A non–RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo

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Abstract

Purpose: Integrins are expressed by numerous tumor types including breast cancer, in which they play a crucial role in tumor growth and metastasis. In this study, we evaluated the ability of ATN-161 (Ac-PHSCN-NH2), a 5-mer capped peptide derived from the synergy region of fibronectin that binds to αvβ3 and αvβ5 in vitro, to block breast cancer growth and metastasis. Experimental design: MDA-MB-231 human breast cancer cells were inoculated s.c. in the right flank, or cells transfected with green fluorescent protein (MDA-MB-231-GFP) were inoculated into the left ventricle of female BALB/c nu/nu mice, resulting in the development of skeletal metastasis. Animals were treated with vehicle alone or by i.v. infusion with ATN-161 (0.05–1 mg/kg thrice a week) for 10 weeks. Tumor volume was determined at weekly intervals and tumor metastasis was evaluated by X-ray, microcomputed tomography, and histology. Tumors were harvested for histologic evaluation. Result: Treatment with ATN-161 caused a significant dose-dependent decrease in tumor volume and either completely blocked or caused a marked decrease in the incidence and number of skeletal as well as soft tissue metastases. This was confirmed histologically as well as radiographically using X-ray and microcomputed tomography. Treatment with ATN-161 resulted in a significant decrease in the expression of phosphorylated mitogen-activated protein kinase, microvesSEL density, and cell proliferation in tumors grown in vivo.

Conclusion: These studies show that ATN-161 can block breast cancer growth and metastasis, and provides a rationale for the clinical development of ATN-161 for the treatment of breast cancer. [Mol Cancer Ther 2006;5(9):2271–80]

Introduction

Integrins are α/β heterodimeric membrane-associated proteins that promote cell adhesion to the surrounding extracellular matrix and activate a number of intracellular signaling pathways that can alter cell behavior (1). It has been hypothesized that selective integrin expression allows tumor cells to metastasize to different organs (2). For example, lung colonization by breast cancer cells is associated with tumor cell expression of αvβ6 whereas the ability of multiple myeloma to grow in the bone microenvironment is dependent on the expression of α4β7 (3, 4). Solid tumors (breast, prostate, and lung) which metastasize to the bone often express high levels of αvβ3 (5). In particular, bone-residing breast cancer cells have been shown to express significantly higher levels of αvβ3 compared with primary breast carcinoma (6). The multistep process of breast cancer metastasis to the bone involves the invasion of tumor cells into the bone marrow cavity. This process is facilitated by the capacity of αvβ3 integrins, expressed by tumor cells, to recognize several plasma and extracellular matrix proteins such as vitronectin, fibronectin, and fibrinogen (7). The expression of αvβ3 by human breast cancer cells is therefore directly associated with an increased ability to promote the osteolytic skeletal metastases that are often associated with breast cancer (8). Similarly, the expression of αvβ3 by osteoclasts may also play a critical role in breast cancer metastasis by mediating the bone resorption that is typically associated with breast cancer metastasis (9). Finally, angiogenesis may also contribute to the growth and metastasis of breast cancer, and the integrin αvβ3 has recently been implicated as a major mediator of tumor angiogenesis (10).

Despite recent advances in early detection and novel hormone therapies for breast cancer, metastasis has continued to be a challenging clinical problem (11). ATN-161 is a capped five-amino acid peptide that has shown antitumor activity as well as the ability to inhibit soft tissue metastasis in models of colon and prostate cancer (12, 13). ATN-161 was derived from the synergy region of fibronectin and has been proposed to antagonize synergy function, suggesting that ATN-161 may interact with integrin αvβ3 (12). Preliminary data has also shown that ATN-161 interacts primarily with the β3 subunit as well as with other β-integrin subunits such as β3 and that these interactions depend on the covalent interaction of ATN-161 with the free sulphydryl residues in these β-integrin
subunits (13). Unlike other integrin-binding peptides, ATN-161 is unique in that it is not based around the arginine, glycine, aspartic acid (RGD) integrin adhesion epitope and, unlike RGD-based antagonists, does not inhibit cell adhesion in vitro. The evaluation of ATN-161 in patients with advanced cancer in a phase I trial has recently been completed and a biomarker-driven phase II trial of ATN-161 in patients with renal cell carcinoma has recently been initiated. However, little is known about the effects of ATN-161 on breast cancer growth and metastasis to bone. In this study, we used MDA-MB-231 breast cancer cells in well-characterized models of tumor growth and metastasis to evaluate the potential of ATN-161 in this therapeutic setting.

