Angiogenin was originally isolated from the conditioned-medium of HT-29 human colon adenocarcinoma cells as a tumor angiogenic protein (1). Its expression is up-regulated in a variety of cancer cells and its concentration in plasma is elevated in many types of cancer patients (2–11). Angiogenin antagonists including monoclonal antibodies (mAb; ref. 12), soluble binding proteins (13), and small molecules (14) have been shown to inhibit the growth of human tumor cells in athymic mice, accompanied with a marked decrease in tumor angiogenesis. Angiogenin is rapidly translocated to the nucleus of endothelial cells (15, 16) where it binds to the promoter region of the rRNA gene (17) and stimulates rRNA transcription (18), a rate-limiting step in the process of ribosome biogenesis (19). Nuclear translocation of angiogenin in endothelial cells is essential for its angiogenic activity (15). It has been shown that angiogenin variants with an altered nuclear localization sequence do not undergo nuclear translocation and are not angiogenic (15), even though their other properties, including the ability to bind to the endothelial cell surface, are unchanged. Nuclear accumulation of angiogenin is required for cell proliferation and angiogenesis induced by a variety of other angiogenic molecules including acidic and basic fibroblast growth factors, vascular endothelial growth factor, and epidermal growth factor (20).

We have previously shown that the aminoglycoside antibiotic neomycin blocks the nuclear translocation of angiogenin and inhibits its angiogenic activity. However, neomycin has not been considered as a favorable drug candidate for clinical development because of its known nephrotoxicity and ototoxicity. The aim of this study is to determine whether neamine, a nontoxic derivative of neomycin, possesses antitumor activity. Experimental Design: The effect of neamine on the nuclear translocation of angiogenin was examined by means of immunofluorescence and Western blotting. The antitumor activity of neamine was determined with three different animal models.

Results: Neamine effectively blocked the nuclear translocation of angiogenin in endothelial cells and inhibited angiogenin-induced cell proliferation. It inhibited the establishment of human tumor xenografts in athymic mice in both ectopic and orthotopic tumor models. It also inhibited the progression of established human tumor transplants, whereas the structurally related antibiotic paromomycin had no effect. Immunohistochemical staining showed that both angiogenesis and cancer cell proliferation are inhibited by neamine.

Conclusion: These results suggest that the nontoxic aminoglycoside antibiotic neamine is an effective inhibitor of nuclear translocation of angiogenin and may serve as an inhibitor for angiogenin-induced angiogenesis and cancer progression.
Materials and Methods

Cell culture. Human umbilical vein endothelial cells (HUVEC) were cultured in human endothelial-SFM basal growth medium (HEM) supplemented with 5% fetal bovine serum and 5 ng/mL of basic fibroblast growth factor. The culture medium was changed every 2 days and the cells were subcultured upon confluence with a 1:3 splitting ratio. Cells between passages three and nine were used in this study.

HT-29 human colon adenocarcinoma cells, MDA-MB-435 human breast carcinoma cells, and A431 human epidermoid carcinoma cells were cultured in DMEM + 10% fetal bovine serum. Cell viability was determined by the trypan blue exclusion method. Cell number was determined with a Coulter counter.

Preparation of neamine. Neamine was prepared from neomycin by methanolysis (25). Briefly, 5 g of neomycin sulfate was dissolved in 600 mL of methanol and 19 mL of concentrated HCl. The mixture was refluxed for 4 hours and cooled in an ice bath. Anhydrous ether, 200 mL, was added to precipitate neamine. The precipitate was collected on a sintered glass filter (fine pore size), washed twice with 10 mL of ether and dried under vacuum over P2O5. Typically, 2.2 g of neamine was obtained from 5 g of neomycin. The purity of neamine was determined by TLC on silica gel with a developing solvent containing 50% n-butanol, 25% acetic acid, and 25% H2O (26).

