 μM) and B-428 (0.5–5.0 μM) on the invasive capacity of Mat B-III and Mat B-III-uPAR cells were examined by Boyden Chamber invasion assays. Both TAM and B-428 were able to cause a significant decrease in the ability of control and experimental Mat B-III cells to invade through the Matrigel in a dose-dependent manner. The number of cells invading through Matrigel following treatment with these reagents was counted and compared with cells treated with vehicle alone. Although an increased number of experimental cells invaded through the Matrigel as compared to control cells as reported previously (19), TAM (0.01–1.0 μM) was equally effective (25–52%) in decreasing the invasive capacity of both control and experimental cells (Fig. 1A). In contrast to this, B-428 (0.5–5.0 μM) was more effective (36–67%) in inhibiting the invasive capacity of Mat B-III-uPAR as compared to control Mat B-III cells (27–52%; Fig. 1B). Furthermore, an additive inhibitory effect on cell invasion was observed in both control and experimental cells that received a combination treatment of TAM (1.0 μM) and B-428 (5.0 μM; Fig. 1C).

Effect of TAM and B-428 on Mat B-III Cell Growth in Vitro. Wild-type untransfected Mat B-III cells (control) and Mat B-III-uPAR cells overexpressing uPAR (experimental) were maintained in culture in the absence or presence of TAM (1.0 μM) and B-428 (5.0 μM) alone.
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pared to either of these agents when tested alone (Fig. 2B). Overall, TAM was equipotent in blocking growth of Mat B-III and Mat B-III-uPAR cells; however, B-428 alone or in combination with TAM was found to be a more effective inhibitor of Mat B-III-uPAR cell growth. The viability of both groups of cells after treatment with these agents was determined by trypan blue assay at each time point. No evidence of cytotoxicity was observed. Furthermore, these concentrations of TAM and B-428 did not alter cell morphology (data not shown).

Effect of TAM and B-428 on uPA and uPAR mRNA Expression, Gene Transcription, and uPAR Protein Production in Vitro. Both control (Mat B-III) and experimental (Mat B-III-uPAR) cells were treated with either TAM (0.1-1.0 μM), B-428 (0.5-5.0 μM), or a combination of TAM and B-428 (1.0 μM TAM and 5.0 μM B-428) for 6 days. Incubation of control and experimental Mat B-III cells with both TAM and B-428 for 6 days had no cytotoxic effects. In our previous study, we have shown that Mat B-III-uPAR cells express 4-fold higher levels of uPAR mRNA as compared to control untransfected cells without altering the level of uPA mRNA expression (19).

and in combination for 1-4 days, and the effect of this treatment on cell proliferation in vitro was examined. Both TAM and B-428 inhibited the growth of control Mat B-III cells for up to 4 days. TAM inhibited Mat B-III cell growth by 30%, whereas a 40% decrease in cell proliferation was seen following treatment with B-428 (Fig. 2A). Treatment of these control cells with TAM and B-428 in combination resulted in a significantly greater (60%) inhibition in cell growth as compared to either of these agents alone (Fig. 2A). Under similar experimental conditions, treatment of Mat B-III-uPAR cells with TAM caused a 29% decrease in cell proliferation, whereas a significantly more marked decrease (55%) in cell growth was seen following treatment of these cells with B-428 (Fig. 2B). Furthermore, the combination of TAM and B-428 had a more pronounced (75%) effect on the growth of these experimental cells overexpressing uPAR as compared to either of these agents when tested alone (Fig. 2B). Overall, TAM was equipotent in blocking growth of Mat B-III and Mat B-III-uPAR cells; however, B-428 alone or in combination with TAM was found to be a more effective inhibitor of Mat B-III-uPAR cell growth. The viability of both groups of cells after treatment with these agents was determined by trypan blue assay at each time point. No evidence of cytotoxicity was observed. Furthermore, these concentrations of TAM and B-428 did not alter cell morphology (data not shown).

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**Fig. 3.** Effect of TAM and B-428 on uPA and uPAR mRNA expression in wild-type Mat B-III cells. Total cellular RNA was extracted from untreated (CTL) and cells treated with TAM (1.0 μM) and B-428 (5.0 μM) alone or a combination of TAM and B-428. Twenty μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a 32P-labeled rat uPA cDNA, rat uPAR cDNA, or with a 32P-labeled 18S cDNA as described in "Materials and Methods." Blots were scanned by laser densitometric scanning, and change in uPA (A) and uPAR (B) mRNA expression was determined by plotting the ratio of uPA or uPAR/18S mRNA and are shown in the lower panels. Results are representative of at least four different experiments; bars, SE. Significant difference from control is represented by asterisks (P < 0.05).