Materials and Methods
Cells and Cell Culture
Human MDA-MB-231 breast cancer adenocarcinoma cells were obtained from American Type Tissue Culture Collection (Rockville, MD) and cells transfected with green fluorescent protein (MDA-MB-231-GFP) were prepared and maintained in culture as previously described (14, 15). ATN-161 and scrambled peptide ATN-165 were manufactured by Peptisyntha (Brussels, Belgium) using solution phase methodologies under cyclic guanosine 3',5'-monophosphate.

Binding Assays
The binding of ATN-161 to MDA-MB-231 tumor cells and to immobilized purified integrins, α5β1 and α4β2, was evaluated using a biotinylated version of ATN-161 (ATN-453; Ac-PHSCNGGK-Biotin). ATN-453 has previously been shown to retain the entire binding activity of ATN-161 (16). Briefly, cells were harvested using trypsin, washed twice in binding buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.1% bovine calf serum, and 2 mmol/L MnCl2] and resuspended at a final concentration of 1 × 106 cells/mL. Cells (1 × 105 per treatment) were incubated for 2 hours at 4°C with various concentrations of ATN-453 in the presence or absence of ATN-161 (Ac-PHSCN-NH2), washed extensively with binding buffer and incubated with streptavidin–horseradish peroxidase for another 30 minutes at 4°C. After additional washes, cells were incubated with o-phenylenediamine substrate and absorbance was recorded at OD 490 nm. Binding to purified integrins was evaluated in a similar manner except that the purified integrins (5 μg/mL in PBS) were first immobilized onto high-protein binding microtiter plates for 1 hour at 37°C followed by blocking with 0.1% bovine serum albumin (1 hour, 37°C). Binding data was analyzed using Prism software (GraphPad Software, San Diego, CA) and binding curves were fit using nonlinear regression approaches.

Cell Proliferation Assay and Western Blotting
MDA-MB-231 cells were plated in triplicate in six-well plates in the presence of 2% fetal bovine serum with vehicle alone or with different concentrations (1–100 μmol/L) of ATN-161 (15, 17). Triplicate wells were trypsinized and counted using a Coulter counter on alternate days (model ZF, Coulter Electronics, Harpenden, Hertfordshire, United Kingdom). Cell culture medium was replenished daily or every other day.

For Western blotting, MDA-MB-231 (1 × 105) cells were plated in 100 mm Petri dishes for 24 hours, then serum-starved overnight before treatment with vehicle or ATN-161 (1–100 μmol/L) for different time periods (15–60 minutes). Western blot analyses were carried out using antibodies against focal adhesion kinase (FAK; Santa Cruz Biotechnology, Inc. Santa Cruz, CA), phosphorylated FAK (P-FAK; BioSource International, Inc. Camarillo, CA), mitogen-activated protein kinase (MAPK), phosphorylated MAPK (P-MAPK; Cell Signaling Technology, Inc., Beverly, MA), and β-tubulin (BD Biosciences, Mississauga, Ontario, Canada) as previously described (17). Western blots were detected using enhanced chemiluminescence detection reagents (Perkin-Elmer Life Sciences, Inc., Boston, MA).

Cell Migration Assay
MDA-MB-231 cells (3 × 105 per well) were plated in a six-well plate. Approximately 48 hours later, when the cells were 100% confluent, the monolayer was scratched using a 1 mL pipette tip. Media and nonadherent cells were aspirated, the adherent cells were washed once, and new medium containing various concentrations of ATN-161 (0.1–100 μmol/L) were added. Cells were observed under the microscope at different times and the inhibition of migration was assessed when wounds in the control treated group were closed.

