Pharmacology of the Novel Antiangiogenic Peptide ATN-161 (Ac-PHSCN-NH₂): Observation of a U-Shaped Dose-Response Curve in Several Preclinical Models of Angiogenesis and Tumor Growth

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Abstract

Purpose: ATN-161 (Ac-PHSCN-NH₂) is an integrin-binding peptide that is currently in phase II trials in cancer patients. This peptide has been shown to have antitumor activity in a number of preclinical models.

Experimental Design: In this study, we examined the binding, biodistribution, and dose and biomarker response of ATN-161 in several animal models.

Results: ATN-161 bound to the β subunit of a number of different integrins implicated in tumor growth and progression, which depended on its cysteine thiol. The peptide had antiangiogenic activity in the Matrigel plug model, and this activity could be reversed by inhibitors of protein kinase A, an effector of α5β1-dependent angiogenesis. A labeled analogue of ATN-161, ATN-453, localized to neovessels but not to preexisting vasculature in vivo. The half-life of the peptide when localized to a tumor was much longer than in plasma. Dose-response studies in the Matrigel plug model of angiogenesis or a Lewis lung carcinoma model of tumor growth showed a U-shaped dose-response curve with 1 to 10 mg/kg given thrice a week, being the optimal dose range of ATN-161. Two additional pharmacodynamic models of angiogenesis (dynamic contrast-enhanced magnetic resonance imaging and measurement of endothelial cell progenitors) also revealed U-shaped dose-response curves.

Conclusions: The presence of a U-shaped dose-response curve presents a significant challenge to identifying a biologically active dose of ATN-161. However, the identification of biomarkers of angiogenesis that also exhibit this same U-shaped response should allow the translation of those biomarkers to the clinic, allowing them to be used to identify the active dose of ATN-161 in phase II studies.

ATN-161 is a five–amino acid peptide that binds to several integrins, including α5β1 and αvβ3, that play a role in angiogenesis and tumor progression (1). It has been shown to inhibit tumor growth and metastasis and extend survival in multiple animal tumor models either when given as a single agent or when combined with chemotherapy (2, 3). ATN-161 has a large therapeutic index, with antitumor effects observed in multiple animal tumor models either when given as a single agent or when combined with chemotherapy (2, 3). ATN-161 is derived from the synergy region (PHSRN) of human fibronectin, in which a cysteine residue replaces an arginine in the original sequence (PHSCN). Fibronectin is an extracellular matrix protein that has been implicated in tumor angiogenesis and metastasis (6, 7). The synergy region of fibronectin has been proposed to interact with the α5β1 integrin and to increase the avidity of this integrin for the arginine-glycine-aspartate (RGD) adhesion sequence in the 10th type III repeat of fibronectin (8). Integrins, through their interaction with extracellular matrix proteins, mediate the migration and survival of tumor cells and angiogenic endothelial cells. To investigate the binding of ATN-161 to integrins, we generated a series of analogues of ATN-161. One of these analogues, ATN-453 (Ac-PHSCNGGK-biotin), has been extended by several amino acids and contains a biotin moiety allowing for the detection of bound ATN-453. The binding of ATN-453 to tumor cells and purified α5β1 and αvβ3 has previously been described (1) and is indistinguishable from ATN-161. In this study, we extend these results to endothelial cells and present data on how this peptide interacts with integrins at the molecular level. In addition, we present data from four different studies evaluating various biomarkers of ATN-161 activity demonstrating that ATN-161 exhibits a U-shaped dose-response curve and is more active at lower doses than at higher...
doses. A recently completed phase I clinical trial evaluating ATN-161 in patients with advanced solid tumors showed a number of patients with prolonged stable disease across a broad range of doses, consistent with a U-shaped dose-response curve (9). In agreement with the preclinical data, a MTD was not achieved. Thus, the results of that phase I study have made establishing a dose to move forward within phase II extremely challenging. The results of this current study identify two biomarkers that might be useful for confirming an active dose in human patients and further define the U-shaped dose-response curve profile of ATN-161.