Treatment of Mat B-III cells with TAM, B-428, or a combination of the two failed to show any significant change in uPA mRNA expression as determined by Northern blot analysis (Fig. 3A). In contrast, TAM and B-428, alone or in combination, caused a significant decrease in the rate of uPAR mRNA expression in the control Mat B-III cells (Fig. 3B). Similarly, no statistically significant change in uPA mRNA expression was seen in Mat B-III-uPAR cells (Fig. 4A), whereas a marked inhibition of uPAR mRNA levels was observed following treatment with TAM and B-428 alone or in combination (Fig. 4B). The effects of B-428 on uPAR mRNA expression were especially more profound in Mat B-III-uPAR.

To explore the molecular mechanisms of action of TAM and B-428, the effect of these agents alone and in combination on uPA/uPAR gene transcription was examined by nuclear runoff assay. Following treatment with TAM (1.0 μM), B-428 (5.0 μM), or a combination, cellular nuclei were isolated from control Mat B-III cells, and the rate of uPA/uPAR gene transcription was determined. TAM and B-428 alone or in combination had no significant effect on uPA gene transcription (Fig. 5A). However, treatment of these cells with either TAM or B-428 caused a marked decrease in uPAR gene transcription, and the effects were additive when these reagents were tested in combination (Fig. 5B). A similar effect on uPA/uPAR gene transcription was observed following treatment of experimental Mat B-III-uPAR cells with TAM and B-428 (data not shown). This decrease in uPAR mRNA expression and gene transcription resulted in a similar lower level of uPAR expression on the Mat B-III cell surface as determined by indirect immunofluorescence and receptor binding assays (data not shown).

To determine if a decrease in uPAR gene transcription and mRNA expression by TAM and B-428 results in a similar lower level of uPAR protein production, Mat B-III cells were treated with either vehicle TAM (1.0 μM) and B-428 (5.0 μM) alone or a combination of TAM and B-428. Treatment of these cells with both TAM and B-428 resulted in a marked decrease in uPAR expression on the Mat B-III cell surface, as assessed by immunofluorescence (Fig. 6). These effects were additive when TAM and B-428 were given in combination and showed decreased uPAR expression at similar levels as seen by Northern blot analysis. A similar decrease in total bonding of 125I-labeled rat uPA was seen in Mat B-III cells following treatment with TAM and B-428 alone or in combination (data not shown).

**Effect of TAM and B-428 on Tumor Growth.** Female Fisher rats were inoculated with Mat B-III (control) and Mat B-III-uPAR (experimental) cells. Animals were divided into four experimental groups receiving vehicle alone, TAM (3 mg/kg/day), B-428 (0.96 mg/kg/day) alone, or TAM and B-428 in combination. Animals were monitored for tumor growth and were sacrificed at day 15 after tumor inoculation.
Fig. 4. Effect of TAM and B-428 on uPA and uPAR mRNA expression in Mat B-III-uPAR cells. Total cellular RNA was extracted from untreated (CTL) and Mat B-III-uPAR cells treated with TAM (1.0 μM) and B-428 (5.0 μM) alone or in combination. Twenty μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a 32P-labeled rat uPA cDNA, rat uPAR cDNA, or with a 32P-labeled 18S cDNA as described in “Materials and Methods.” Blots were scanned by laser densitometric scanning, and change in uPA (A) and uPAR (B) mRNA expression was determined by plotting the ratio of uPA or uPAR/18S mRNA in the lower panels. Results are representative of at least four different experiments. Significant difference from control is represented by asterisks (P < 0.05). Bars, SE.

for evaluation of tumor metastasis. Animals receiving Mat B-III (Fig. 7A) and Mat B-III-uPAR (Fig. 7B) treated with either TAM or B-428 alone showed a marked decrease in tumor volume as compared with the control group of animals receiving vehicle alone. Consistent with our in vitro observations of the effect of TAM on tumor cell growth, TAM was equipotent in inhibiting tumor growth in animals receiving Mat B-III (control) and Mat B-III-uPAR (experimental) cells. In these studies, B-428 was equally as effective as TAM in blocking tumor growth of animals inoculated with control cells alone. However, infusion of B-428 into animals inoculated with experimental Mat B-III-uPAR cells resulted in a significantly greater decrease in tumor volume as compared with TAM alone (Fig. 7A). Most significantly, although combination therapy with TAM and B-428 showed an additive effect on tumor reduction in animals inoculated with control cells (Fig. 7A), these effects were more pronounced in animals receiving experimental Mat B-III-uPAR cells. Furthermore, infusion of TAM and B-428 alone or in combination was well tolerated by experimental animals without any noticeable side effects.