Animal Protocols
For xenograft studies, 5-week-old (15–20 g) female BALB/c nu/nu mice (Charles River, St. Constant, Quebec, Canada) were used throughout (18, 19). Prior to inoculation, MDA-MB-231 cells grown in serum containing culture medium were washed with Hank’s balanced buffer and centrifuged at 1,500 rpm for 5 minutes. Cell pellets (5 × 105 cells/mouse) were resuspended in 100 μL of Matrigel (Becton Dickinson Labware, Mississauga, Ontario, Canada) and saline mixture (20% Matrigel), and injected s.c. into the right flank of mice. All animals were numbered and kept separately in a temperature-controlled room on a 12-hour light/dark schedule with food and water ad libitum. Alternatively, MDA-MB-231-GFP (1 × 105) cells were injected in 0.1 mL of cell suspension into the left ventricle of the heart (intracardiac) using a 26-gauge needle (17, 19). In the case of animals inoculated with MDA-MB-231 cells via the s.c. route, tumors were allowed to grow to 20 to 40 mm3. At this stage, animals were randomized and treated with vehicle alone or with different doses of ATN-161 (i.v., 0.05–1 mg/kg/d, thrice a week for 6 weeks). Tumor volumes were determined from caliper measurements obtained weekly. For animals inoculated with MDA-MB-231-GFP cells via the intracardiac route, animals were treated with vehicle alone or with ATN-161 (i.v., 1 mg/kg/d, twice a week) from the day of tumor cell inoculation for 10 weeks (17, 19–21). In other studies, tumor bearing animals were also treated with scrambled peptide ATN-165 as a control. All animal protocols were in accordance with and approved by the institutional review board.
Radiologic and Histologic Analysis

High-resolution total body radiologic analysis were carried out as previously described (17, 19–21). The area and number of skeletal lesions (mm²) was determined in both femora and tibia from all animals by using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis Corporation, Nashville, TN). All radiographs were carefully evaluated by at least three investigators (including one radiologist) who were blinded to experimental protocols (17, 19–21). Microcomputed tomographic scans on femora were done on a standard desktop micro-CT instrument (model 1072, SkyScan, Aartselaar, Belgium). Images were captured using a 12-bit, cooled CCD camera.

Following completion of the experimental metastasis model (using intracardiac inoculation of cells), mice were sacrificed and their lungs, liver, and spleen were removed. Fresh tissue was sliced at 1 to 5 mm thickness and observed directly under a fluorescence microscope. The number of tumor cells per field of examination from 10 random sites were counted, photographed, and plotted as described previously (14, 15). In addition, radiologically affected and unaffected long bones were excised, fixed, decalcified, and paraffin-embedded (17, 19–21). Sections of 5-µm thickness were prepared for immunohistochemistry and histologic analysis. Histologic measurement of total tumor volume was done in representative sections in the mid-portions of the bone with maximum tumor burden of the tibia from all animals. The tumor volume/tissue volume was measured with BioQuant image analysis software (BioQuant Image Analysis Corporation) and the percentage of tumor volume/tissue volume was calculated as previously described (17, 19–21).

Immunohistochemical staining was done on tumors harvested from the s.c. model as previously described (17, 19, 22). The staining was done following the protocol described in the Vectastain ABC-AP kit (Vector Laboratories, Inc., Burlingame, CA). The antibodies used were against FAK, P-FAK, MAPK, and P-MAPK as listed above. Anti-Ki-67 (clone MIB-1) was from DAKO Cytomation, Inc. (Mississauga, Ontario, Canada). Immunostaining was quantified by using BioQuant image analysis software as previously described (17, 19, 22). Determination of mitotic index was carried out on H&E-stained sections of control and experimental tumors. Mitotic figures in tumor cells were counted in 10 randomly selected fields under high magnification (×400). Total mitotic index was calculated as the percentage of total tumor cells (23). The Ki-67 index was expressed as the ratio of positive cells to all tumor cells (17, 19).

Statistical Analysis

All results are expressed as mean ± SE. The statistical significance of the difference in numbers of osteolytic metastases and tumor volume between control and ATN-161–treated groups were analyzed by Mann-Whitney test for nonparametric samples. Statistical comparisons of tumor progression data from image analysis of sections were made using Student’s t tests or ANOVA. P < 0.05 was considered statistically significant.