Materials and Methods

Cell lines and reagents. Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC) were purchased from Cambrex. HT-29 and Lewis lung carcinoma (3LL) cells were purchased from American Type Culture Collection. MDA-MB-231 cell line was kindly provided by Dr. Janet E. Price (M. D. Anderson Cancer Center University of Texas). ATN-161 (Ac-PHSCNGGK-NH₂) was manufactured using solution phase methodologies under cyclic guanosine 3′,5′-monophosphate by Peptisyntha. ATN-453 (Ac-PHSCNGGK-NH₂-biotin), a biotinylated analogue of ATN-161 that had previously been shown to retain the full binding activity of ATN-161 (1), was synthesized using solid phase methods (Pepsysyntha). Purified integrins and antiintegrin antibodies, including P1D6 and AB1958 (anti-αv), CBL479 (anti-β3), 2251 (epitope amino acids 657-670 of β1), 2252 (epitope amino acids 15-54 of β1), and 1952 (anti-β3) were purchased from Chemicon, and goat anti-mouse horseradish peroxidase antibody was from Fisher. Matrigel was purchased from BD Biosciences.

In vitro binding assays. We have previously shown the direct binding of ATN-453 to purified αvβ3 and αvβ5, as well as to MDA-MB-231 cells (1). The binding of ATN-453 could be competed with ATN-161 with an IC₅₀ that was similar to the Kd measured in the direct binding assay. Similar methods were used to show the binding of ATN-453 to HUVECs and HMVECs. Briefly, endothelial cells were grown to ~70% confluence and harvested using trypsin. After neutralization of trypsin activity in media containing 10% FCS, cells were pelleted by centrifugation and washed twice in ice-cold binding buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.1% FCS, and 2 mmol/L MnCl₂] before being resuspended at 1 × 10⁶ cells/mL. ATN-453 was added to 1 × 10⁶ cells at a final concentration of 2 μmol/L with and without a 50-fold molar excess of competitor peptide. In some experiments, antibodies (final concentration, 10 μg/mL) specific for various integrins and integrin subunits were included as competitors. After incubation for 2 h at 4°C, cells were pelleted by centrifugation at 13,000 rpm for 30 s and washed thrice with 1 mL of binding buffer. Cells were incubated for an additional 30 min at 4°C in binding buffer containing streptavidin–horseradish peroxidase (Southern Biotech). After washing as described above, cells were incubated with 100 μL of horseradish peroxidase substrate (SIGMA FAST OPD tablets, Sigma-Aldrich) for 10 min, and the reaction was stopped by the addition of 100 μL H₂SO₄. Supernatants were transferred to 96-well plates, and the absorbance at A490 nm was recorded.

Binding to purified integrins and localization of ATN-453 to a particular integrin subunit was carried out as follows. αvβ3-integrin (Chemicon; 0.2 μmol/L), ATN-453 (10 μmol/L), and 2 mmol/L MnCl₂ in 50 mmol/L HBS were incubated at room temperature for 2 h Samples were then resolved under nonreducing conditions on a 4% to 12% NuPAGE Bis-Tris gel, then transferred to a polyvinylidene difluoride membrane by Western blot, and probed with Streptavidin–horseradish peroxidase to identify the ATN-453–bound band. Separate blots were also probed with either an anti-αv, an anti-β3, or an anti-β3 antibody followed by the appropriate secondary antibody to identify the subunit that interacts with ATN-453. Alternatively, purified αvβ3 was incubated with anti-β3 antibodies followed by incubation with ATN-453, as previously described (1). Under nonreducing conditions, the αv subunit runs at ~160 kDa and β3 at ~110 kDa.

Pharmacokinetic studies of mouse plasma and tumor tissue. Pharmacokinetic studies of mouse plasma using LC/MS/MS were done using a validated method identical to the method previously reported for human plasma (9), with a modification that allowed the measurement of free ATN-161 in addition to total ATN-161. Briefly, mice were injected with ATN-161 (1, 10, and 50 mg/kg) and blood collected by terminal bleed at different time points in tubes containing citrate to prevent coagulation and Tris (2-carboxy-ethyl)-phosphine–HCl to release any protein-bound ATN-161. After preparation of plasma, samples were stored frozen at -20°C until analysis for total ATN-161 by LC/MS/MS as previously described (9). To determine free ATN-161, samples were treated in a similar manner but without pretreatment with Tris (2-carboxy-ethyl)-phosphine–HCl.