Nuclear translocation of angiogenin. HUVECs were seeded at a density of 5 x 10^3 cells/cm^2 on a coverslip placed in a 35 mm culture dish. The cells were cultured in HEM + 5% fetal bovine serum + 5 ng/mL basic fibroblast growth factor for 24 hours, washed thrice with serum-free HEM, and incubated with 1 μg/mL angiogenin at 37°C for 1 hour. Test compounds were added 10 minutes prior to the addition of angiogenin. At the end of the incubation, cells were washed thrice with PBS and fixed with methanol at -20°C for 10 minutes. The fixed cells were blocked with 30 μg/mL bovine serum albumin in PBS and incubated with 30 μg/mL of antiangiogenin mAb 26-2F for 1 hour, washed thrice and incubated with Alexa 488-labeled goat F(ab')2, anti-mouse IgG at a 1:250 dilution for 1 hour. The cells were then washed, mounted in 50% glycerol and examined with a Nikon Labphot epifluorescent microscope (27).

Cell proliferation. HUVECs were seeded at 4 x 10^4 cells/cm^2 in attachment factor–coated 35 mm dishes in serum-free HEM, and incubated with 1 μg/mL angiogenin in the presence or absence of test compounds at 37°C for 48 hours. Cells were detached by trypsinization and counted with a Coulter counter.

Ectopic growth of human cancer cells in athymic mice. HT-29 cells, 1.25 x 10^5 per mouse, were injected s.c. into the left shoulder of male athymic mice. The animals were treated with local s.c. injections of PBS (control), neomycin, or neamine daily for 20 days, and then every other day until day 56. The dosages of neomycin and neamine were 60 and 30 mg/kg, respectively (roughly equivalent molar terms: the molecular weights of neomycin and neamine are 614 and 321, respectively). Mice were palpated daily for the first sign of tumor appearance, after which tumor size was estimated twice a week with a microcaliper and recorded in mm^3 (length x width^2).

Orthotopic growth of human cancer cells in athymic mice. MDA-MB-435 human breast carcinoma cells were injected directly into the surgically exposed mammary fat pad of female athymic mice. For this procedure, the mice were anesthetized with i.p. injection of ketamine and xylazine (100 and 10 mg/kg body weight, respectively) and allowed to stabilize under anesthesia for 15 minutes. An incision of 6 mm was made through the skin in the area of the left lateral thorax behind the left front leg and the mammary fat pad was exposed. MDA-MB-435 cells, 1 x 10^4 in 20 μL, were injected into the fat pad and the incision was closed. Treatment with neomycin (60 mg/kg body weight), neamine (30 mg/kg

Table 1. Relative nephrotoxicity and ototoxicity of aminoglycoside antibiotics

<table>
<thead>
<tr>
<th>Aminoglycosides</th>
<th>Nephrotoxicity*</th>
<th>Ototoxicity*</th>
<th>Antiangiogenicity*</th>
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</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>100</td>
<td>100</td>
<td>+</td>
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<tr>
<td>Tobramycin</td>
<td>43</td>
<td>62</td>
<td>–</td>
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<tr>
<td>Gentamicin</td>
<td>31</td>
<td>62</td>
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<tr>
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<td>25</td>
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<tr>
<td>Amikacin</td>
<td>5</td>
<td>23</td>
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<tr>
<td>Kanamycin</td>
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<td>22</td>
<td>–</td>
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<tr>
<td>Neamine</td>
<td>5</td>
<td>6</td>
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</tr>
<tr>
<td>Streptomycin</td>
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<td>not determined</td>
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<tr>
<td>Spectinomycin</td>
<td>not determined</td>
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*Data from refs. 22 and 23.

†Determined by the chick chorioallantoic membrane assay (50).
body weight), or PBS (control) started on day 1 and was given s.c. daily for 20 days and then every other day for another 30 days.