Results

Effect of ATN-161 on MDA-MB-231 Cell Growth

An analogue of ATN-161 (ATN-453; Ac-PHSCNGGK-biotin-NH₂) binds to MDA-MB-231 cells in vitro, and this binding can be fit to a two-site binding model with a high-affinity site having a Kd = 2.0 ± 0.9 μmol/L, and a low-affinity site with a Kd = 100.5 ± 32.8 μmol/L (Fig. 1A). This binding can be competed using ATN-161 with an IC₅₀ of ~1 μmol/L (data not shown), indicating that extending the COOH terminus of ATN-161 in order to create a binding tracer (ATN-453) has no significant effect on the binding activity. The use of ATN-453 (which is detected using a colorimetric detection system) yields binding data such that a Kd can be determined, but the number of binding sites cannot. The binding of ATN-453 was dependent on the presence of Mn²⁺ (Fig. 1B), which is known to fully activate integrins (24). Very little specific binding was observed in the absence of Mn²⁺ (Fig. 1B) or when Mg²⁺ or Ca²⁺ (data not shown) were used to activate integrins prior to initiating the binding assay. The binding to purified integrins was best-fit using a single-site binding model. The Kd observed for the binding of ATN-453 to either purified immobilized α5β₃ (Fig. 1C) or αvβ₅ (Fig. 1D) was 1.0 ± 0.2 and 0.69 ± 0.1 μmol/L, respectively, consistent with the high-affinity site observed on MDA-MB-231 cells. It should be noted that ATN-161 ultimately forms a disulfide bond with its integrin target and that the Kd represents the reversible portion of the “pulled” binding reaction.

Integrins have been implicated in cell proliferation and migration (25). Thus, we evaluated the effect of ATN-161 on MDA-MB-231 cell proliferation and migration in vitro. For the proliferation assay, cells were seeded in six-well plates and incubated in the absence or presence of 2% fetal bovine serum or 2% fetal bovine serum + different doses (1–100 μmol/L) of ATN-161, which was added daily or every other day for 5 days. Cells from triplicate wells in each group were trypsinized and counted at different time points. ATN-161 treatment up to 100 μmol/L showed no significant effect on tumor cell proliferation compared with the vehicle-treated control group of cells (Fig. 2A). Migration assays were done as described in Materials and Methods, and concentrations of ATN-161 up to 100 μmol/L were also evaluated with no observed effect (data not shown). We also evaluated the effect of ATN-161 on integrin-mediated signaling in vitro. Treatment of MDA-MB-231 cells with various doses (1–100 μmol/L) of ATN-161 for different time points (15–60 minutes) was followed by the extraction of cellular proteins and Western blot analysis using antibodies directed against FAK and P-FAK. No effect on FAK or P-FAK levels was observed (Fig. 2B). However, ATN-161 significantly inhibited MAPK phosphorylation with maximal effects observed at 20 μmol/L of ATN-161 after 30 minutes of treatment (Fig. 2B).

Effect of ATN-161 on MDA-MB-231 Tumor Growth

Despite the lack of direct antitumor cell activity in vitro, ATN-161 has shown potent antitumor activity in both s.c. and orthotopic mouse models of tumor progression...
In order to evaluate the effect of ATN-161 on MDA-MB-231 tumor growth, \(5 \times 10^5\) cells (in 20% Matrigel) were inoculated s.c. into the right flank of female BALB/c nu/nu mice. Tumors were allowed to reach a palpable stage (20–40 mm\(^3\)) approximately 4 weeks post-tumor cell inoculation. Animals were then randomized and treated with vehicle alone or with different doses of ATN-161 (i.v., 0.05–1 mg/kg/d thrice a week) for 6 weeks. Previous
studies showed that a scrambled peptide version of ATN-161, Ac-HSPNC-NH₂, was inactive in vivo and behaved identically to vehicle alone (12, 26). Thus, vehicle alone was used as a control for all the animal studies presented here. Tumor volume was determined at weekly intervals. Treatment with ATN-161 showed a dose-dependent effect in reducing tumor volume as compared with control groups of animals receiving vehicle alone (Fig. 3). Although both 0.1 and 1 mg/kg of ATN-161 had a marked effect on reducing tumor volume, no statistically significant increment in these antitumor effects were seen between doses of 0.1 and 1 mg of ATN-161. It should be noted that the plasma C_{max} of ATN-161 measured in preclinical pharmacokinetic studies in mice at a 1 mg/kg dose was ~2 to 5 μmol/L; this is high enough to bind to αⅡβ3 and αⅠβ3 in the host, as well as to the high-affinity binding site on MDA-MB-231 cells. Higher doses of ATN-161 did not improve the antitumor activity that was observed at the 1 mg/kg dose, which was subsequently selected for all future in vivo studies. Treatment of tumor-bearing animals with scrambled peptide ATN-165 had no significant effect on tumor volume (data not shown). In addition, no significant differences in animal weight were observed between control and treated groups indicating that ATN-161 treatment was well-tolerated (data not shown).