Matrigel plug angiogenesis model. Matrigel plugs were carried out as previously described (10). Briefly, Matrigel (500 μL) on ice was mixed with heparin (50 mg/mL), fibroblast growth factor 2 (FGF-2; 800 ng/mL), and vascular endothelial growth factor (VEGF; 300 ng/mL). The compounds to be tested were added either directly to the plug or injected i.v. The Matrigel mixture was injected s.c. into a 4-wk-old to 8-wk-old female BALB/c nude mice. Animals were sacrificed, and the plugs recovered 5 d postinjection. Quantitation of the level of angiogenesis after plug removal was performed by either measuring the hemoglobin content or fluorescence measurements after injecting 100 μL of 2 mg/mL FITC-dextran solution (Sigma). In some experiments, protein kinase inhibitors HA1004 or KT5720 were also added to the Matrigel plugs. These inhibitors were prepared as 10 mmol/L stock solutions in DMSO and then diluted at least 1:1,000 into Matrigel. The small amount of DMSO cosolvent had no effect on its own in any of the control studies.

Fluorescence detection of ATN-453 biodistribution. Mice were inoculated with 1 × 10⁶ 3LL cells in Matrigel (0.5 mL). After 5 d, the mice were injected with either 25 μg of ATN-453 or saline via tail vein. After 2 h to clear nonbound peptide, the animals were sacrificed and the tumors were retrieved. The tumors were placed in zinc fixative for 24 h, and 4 μm paraffin-embedded sections were prepared. Slides were deparaffinized in xylene, rehydrated, and blocked in 1% bovine serum albumin/3% goat serum for 30 min. The slides were then incubated with a rat monoclonal antibody against CD31 (BD Biosciences) at 1:50 dilution in PBS followed by a goat anti-rat IgG–rhodamine–conjugated antibody (BD Biosciences) at 1:500 dilution in PBS. An antibod y mouse monoclonal FITC-conjugated antibody (Sigma) at 1:500 dilution in PBS was used for ATN-453 detection. For the time-based biodistribution studies, mice were injected with 25 μg of ATN-453 and tumors were harvested at 0.5, 1, 2, 4, and 24 h. Samples were processed as indicated above.

Tumor model for evaluating ATN-161 dose-response curve. A syngeneic 3LL (Lewis lung adenocarcinoma) model in C57/bl mice was used to assess the effect of ATN-161 on tumor growth, as previously described (11). ATN-161 dose-response curve was assessed over the range of ATN-161 doses from 0.2 to 200 mg/kg given i.v. by tail vein injection thrice a week. Growth rate was monitored by twice weekly caliper measurements, and a final tumor volume was measured just before euthanasia and used to calculate treated versus control. For circulating endothelial progenitor (CEP) studies, 2 × 10⁶ MDA-MB-231 cells were inoculated into the inguinal mammary fat pad of anesthetized 6-wk-old to 8-wk-old CB-17 SCID mice (Charles River). Wounds were secured with surgical staples. When tumors reached 200 mm³, mice were treated with escalating doses of ATN-161 for a 1-wk period.

Evaluation of circulating endothelial cells and CEPs by flow cytometry. Blood was obtained from anesthetized mice by either cardiac puncture or retro-orbital sinus bleeding. Viable CEPs were counted using four-color flow cytometry, as described previously.
Briefly, monoclonal antibodies specific for CD45 were used to exclude CD45+ hematopoietic cells, and circulating endothelial cells (CECs) were detected using the murine endothelial markers fetal liver kinase 1/flk-1/VEGF receptor 2 and CD13 (aminopeptidase N). CECs were defined as CECs, which were also positive to the CD117 (c-kit) surface marker (BD PharMingen, BD Biosciences; refs. 14, 15). Nuclear staining (Procount, BD Biosciences) was used in some experiments to exclude platelets or cellular debris (14, 16). After red cell lysis, cell suspensions were analyzed on a FACSCalibur II (BD Biosciences). After acquisition of at least 100,000 cells per sample, analyses were considered informative when an adequate number of events (i.e., >25, typically 50-150) were collected in the CEC/CEP enumeration gates in untreated control animals. Percentages of stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining, and 7-aminoactinomycin D was used to distinguish apoptotic and dead cells from viable cells (17).