Inhibition of established A431 tumor xenografts. A431 human epidermoid carcinoma cells underwent three serial passages in athymic mice before they were used for experiments. The cells (5 × 10⁶) were inoculated s.c. into the right flank of a 5-week-old athymic mouse. When the tumor reached a size of ∼900 mm³, it was removed, homogenized, and resuspended in saline. A fraction of the tumor homogenate (5 × 10⁷ cells) was injected into another mouse. After three passages in this manner, the tumor transplant was homogenized and the cell suspension was used for experiments. Cells, 2 × 10⁶ in 0.2 mL saline, were injected s.c. into the left flank of each mouse. All mice developed a single palpable tumor of approximately 20 mm³ 8 days after cell inoculation. The mice were separated into four groups of six and given daily i.v. injections of paromomycin, neomycin, or neamine (60, 60, and 30 mg/kg body weight, respectively), or PBS for 14 days. Tumor size was measured every 3 days as described above. The animals were sacrificed at day 24 and the tumors were removed and weighed.

Immunohistochemistry. Tumor tissues were fixed in formalin and embedded in paraffin. Tissue sections of 4 μm were cut and deparaffinized with xylene, rehydrated in ethanol, and microwaved for 15 minutes in 10 mmol/L citrate (pH 6.0). Endogenous peroxidase was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 minutes. To determine the angiogenesis index, the sections were blocked in 5% dry milk for 10 minutes, and incubated with an anti-CD31 mAb at a 1:200 dilution at 4°C for 16 hours. Bound antibody was detected with Dako’s (Carpinteria, CA) EnVision system. The sections were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibody. CD31-positive vessels in each tumor were counted in the five most vascularized areas at ×200 magnification (0.785 mm²/field), and the average was calculated. Vessel density (vessels/cm²) was shown as mean ± SD for each group. Angiogenin was stained with mAb 26-2F as the primary antibody at the concentration of 10 μg/mL. Proliferating cells were stained with an anti-proliferating cell nuclear antigen IgG.

Results

Neamine inhibits the nuclear translocation of angiogenin in human umbilical vein endothelial cells. Immunofluorescence was used to monitor nuclear translocation of angiogenin in HUVEC. As shown in Fig. 2A, after 30 minutes of incubation in the absence of test compounds, the majority of cell-associated angiogenin was in the nucleus, where it accumulates in the nucleolus. In the presence of 100 μmol/L neomycin (Fig. 2B) or neamine (Fig. 2C), the amount of nuclear angiogenin was decreased markedly. Instead, strong staining on plasma membranes was observed, suggesting that angiogenin still bound to the cell surface but that it was trapped there in the presence of neomycin and neamine. Paromomycin had no effect on nuclear translocation of angiogenin (Fig. 2D), suggesting the importance of the amino group at the C6 position of the D-glucopyranosyl ring (Fig. 1). Because neamine consists of the first two rings (2,6-diamino-2,6-dideoxy-α-D-glucopyranose and 2-deoxystreptamine) of the four-ring structure of neomycin (Fig. 1), these results also indicate that the third and fourth rings (β-D-ribofuranose and 2,6-diamino-2,6-dideoxy-α-L-idopyranose, respectively) of the neomycin molecule are not required for inhibitory activity. However, 2,6-diamino-2,6-dideoxy-α-D-glucopyranose (the first hexose ring) had no activity by itself, indicating that the 2-deoxystreptamine moiety has an important role (data not shown).

Western blotting analysis showed that angiogenin was detectable in nuclear proteins extracted from control- and paromomycin-treated cells, but not in those from neomycin- and neamine-treated cells (Fig. 2E). The amount of angiogenin protein in the cytoplasmatic fractions was too low to be detected by Western blotting. However, the total amounts of angiogenin protein in whole cell lysates did not differ among the samples (Fig. 2F), suggesting that neomycin and neamine act at a step(s) downstream of cell surface binding in the nuclear translocation process. These results are consistent with the immunofluorescence results and with our previous observation that neomycin does not interfere with angiogenin-stimulated Erk1/2 phosphorylation (28).