Effect of ATN-161 on MDA-MB-231 Skeletal Metastasis

Because skeletal metastasis is a common complication associated with human breast cancer, we examined the effect of ATN-161 in a xenograft model of experimental skeletal metastasis using MDA-MB-231 cells transfected with GFP. We have extensively characterized these experimental cells both in vitro and in vivo (14, 15, 19). The use of these MDA-MB-231-GFP cells allowed us to evaluate the effect of ATN-161 on tumor metastasis to both skeletal and nonskeletal sites. In these studies, MDA-MB-231-GFP cells were inoculated into the left ventricle of 5-week-old female BALB/c nu/nu mice. This route of tumor cell inoculation routinely results in the development of tumor metastasis to the long bone (tibia, femur), which can be visualized by radiologic examination (17, 19–21). From the day of tumor cell inoculation, animals were treated with vehicle alone or ATN-161 (i.v., 1 mg/kg, thrice a week) for 10 weeks. Radiologic analysis of animals treated with vehicle alone showed the presence of lesions in the tibia and femur from week 5 post-tumor cell inoculation, and the size and number of these skeletal lesions continued to increase over time. Animals in both control and experimental groups were evaluated by radiographic analysis at weekly intervals. In these studies, 10% of animals developed tumors in their heart due to the inoculation of tumor cells in the cardiac muscle and died by week 3 post-tumor cell inoculation. These animals were not included in the statistical analysis to evaluate the antitumor effects of ATN-161 (data not shown). In the control group of animals receiving vehicle alone, >90% animals (n = 22/24) showed the presence of distinct skeletal lesions at week 5, and continued to show progressive increases over time. At week 10 post-tumor cell inoculation, all control animals showed radiologic or histologic evidence of skeletal lesions. In contrast, at week 10 post-tumor cell inoculation, only 70% (n = 17/24) of the animals receiving ATN-161 (1 mg/kg/d) showed radiologic evidence of skeletal lesions. In addition, the skeletal lesion area was markedly lower in ATN-161–treated animals as compared with the control group of animals receiving vehicle alone. However, at this time point, no radiologic evidence of skeletal lesions was observed in 30% of the animals in the ATN-161–treated group. Statistical analysis of lesion in the long bones of control and ATN-161–treated groups of animals showed a significant decrease in the frequency and incidence of skeletal lesions following treatment with ATN-161 (Fig. 4A). Animals were sacrificed at week 11 and their long bones removed and subjected to further analysis by microcomputed tomography. These studies showed that animals receiving vehicle alone had significantly larger lesion areas than animals receiving ATN-161 (Fig. 4B). Following microcomputed tomography analysis, long bones were decalcified, fixed, and subjected to histologic analysis, which showed the presence of tumor cells in the bone marrow cavity and the destruction of both trabecular and cortical bone. A significant decrease in tumor volume / tissue volume ratio was also observed in the ATN-161–treated animals as compared with controls (Fig. 4C).

4 Unpublished results.
Effect of ATN-161 on Soft Tissue Metastases

The use of MDA-MB-231-GFP cells allowed us to detect the presence of tumor cells in different organs by quantifying the number of GFP-positive tumor cells. Following 10 weeks of treatment with vehicle alone or of ATN-161 (i.v., 1 mg/kg/d thrice a week), animals were sacrificed and different organs (lungs, liver, and spleen) were removed. Evaluation of these organs for the presence of microscopic tumor foci revealed the presence of disseminated GFP-positive tumor cells. The number of these metastatic tumor cells was significantly lower in animals treated with ATN-161 as compared with the cohort of animals receiving vehicle alone (Fig. 5).