Small animal dynamic contrast-enhanced magnetic resonance studies in tumor bearing mice. Male scid mice were inoculated with 5 × 10⁵ HT-29 human colon cancer cells in the right hind flank, an injection site that was chosen to minimize motion artifacts during magnetic resonance imaging (MRI) data acquisition. Palpable tumors developed in ~10 d and reached a convenient size for imaging (~200 mm³) in ~3 wk. Mice were given a dynamic contrast enhanced (DCE)-MRI scan, treated immediately with either 1 mg/kg ATN-161 dissolved in a citrate/glycine buffer, vehicle, or a dose of 50 mg/kg and then given a second DCE-MRI scan 3 h later 0.2 mL of Magnevist, diluted 20:1, given via a 27-gauge butterfly needle that had been inserted into the tail vein before imaging and held in place with removable cyanoacrylate veterinary adhesive (Vetbond, Penn Veterinary Supplies).

The scanning protocol consisted of the acquisition of a set of scout scans and a T₁-weighed scan (Tₑ = 1,200 ms, Tᵣ = 26 ms) to define the position of the tumor unambiguously and followed by an injection of the small molecule contrast agent Gd-DTPA The DCE-MRI scans consisted of a series of T₁-weighed gradient echo images. Image sets consisting of eight contiguous slices were acquired with a multislice gradient echo pulse sequence with the following scan variables: repetition time (Tᵣ) = 100 ms, echo time (Tₑ) = 3.3 ms, 90°
A, ATN-161 inhibits angiogenesis in a Matrigel plug model. Different concentrations of ATN-161, VEGF (300 ng/mL), and FGF-2 (800 ng/mL) were added directly to the Matrigel as described in Materials and Methods. ATN-161 showed a dose-dependent inhibition of angiogenesis that was statistically significant at concentrations of 1 and 10 μmol/L, respectively (**, P = 0.0017; *** , P = 0.0004). B, the PKA inhibitor HA1004 reverses the inhibition of angiogenesis by ATN-161. HA1004 was dissolved in DMSO as a 10 mmol/L stock and then diluted into the Matrigel to a final concentration of 5 μmol/L, along with ATN-161, FGF-2, and VEGF. The model was carried out as described in Materials and Methods (**, P = 0.0001); C, the PKA inhibitor KT5720 reverses the inhibition of angiogenesis by ATN-161. KT5720 was dissolved in DMSO and then diluted into the Matrigel to a final concentration of 10 μmol/L, along with ATN-161, FGF-2, and VEGF. The model was carried out as described in Materials and Methods (*, P = 0.03).

Results

ATN-453 binds to endothelial cells. We previously showed that ATN-453 could bind to purified α5β1 and αvβ3, as well as to tumor cells expressing these integrins (1). In this study, we evaluated the ability of ATN-453 to bind to venous (HUVEC) and microvascular (HMVEC) endothelial cells. ATN-453 bound to both cell types and this binding could be competed by a 50-fold molar excess of ATN-161 (Fig. 1A and B). To identify the integrin subunit and binding site for ATN-453, several subunit-specific antibodies were used. Antibody 2252, which recognizes the activated conformation of the β1 subunit and binds an epitope between amino acids 15 to 54, could inhibit the binding of ATN-161, whereas an antibody to a different epitope in β1 (antibody 2251) or to the α5 subunit (antibody P1D6) of α5β1 had no effect on binding (Fig. 1C). The binding of ATN-453 to the β1 subunit of purified α5β1 was observed by Western blotting (110 kDa; Fig. 1D). A small amount of nonspecific binding is observed to the α5 subunit (160 kDa), but the predominant interaction of ATN-453 with α5β1 seems to be with the β1 subunit. Similarly, the binding of ATN-453 to the β3 and β5 subunits of αvβ3 and αvβ5, respectively, could be shown directly using antibodies specific for β3 or αvβ5 (data not shown). The binding of ATN-453 to these integrin subunits could only be observed under nonreducing conditions and reduction with β-mercaptoethanol before SDS-PAGE abolished this interaction (data not shown). However, despite the fact that ATN-161 seems to bind to integrins, it does not affect cell attachment to a variety of matrix proteins.