Neamine inhibits angiogenin-induced human umbilical vein endothelial cell proliferation. We have reported previously that HUVEC cultured in serum-free HEM do not divide spontaneously but can be induced to proliferate by adding angiogenin or other angiogenic factors (29). We have also shown that neomycin inhibits angiogenin-stimulated cell proliferation (21). We now find that neamine has a similar effect. It inhibits angiogenin-stimulated HUVEC proliferation in a dose-dependent manner (Fig. 3) with an apparent IC₅₀ of ∼5 μmol/L. Paromomycin had no effect at a concentration up to 200 μmol/L (Fig. 3), consistent with our
injected into the surgically exposed mammary fat pads of athymic mice. Next, we assessed the antitumor activity of neomycin and neamine in an orthotopic breast cancer model. Treatment with neomycin and neamine inhibited the establishment and growth of HT-29 cell tumors. By day 39, all of the animals in the PBS control group had developed tumors with a size of \(\sim 20\, \text{mm}^3\). As shown in Fig. 5, daily i.v. administration of neomycin and neamine at doses of 40 and 20 mg/kg, respectively, significantly decreased the tumor growth rate, as compared with that observed in the PBS control group. Paromomycin had no effect. The average tumor weights were 550 ± 80, 720 ± 63, 180 ± 40, and 200 ± 30 mg, respectively, in the groups of mice treated with PBS, paromomycin, neomycin, and neamine, respectively (Fig. 5B). Thus, neomycin and neamine inhibited the growth of established A431 tumors in athymic mice by 67% and 64%, respectively.

Neomycin inhibits both angiogenesis and tumor cell proliferation. Immunohistochemical staining of human angiogenin in the tumors from the MDA-MB-435 orthotopic model (Fig. 4E) showed that angiogenin was predominantly in the nucleus in the control tumors (Fig. 6A). Nuclear angiogenin was decreased in neomycin-treated (Fig. 6B) and neamine-treated (Fig. 6C) tumors with a concomitant increase of extracellular angiogenin, indicating that neomycin and neamine inhibited nuclear translocation of angiogenin not only in endothelial cells but also in tumor cells. The percentage of proliferating cell nuclear antigen–positive cells decreased from 83 ± 7% in the control tumors (Fig. 6D) to 27 ± 6% and 31 ± 5% respectively, in tumors from the neomycin (Fig. 6E) and neamine (Fig. 6F) treatment groups. These results suggested that neomycin and neamine might also directly inhibit tumor cell proliferation. Consistently, in vitro assay confirmed that proliferation of MDA-MB-435 cells was inhibited by 35% and 30% in the presence of 100 \(\mu\text{mol/L}\) neomycin and neamine, respectively (data not shown).

Neovessel staining with an anti-CD31 antibody (Fig. 6G-I) showed that the angiogenesis indexes in the central regions of the neomycin- and neamine-treated tumors were 20.0 ± 3.1 and 13.9 ± 2.8, 7.1 ± 1.5 and 3.7 ± 1.1, and 8.0 ± 2.3 and 4.8 ± 0.9 vessels/mm\(^2\), respectively (Fig. 4C). These results show that both neomycin and neamine inhibited the establishment and growth of HT-29 cells in athymic mice as well as tumor angiogenesis.

Inhibition of orthotopic growth of human breast cancer cells in athymic mice. Next, we assessed the antitumor activity of neamine and neomycin in an orthotopic breast cancer model in which MDA-MB-435 human breast cancer cells are injected into the surgically exposed mammary fat pads of female athymic mice. Figure 4D shows that all of the animals in the PBS control group had developed palpable tumors by day 24, but 80% and 75% of the mice in the neomycin- and neamine-treated groups, respectively, were still tumor-free. Half of the animals never developed tumors in the entire period of the experiment (56 days). The average tumor weight (Fig. 4E) and the angiogenic indexes (Fig. 4F) were significantly lower in neomycin- and neamine-treated mice than in the control group. Thus, neomycin and neamine inhibit both the ectopic growth of HT-29 cell tumors and the orthotopic growth of MDA-MB-435 cell tumors in athymic mice.