Effect of ATN-161 on Tumor Vascularization and Tumor Cell Proliferation In vivo

Because integrins are known to play a major role in tumor angiogenesis, a key event in tumor metastasis, we evaluated the effect of ATN-161 on tumor vascularization. Immunohistochemical analysis of primary tumors treated with vehicle alone or ATN-161 (1 mg/kg/d) showed significantly decreased (43%) microvessel density following treatments with ATN-161 (Fig. 6A). In addition, we evaluated the effect of ATN-161 on MDA-MB-231 proliferation in vivo. Primary tumors were subjected to H&E staining and evaluated for the number of mitotic cells. These studies showed a significantly lower number of mitotic cells in experimental tumors from animals treated with ATN-161 (Fig. 6A). In order to establish greater specificity for the antiproliferative response observed in ATN-161–treated animals, tumor sections were analyzed by immunohistochemistry using an antibody directed against Ki-67. The Ki-67 immunostaining was significantly lower in tumor sections from animals treated with ATN-161 as compared with controls, confirming the mitotic index results (Fig. 6A). Immunostaining for FAK, P-FAK, MAPK, and P-MAPK showed no differences in the levels of FAK or P-FAK in tumors in vivo, but showed decreased expression of P-MAPK, consistent with the in vitro results (Fig. 6B).

Discussion

During the past decade, despite numerous advances in the identification of agents with activity in the advanced breast cancer setting, metastatic breast cancer continues to represent a huge unmet medical need. The effective treatment of tumors that metastasize to the bone will likely require targeting several compartments, including the tumor cells themselves, the neovessels that are formed to support the tumor cells, and the osteoclasts that are involved in bone remodeling and the formation of osteolytic metastatic lesions. Recent studies have implicated integrins, and specifically, α6β3 as having a causal role in the metastasis to bone, and more precisely, in the osteolytic variant that is usually observed in patients with breast cancer. In addition, breast cancer metastases induces angiogenesis locally when arrested in the bone, which may also fuel the growth of the metastatic lesions (27). Although the integrin α5β1 has not been specifically implicated in angiogenesis arising from the metastasis of breast cancer.
cells, it has been shown to be critical for tumor angiogenesis in general as well as for the metastasis of other tumor types (27–29). Thus, we hypothesized that pharmacologic targeting of both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ would lead to significant effects on the establishment and outgrowth of breast cancer metastasis in bone and in soft tissue.

In order to test this hypothesis, we used an integrin-binding peptide, ATN-161, derived from the synergy region of fibronectin, which binds to both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in vitro. The synergy region of fibronectin has been shown to interact with $\alpha_5\beta_1$ and mediates the high-affinity RGD-dependent adhesion of cells to fibronectin (30). Although several integrin binders have been described in the literature, ATN-161 is unique because it is not an RGD-based sequence and does not affect or alter cell adhesion, indicating that it may affect integrin signaling in a manner different and more subtle than the RGD-based integrin antagonists (31, 32).

Despite the fact that ATN-161 was able to bind to MDA-MB-231 cells with an affinity consistent with the binding to purified $\alpha_5\beta_1$ and $\alpha_v\beta_3$, ATN-161 did not seem to affect serum-driven MDA-MB-231 cell proliferation in vitro. At least one report has implicated $\alpha_v\beta_3$ as being important to the proliferation of MDA-MB-231 cells (33), which raises the issue of the target for ATN-161 on MDA-MB-231 cells. However, there is some controversy in the literature as to the level of expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in parental MDA-MB-231 cells. Isolated reports claim high levels of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ expression in these cells (34), whereas other studies have shown low-level expression of these integrins with increased expression observed in subclones isolated from metastatic lesions (8). In general, the expression levels of these integrins in breast cancer and other cell lines have been analyzed using semiquantitative methods such as Western blotting, immunohistochemistry, and flow cytometry. There is also a striking lack of quantitative binding data in the literature that measures the receptor copy number of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ on cells or in tumor tissues. Our binding assay does not allow the determination of the number of binding sites for ATN-161 on MDA-MB-231, making it impossible to compare the expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ observed in these cells by other investigators. Thus, the lack of an antiproliferative effect of ATN-161 on MDA-MB-231 cells in vitro could be explained by the low copy number of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ or the binding of ATN-161 to an alternate target and studies to resolve these issues are currently under way. To that end, one feature of ATN-161 that initially concerned us was the presence of a free cysteine residue that could lead to promiscuous binding to a number of cysteine or disulfide-containing proteins. However, a preliminary analysis of the binding of ATN-453 to blood cells and plasma proteins has shown.