Inhibition of angiogenesis and localization to neovessels in vivo. The ability of ATN-161 to inhibit angiogenesis in vivo was investigated using the Matrigel plug model of angiogenesis (10). In the first study, ATN-161 at different concentrations was added directly to the Matrigel using FGF-2 and VEGF as angiogenesis inducers, as described in the Materials and Methods. In this study, ATN-161 was able to inhibit angiogenesis in a dose-dependent manner (Fig. 2A). Recently, Kim et al. showed that antagonists of integrin α5β1 activate protein kinase A (PKA) leading to the induction of apoptosis and the inhibition of angiogenesis (18). Conversely, a cell permeable inhibitor of PKA, HA1004, substantially suppressed the apoptosis induced by α5β3, integrin antagonists. Because ATN-161 binds to integrins in vitro, including α5β1, we tested the hypothesis that ATN-161 inhibited angiogenesis via a PKA-mediated pathway, as described above. HA1004 did not affect angiogenesis in the Matrigel plug when tested alone (data not shown). However, it did reverse the antiangiogenic activity of ATN-161, indicating that at least part of the antiangiogenic activity of ATN-161 was being mediated by PKA (Fig. 2B). Similar results were obtained using another PKA inhibitor (KT5720; Fig. 2C). These results support the hypothesis that the antiangiogenic activity of ATN-161 may be mediated by modulating integrin signaling in a PKA-dependent manner.
ATN-453 was then used to evaluate the biodistribution of ATN-161 binding sites in a Matrigel tumor model. In addition to binding to integrins and cells in a manner that was indistinguishable from ATN-161, ATN-453 had similar activity to ATN-161 in the Matrigel plug model, demonstrating that it behaves analogously to ATN-161 (data not shown). In this study, 3LL cells were added to the Matrigel in lieu of FGF-2 and VEGF, as described in the Materials and Methods. ATN-453 bound only to neovessels in the tumor (Fig. 3A) and did not bind to any appreciable extent to vasculature or other cell types in any other organ (Fig. 3B). ATN-453 binding to neovessels in the tumor could still be observed as long as 24 hours after a single injection of the peptide (data not shown). This long-lived association with neovessels may underlie the efficacy of ATN-161 when used in a thrice-a-week schedule in animal tumor models, and more frequent dosing does not improve the antitumor activity despite the fact that ATN-161 is a peptide with a fairly short plasma half-life. To further investigate this, we compared the plasma pharmacokinetics of ATN-161 to its tumor pharmacokinetics. Despite the rapid clearance of ATN-161 from the plasma, ATN-161 was cleared from the tumor with much slower kinetics supporting the hypothesis that this peptide exerts its effects through a durable interaction with its target(s) in the tumor (Fig. 3C).

**ATN-161 exhibits a U-shaped dose-response curve in models of angiogenesis and tumor growth in vivo.** The dose-response curve of ATN-161 was evaluated in several in vivo models. In the Matrigel plug model of angiogenesis, ATN-161 given i.v. potently inhibited neovessel formation (Fig. 4A). The optimal systemic antiangiogenic dose range was 1 to 10 mg/kg, and the dose-response curve exhibited a U-shaped (or bell-shaped) curve, with doses above 50 mg/kg or below 0.2 mg/kg, having little or no effect in this model. The dose-response curve in a tumor model was also evaluated using the 3LL lung adenocarcinoma model when a similar dose-response curve and optimal dose range were observed (Fig. 4B). The U-shaped dose-response curve was confirmed in several other tumor models, including HT29 colon carcinoma, B16 melanoma, PC-3 prostate, and MDA-MB-231 breast cancer models (data not shown).

Two biomarkers of angiogenesis exhibit a U-shaped dose-response curve to ATN-161. During the preclinical development of ATN-161, the effects of this peptide on two biomarkers of angiogenesis, such as CEPs and blood flow measured by DCE-MRI, were studied. At optimal doses, ATN-161 decreased the number of viable CEPs (Fig. 4C) and viable CECs (Fig. 4D) in tumor-bearing mice. The ATN-161 dose-response curve followed a U-shape for both cell types, similar to what was observed in the Matrigel plug and 3LL models. A similar U-shaped dose-response curve was also observed in nontumor bearing animals (data not shown). Finally, when blood flow and tumor perfusion were evaluated in the tumors of tumor-bearing mice using DCE-MRI, a lower dose of ATN-161 had significant effects on blood flow whereas a higher dose had no effect (Table 1). Although a full dose-response curve was not evaluated in this study, the fact that a lower dose worked better than a higher dose also implies a U-shaped dose effect.