**Effect of TAM and B-428 on Tumor Metastasis.** Comparison of tumor metastases in animals inoculated with wild-type control Mat B-III cells and experimental cells overexpressing uPAR confirmed our previous observations that animals receiving cells overexpressing uPAR developed more extensive macroscopic tumor metastases to the lungs, livers, and auxiliary lymph nodes as compared to animals receiving wild-type Mat B-III cells (19). On day 15 after tumor inoculation, animals inoculated with Mat B-III and Mat B-III-uPAR cells receiving either vehicle, TAM, B-428, or TAM and B-428 in combination, which showed marked effects of these agents in decreasing tumor volume, were sacrificed and evaluated for the presence of macroscopic tumor metastasis. TAM treatment had no significant effect on the development of tumor metastasis in animals inoculated with Mat B-III or Mat B-III-uPAR cells. In contrast, infusion of B-428 into animals receiving Mat B-III or Mat B-III-uPAR cells resulted in a significant reduction in tumor metastasis, with an even greater reduction in those animals receiving Mat B-III-uPAR cells (Table 1). Treatment of these groups of animals with a combination of TAM and B-428 failed to show any additive effect in preventing tumor metastasis in either group as compared to infusion of B-428 alone (Table 1).

**DISCUSSION**

The efficacy of currently available therapies for breast cancer is restricted by the disseminated nature of the disease, which is charac-
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A

uPA

18S

B

uPAR

18S

Fig. 5. Effect of TAM and B-428 alone and in combination on uPA (A) and uPAR (B) gene transcription in Mat B-III cells. Nuclear runoff assays were performed as described in “Materials and Methods.” 32P-labeled runoff transcripts were prepared from Mat B-III cell nuclei following treatment with TAM, B-428 alone, or in combination. Probes used were uPA, uPAR, and 18S cDNAs. All blots were scanned by laser densitometry. The fold stimulation of the rate of gene transcription of uPA and uPAR relative to 18S was determined. Significant difference from control is represented by asterisks. Bars, SE.

terized by the progression of the majority of tumors to a phenotype that is resistant to both cytotoxic and hormonal therapies, and the inability of these therapies to effectively control tumor spread to various metastatic sites (3). Therefore, the development of a complementary approach that involves modifying the tumor microenvironment and reducing the propensity for tumor cell invasion, neovascularization, and metastasis is required. The role of cell-associated uPA and its cell surface receptor (uPAR) in extracellular matrix degradation and cellular invasiveness and tumor progression is well documented in clinical and experimental studies (12–19, 30, 31). Inhibition of uPA activity and interruption of uPA/uPAR interaction is, therefore, an attractive target for blocking cellular invasiveness in cancer (19, 20). Due to the species specificity of uPA and uPAR interaction, a syngeneic model of breast cancer is best suited for evaluation of certain therapeutic strategies aimed at the plasminogen activator system (15, 19). Using this model, in which uPA/uPAR plays a key role, we have evaluated the anti-invasive and antimetastatic abilities of the recently developed synthetic uPA active site inhibitor B-428 alone and in combination with TAM.

We have previously reported the ability of B-428 to block tumor progression in our syngeneic model overexpressing uPA (21). The availability of control and experimental Mat B-III cells overexpressing uPAR in the current study allowed us to evaluate the usefulness of not only the antiestrogen agent TAM but also the efficacy of any adjuvant antiproteolytic therapy against uPA. In breast cancer (and certain other cancers), uPA may not be expressed directly by the tumor cells but rather recruited from the surrounding stroma (32). Furthermore, overexpression of uPAR by the tumor cells themselves creates a system of localization of uPA to the cell surface. Therefore, although the tumor cells themselves do not express high levels of uPA, an anti-uPA antagonist would nevertheless be potentially useful due to the localization of uPA in the tumor milieu. This model may also prove to be of benefit in assessing the usefulness of inhibitors of other proteases (e.g., MMP, cathepsin, and cadherins) and growth factors (e.g., EGF) implicated in tumor progression (12, 33–35).