![Figure 5. Effect of ATN-161 on MDA-MB 231-GFP breast cancer micrometastases.](image-url)

To evaluate the effect of ATN-161 on micrometastasis to various organs, female BALB/c nu/nu mice were inoculated with human breast cancer cells ($1 \times 10^5$) transfected with green fluorescent protein (MDA-231-GFP) into the left ventricle (intracardiac). Animals were treated with vehicle alone or ATN-161 (i.v., 1 mg/kg/d thrice a week) for 10 wk as described in “Materials and Methods.” Animals were sacrificed at week 11 post-tumor cell inoculation and different organs were collected. One-millimeter-thick slices of lungs, liver, and spleen were cut, placed on glass slides, and examined directly under a fluorescent microscope for the presence of GFP-expressing tumor foci. Tumor foci in 10 random fields per slide (five slides per organ) were counted to determine the average number of tumor foci in these organs. Results are representative of 12 animals in each group from two different experiments. Columns, mean of at least 12 animals in each group from two different experiments; bars, ±SE. *, $P < 0.05$, significant changes in tumor foci per field.
substantial specificity, with appreciable binding occurring only to platelets (which display αvβ3) and albumin. Additional studies attempting to isolate the target in MDA-MB-231 and endothelial cells are also under way.

Serum-starved conditions were used to isolate the effect of ATN-161 on integrin ligated cells in the absence of other pro-proliferative or pro-migratory stimuli. In these experiments, MDA-MB-231 cells are initially plated in the presence of serum, which is abundant in fibronectin and vitronectin, and these serum proteins ligate cell surface integrins in the MDA-MB-231 cells, leading to the adhesion of these cells. The cells are then transferred to serum-free conditions. Because ATN-161 does not inhibit integrin-mediated adhesion, it was not surprising that it did not affect FAK activation under the conditions of our assay. In contrast with FAK, MAPK phosphorylation was inhibited in vitro. Constitutive MAPK activation is known to occur in tumor cells and high basal levels of activated MAPK have been described, specifically, in MDA-MB-231 (35) although adhesion of these cells could also up-regulate MAPK activation (36). However, the inhibition of MAPK by ATN-161 in MDA-MB-231 tumor cells in vitro was insufficient by itself to affect the proliferation or migration of these cells, and some studies have shown that the simultaneous inhibition of both FAK and MAPK signaling is required to attenuate integrin-mediated migration or proliferation (36). In addition to low copy numbers or an alternate receptor for ATN-161, this could also explain the lack of an effect on proliferation of MDA-MB-231 cells in vitro. In contrast, ATN-161 had significant effects on the proliferation of MDA-MB-231 tumors in vivo (Fig. 6). A significant inhibition of angiogenesis was also observed in the ATN-161-treated animals, suggesting the possibility that the inhibition of proliferation observed in tumor

Figure 6. Effect of ATN-161 on vascularization, mitotic index, and integrin-mediated signaling pathway in primary MDA-MB-231 tumors. A, expression of CD31 as an index of tumor vessel density, mitotic index, and Ki-67 immunohistochemical analysis as an index of tumor cell proliferation in vivo was determined in MDA-MB-231 tumors as described on “Materials and Methods.” B, expression of FAK, P-FAK, MAPK, or P-MAPK was determined and quantified on histologic sections of MDA-MB-231 tumors from animals treated with vehicle control or ATN-161 as described in “Materials and Methods.” Quantitative analysis was carried out in 10 high-power fields in control and experimental sections (arrowheads, mitotic cells; magnification, ×400). Columns, mean of six animals in each group from two separate experiments; bars, ±SE. *, P < 0.05, significant difference from controls (right).
xenografts is indirect and results from decreased neo-
vascularization of the tumor. The indirect effects of
inhibiting angiogenesis have also been observed on
extracellular signal-regulated kinase phosphorylation in
other tumor types, raising this possibility for the MDA-MB-
231 tumors used in this study (37). This inhibition of
extracellular signal-regulated kinase phosphorylation could
account for the decreased proliferation of MDA-
MB-231 tumors because activated extracellular signal-
regulated kinase is known to mediate the proliferation of
this tumor cell line. Finally, the lack of inhibition of
proliferation of MDA-MB-231 cells treated with ATN-161
in vitro is consistent with the hypothesis that the effects of
ATN-161 on proliferation in vivo are indirect and require
the inhibition of angiogenesis.