**Discussion**

We have previously described the activity of a novel peptide cancer therapeutic agent, ATN-161, which bound to purified integrins $\alpha_5\beta_1$ and $\alpha_6\beta_3$ and inhibited tumor growth in vivo (1). In this study, we further characterize the biological activity of ATN-161, which is currently advancing into phase II testing in patients with glioma. ATN-161 binds to the $\beta$ subunits of several integrin heterodimers, including $\alpha_5\beta_1$, $\alpha_6\beta_3$, and $\alpha_6\beta_2$. The interaction of ATN-161 with these integrins potentially localizes ATN-161 to neovessels and inhibits angiogenesis.
This inhibition of angiogenesis, as well the antitumor activity of ATN-161, both display U-shaped dose-response curves (the antiangiogenic activity may in fact underlie the antitumor effects observed with ATN-161 in vivo), making the identification of a biologically optimal dose of this peptide in patients particularly challenging in light of the fact that an MTD was not reached in the phase I clinical evaluation of ATN-161 (9).

Competition binding experiments revealed that antibody 2252, specific for the activated integrin β1 subunit, inhibited binding of ATN-453 to HUVEC and HMVEC. Previous studies using the integrin β3 subunit have revealed that, upon integrin activation, a conformational change that involves the formation of a long-range disulfide bond between Cys5 and Cys453 in cysteine-rich repeat 1 of the β3 subunit occurs (19, 20). Similarly, the epitope recognized by antibody 2252 is revealed upon integrin activation, and we hypothesize that this too is due to the formation of a long-range disulfide bond between a cysteine residue in or close to the antibody 2252 epitope and the cysteine-rich repeat 1 of β1. Indeed, there is significant homology between these regions of the β1 and β3 integrin subunits, and a homologous sequence is also present in β1 and β3 (Table 2). Thus, because ATN-161/ATN-453 bind to integrin β subunits and contain a free cysteine and binding can be disrupted by reduction, we hypothesize that ATN-161 may be exerting its activity by binding to this epitope via a disulfide bond and somehow perturbing the activation of the integrin. Additional studies to further characterize the interaction of ATN-161 with integrin β subunits will be required to further investigate this hypothesis.

The concept of a U-shaped or bell-shaped dose-response curve, also called hormesis, is not novel in biology and medicine. Numerous agents, including chemotherapeutics (21, 22), IFN (23–25), other cytokines (26), and most recently rosiglitazone (27) and endostatin (28), have all displayed a U-shaped dose-response curve in both clinical and preclinical studies. Even the recently approved anti-VEGF therapy, bevacizumab, has shown greater activity at lower doses of ATN-161/ATN-453 to HUVEC and HMVEC. Each dose was tested in duplicate, and the experiment was repeated thrice with similar results. The curve presented is representative of these three experiments.

ATN-161 exhibits a U-shaped dose-response curve in the percentage of viable CECs in MDA-MB-231 tumor-bearing mice.

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**Fig. 4.** ATN-161 exhibits a U-shaped dose-response curve. A, dose-response curve obtained in the Matrigel plug model of angiogenesis. The Matrigel plug model was used to evaluate the antiangiogenic effects of ATN-161 given systemically (i.v.) at different doses of peptide (0.025-150 mg/kg), as described in Materials and Methods. Each dose was tested in duplicate, and the experiment was repeated thrice with similar results. The curve presented is representative of these three experiments. B, dose-response curve obtained in the 3LL model of s.c. tumor growth. 3LL cells (1 × 10^6 cells in 100 μL) were injected s.c. in C57/bl mice, and treatment with different doses of ATN-161 was initiated the day after tumor cell inoculation as described in Materials and Methods. Tumor volumes were measured just before euthanasia, and the ratio of tumor volume in treated mice to tumor volume in control mice was calculated to determine treated versus control; **, *P* = 0.001 (dose at 1 mg/kg compared with 0.2 mg/kg). C, ATN-161 exhibits a U-shaped dose-response curve in the percentage of viable CEPs. The optimal dose range is between 1 and 10 mg/kg, consistent with the results observed in the angiogenesis and tumor growth models. D, ATN-161 exhibits a U-shaped dose-response curve in the percentage of viable CECs in MDA-MB-231 tumor-bearing mice.
doses compared with higher doses against some tumor types (29). The hypothesized mechanisms for these U-shaped dose-response curves may be numerous. A trivial explanation for the U-shaped dose-response curve is that ATN-161 dimerizes or forms a complex with free thiol-containing proteins in plasma, such as albumin. However, pharmacokinetic experiments showed no dose dependence to the extent of ATN-161–protein complex formation, and ATN-161 dimer was not detected in any large extent in vitro or in vivo. Biochemical reasons for the U-shaped response include crossover from antagonist to agonist due to presence of a second lower affinity receptor for a particular ligand, down-regulation of receptors for a particular ligand after exposure to saturating concentrations of that ligand, and up-regulation of clearance mechanisms at high concentrations of ligand that feedback to modulate the activity of the ligand, e.g., the induction of SOCS proteins that negatively regulate cytokine activity (30).