In the present study, both TAM and B-428 decreased the invasive capacity of control and experimental Mat B-III cells in a dose-dependent manner. Combination TAM and B-428 treatment demonstrated an additive anti-invasive effect that was more marked in experimental cells. Although B-428 has previously been shown to be an anti-invasive agent in prostate cancer cells, this is the first report demonstrating the antiproliferative effects of this protease inhibitor (21). TAM and B-428 were also antiproliferative in the absence of cytotoxicity, suggesting a role for these inhibitors in tumor cell signal transduction. The molecular mechanism of these effects was further examined by Northern blot analysis and nuclear runoff assays for uPA/uPAR (36). Although neither TAM or B-428 had any effect on uPA mRNA expression, these agents caused a significant decrease in uPAR mRNA expression and protein production in control and experimental cells. These results suggest several potential mechanisms of action for B-428; direct inhibition of the uPA active site can down-regulate the uPA-dependent cell surface proteolytic cascade,
Fig. 6. Effect of TAM and B-428 alone and in combination on uPAR protein production in Mat B-III cells. Expression of uPAR on Mat B-III cell surface following treatment with vehicle alone (CTL), TAM, B-428, and a combination of TAM and B-428 was examined by indirect immunofluorescence as described in "Materials and Methods." Results are representative of four different experiments.

which can ultimately activate latent growth factors via the action of plasmin or metalloproteases (10). Blocking the start of this cascade leads to lower growth factor activity in the tumor milieu, resulting in decreased proliferation and invasion of the tumor. Decreased growth factor activity may act in trans on uPAR expression, resulting in down-regulation of its expression as well. Alternatively, the observed effects could be a direct result of uPAR down-regulation. The increased proliferative response of tumors formed in vivo by Mat B-III-uPAR could reflect the direct signal-transducing role of uPAR, which is mediated at least partially by the binding of uPA. uPA/uPAR is endocytosed in some cells by virtue of the cell surface uPA complexing with plasminogen activator inhibitor-1. It is not clear what the downstream consequences of this internalization are. In the presence of B-428, it is possible that plasminogen activator inhibitor-1 may not complex to uPA, thereby creating a long-lived cell surface uPA/uPAR complex, which could impact signal transduction both in cis and trans. It is interesting that the magnitude of the effect observed in the presence of B-428 is greater in the transfected Mat B-III-uPAR. This observation suggests that these effects are due to down-regulation of the proteolytic cascade, resulting in decreased growth factor effects. Finally, a novel protein has been recently identified that regulates uPAR expression at a posttranscriptional level by modulating the half-life of uPAR mRNA (37). This uPAR mRNA binding protein binds a 51-nucleotide segment within the coding region, and it is possible that B-428 has an effect on the expression of this protein, which could also explain the ability of B-428 to down-regulate uPAR levels to a similar degree in control (Mat B-III) and transfected (Mat B-III-uPAR) cells.

The cytotoxic effects of TAM have been reported to result from the ability of TAM to inhibit cyclin-dependent kinases, resulting in cell cycle arrest at the G0-G1 checkpoint (8). In addition to these ER-mediated effects, TAM has also been found to act on several other targets implicated in breast cancer progression including TGF-β, TGF-α, and insulin-like growth factor-I and to reduce cell-matrix adhesion (38-40). The present study, however, is the first report describing the effects of TAM on the plasminogen activator system and its ability to decrease invasiveness by down-regulating uPAR expression.

Using doses established previously, TAM and B-428 caused a significant decrease in tumor growth of animals inoculated with control and experimental Mat B-III cells (21). Tumor progression and metastasis in vivo requires neovascularization of the tumor. Previous studies have implicated uPA/uPAR expression on endothelial cells with angiogenesis (41). Therefore, in vivo administration of B-428
could have an effect not only on the invasiveness of tumor cells but also on endothelial cell-dependent capillary tube morphogenesis. In addition, breast cancer is known to be highly vascularized, and numerous recent studies have attempted to correlate the degree of neovascularization with prognosis (42). Therefore, one potential mechanism of action for TAM and B-428 could be the inhibition of angiogenesis as a result of uPAR down-regulation (as well as a direct mechanism of action for TAM and B-428 could be the inhibition of dual actions of decreasing uPAR expression and anticatalytic activity of uPA, leads to both anti-invasive and antitumorigenic characteristics. Overall, these studies point to the potential use of combination therapies to inhibit tumor angiogenesis, primary tumor growth, local invasion, and metastases to the secondary sites (43). Further studies are in progress to elucidate the mechanism of B-428 in regulating uPAR expression and the role of this expression in angiogenesis.

In summary, these results not only provide support for the notion that inhibiting plasminogen activator-mediated cellular invasiveness is an effective therapeutic intervention but also demonstrate the effectiveness of a combination regimen aimed at targeting different steps of breast cancer progression. With recent advances demonstrating the need for combination therapy for malignancies and viral diseases, a strategy that adds B-428 and other anti-uPA/αuPAR compounds will lead to developing novel therapeutic regimens that approach the ultimate goal of total suppression of mammary carcinogenesis and progression.

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