In addition to direct effects on MDA-MB-231 cells, the
antitumor effects of ATN-161 could also be indirect
through effects on osteoclast function or angiogenesis.
ATN-161 has previously been shown to inhibit angiogen-
esis, although in that study, the inhibition of angiogenes
alone was insufficient to affect tumor growth and soft
tissue metastasis (26). ATN-161 could affect endothelial
cells directly or angiogenesis indirectly. For example, the
inhibition of MAPK activation in MDA-MB-231 cells has
been shown to down-regulate vascular endothelial growth
factor expression, which would be expected to attenuate
angiogenesis (38). Direct inhibitors of osteoclast function
have also been shown to inhibit the formation of bone
metastasis (39, 40). Consistent with the lack of promiscuous
binding described above, a significant amount of ATN-161
(~ 40%), which was not distributed to the tissue, remains
free in plasma even 2 hours after i.v. injection and is
presumably available for binding to its target in a tumor. 4

A recently completed phase I study of ATN-161 showed
that at doses >0.5 mg/kg in man, a Cmax of ~10 μmol/L or
greater can be achieved, which exceeds the in vitro IC50 for
integrin binding, indicating that it is possible to reach a
sufficiently high enough concentration of the peptide in
plasma to nearly saturate its targets (41). Furthermore,
ATN-161 antitumor activity has been described in several
in vivo studies, confirming that sufficient peptide is able to
remain free to elicit an antitumor effect (12, 13). Thus, we
evaluated whether ATN-161 could inhibit MDA-MB-231
growth and metastasis in nude mice. ATN-161 significantly
inhibited the growth of primary MDA-MB-231 tumors.
Immunohistochemical analysis of sections obtained from
these tumors indicated that MAPK activation was
inhibited, whereas FAK was not, consistent with the
in vitro results. In addition, there was a significant decrease in
the proliferative index measured by Ki-67 immunostain-
ing in the tumors from ATN-161–treated animals. Because
the inhibition of MAPK activation in vitro was insufficient
to directly inhibit the proliferation of these cells, it is likely
that the inhibition of angiogenesis observed using CD31
immunostaining contributed to the inhibition of prolifera-
tion observed in vivo, and that a combined inhibition of
angiogenesis/tumor cell MAPK activation resulted in the
observed antitumor effects. Despite the inhibition of
proliferation observed in vivo, there did not seem to be
any effect on cell survival as no difference in terminal
nucleotidyl transferase (TdT)–mediated nick end labeling
staining was observed between the treated and untreated
groups (data not shown).

The observation of antitumor activity in s.c. MDA-MB-
231 tumors was extended in a model of MDA-MB-231
experimental metastasis. Inoculation of tumor cells via the
intracardiac route results in metastasis to the bone, as well
as to soft tissue, and ATN-161 significantly inhibited the
outgrowth of metastases at all metastatic sites. In some
animals, ATN-161 completely inhibited the formation of
bone metastases in a model which is historically refractory
to systemic antitumor treatments. This observation was
extremely encouraging in that very few agents, especially
noncytotoxic agents, have any effect on the formation of
metastatic lesions in the bone. The results presented herein,
combined with previously published results of the syner-
gistic activity of ATN-161 with chemotherapy (12), provide
a basis for future studies of ATN-161 in breast cancer
models in combination with chemotherapeutic agents such
as docetaxel. The demonstration of enhanced antitumor
activity against bone metastasis with a combination
treatment of ATN-161 and chemotherapy would provide
the rationale for developing ATN-161 combinations for the
treatment of patients with advanced breast cancer.

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function with a small peptide (ATN-161) plus continuous 5-FU infusion


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