From a pragmatic standpoint, compounds that exhibit a U-shaped dose-response curve, such as ATN-161, present a unique challenge to clinical development, as previously stated. Because of the presence of this U-shaped response, pushing dosing to MTD is not desirable and likely does not correlate with the activity of ATN-161. Thus, other strategies must be used to identify an optimal biological dose of this peptide to take forward into phase II trials. In addition, despite the challenges presented to identifying the optimal biological dose in patients, the presence of a U-shaped response may be advantageous in that it is unlikely that severe toxicities will be encountered at the biologically active dose, because this dose will likely be substantially lower than the MTD. Similarly, oncology compounds that have been used historically at MTD, such as cyclophosphamide and paclitaxel, are showing more robust antitumor activity when given more frequently, but at a sub-MTD dose, and early data suggest that biomarkers, such as viable CECs, are extremely useful in confirming the biologically active dose of these agents and furthermore seem to correlate with clinical benefit (32). Thus, the identification of biomarkers that can guide dose finding studies in the absence of pushing the dose to MTD is currently an area of intense discovery in oncology drug development.

In this study, we have identified several biomarkers of angiogenesis that also adhere to the same U-shaped dose-response curve observed in studies evaluating the antiangiogenic and antitumor effects of ATN-161. These pharmacodynamic markers can be followed as a way of identifying a biologically active dose of this peptide in cancer patients. ATN-161 showed the ability to alter blood flow in a tumor in the HT29 colon carcinoma model, which is one indicator of antiangiogenic activity. Methods for evaluating angiogenesis in patients using DCE-MRI may have become standardized in the past few years, and this approach has been used as a biomarker of angiogenesis for the early evaluation of antiangiogenic drugs in early clinical studies, including compounds, such as Canto 95 (33), PTK787 (34), bevacizumab (35), and others. Similarly, based on the data presented herein, DCE-MRI may have utility in establishing the biologically active dose of ATN-161 in cancer patients as well.

The measurement of viable CECs and CEPs as biomarkers of antiangiogenic activity is also gaining traction in oncology drug development. These cells have been shown to have utility in identifying the biologically active dose and schedule for various antiangiogenic compounds in preclinical models (12–15), as well as in cancer patients (16). In our study, simple quantitation of total CEP or CEC numbers did not correlate with the dose-response curve to ATN-161 in either normal or tumor-bearing mice. However, when CEPs and CECs were segregated based on whether or not they were viable, the percentage of viable CEPs, as well as CECs, clearly correlated with the ATN-161 U-shaped dose-response curve. Thus, CEPs and CECs represent another potential biomarker for ATN-161.

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<th>Group</th>
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<th>P</th>
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Table 2. Alignment of different human β subunits

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<td>Human integrin β6</td>
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NOTE: Five tumor-bearing animals were studied per group, with an average tumor volume of 178 mm³. There were no significant differences in the average tumor volumes between groups. There was also no significant difference between the area under the curve posttreatment/pretreatment ratios between the vehicle and high dose groups (P = 0.97).

Abbreviation: AUC, area under the curve.

†Compared with the 1 mg/kg dose with Student’s t test.

5 A. Mazar, personal communication.
that can be measured in cancer patients to establish the biologically active dose of ATN-161.

Taken together, the data presented in this study show that ATN-161 exhibits a U-shaped dose-response curve and that several pharmacodynamic biomarkers of its activity have been identified in preclinical studies. These biomarkers are in the process of being translated to the clinic and will be used to establish a biologically active dose of ATN-161 in phase II studies that will then be advanced for future trials in cancer patients in combination with chemotherapy. The tissue selectivity and concomitant lack of toxicity observed thus far in the development of ATN-161 may make it especially useful in combination regimens where overlapping toxicities are often a major concern.

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