Effect on established human tumors in athymic mice. A431 human epidermoid carcinoma cells were used to assess the inhibitory activity of neomycin and neamine on pre-established tumors. In order to minimize any interanimal variations in tumor growth rate, A431 cells were grown in athymic mice for three successive passages before they were used for experiments. A more homogeneous tumor population among the animals was obtained by inoculating with A431 tumor tissue homogenates rather than with cultured cells. Treatments began at day 8, when all of the mice had developed tumors with a size of \(\sim 20\, \text{mm}^3\). As shown in Fig. 5, daily i.v. administration of neomycin and neamine at doses of 40 and 20 mg/kg, respectively, significantly decreased the tumor growth rate, as compared with that observed in the PBS control group. Paromomycin had no effect. The average tumor weights were 550 ± 80, 720 ± 63, 180 ± 40, and 200 ± 30 mg, respectively, in the groups of animals treated with PBS, paromomycin, neomycin, and neamine, respectively (Fig. 5B). Thus, neomycin and neamine inhibited the growth of established A431 tumors in athymic mice by 67% and 64%, respectively.
translocation of angiogenin occurs in both endothelial (20) and cancer (30) cells, and that angiogenin plays a role in cell proliferation of both cell types.

**Discussion**

The antiangiogenesis activity of neomycin was discovered from our mechanistic studies of angiogenin, an angiogenic RNase that has been shown to play a role in tumor angiogenesis (20). Angiogenin undergoes nuclear translocation and plays a role in ribosome biogenesis (20, 30), in part through stimulation of rRNA transcription. Neomycin blocks the nuclear translocation of angiogenin, thereby inhibiting endothelial cell proliferation and angiogenesis (21). However, we have not considered this agent to be a favorable drug candidate for further development because of its known nephrotoxicity and ototoxicity. Here, we have presented data showing that neamine, a nontoxic derivative of neomycin, is an equally potent inhibitor of nuclear translocation of angiogenin. Both neomycin and neamine inhibit the establishment and growth of human tumor xenografts in ectopic and orthotopic athymic mouse models. More importantly, they inhibit the progression of already established human tumor transplants in athymic mice.

Neomycin is an aminoglycoside antibiotic (31) that binds to prokaryotic ribosomes, causing misreading of mRNA (32, 33).

In contrast with the structurally related compound geneticin G418 (34, 35), it does not bind to the ribosomes of eukaryotic cells and therefore are not toxic to most eukaryotic cells. However, like many of the other aminoglycoside antibiotics, neomycin is toxic to the proximal tubular cells of the kidney and the hair cells of the cochlea. In both cases, interactions of the drugs with membrane constituents have been suggested to play a major role in the onset of these toxic phenomena (36–38). In addition, aminoglycosides are also taken up by proximal tubular cells of the renal cortex and accumulate in the lysosomes (39). This accumulation inhibits the activities of lysosomal phospholipases leading to phospholipidosis (40), which then triggers cell necrosis and renal failure (41). Among the common aminoglycoside antibiotics, neomycin has the most severe nephrotoxicity and ototoxicity (22, 23). Parenteral use of neomycin has largely been abandoned for this reason. Therefore, the finding that neamine also potently inhibited angiogenesis and tumor growth revived our interest in this class of compounds.

Neamine is a degradation product of neomycin (26), although there is some evidence that it is also produced in small amounts by Strotomyces fradiae (42). It is comparatively nontoxic, with an acute LD$_{50}$ in mice (1,250 mg/kg) that is 6-fold higher than that of neomycin (220 mg/kg; ref. 43). Moreover, the nephrotoxicity and ototoxicity of neamine is
only 5% and 6% (22, 23), respectively, of that of neomycin (Table 1). Therefore, neamine might serve as a lead compound in this class of antiangiogenesis and antitumor agents to be further developed for potential clinical applications. The antitumor activity of neomycin and neamine is related to the inhibition of nuclear translocation of angiogenin. Accumulating evidence suggest that angiogenin secreted by tumor cells plays an essential role in the process of tumor angiogenesis. For example, the administration of antiangiogenin mAb 26-2F that does not cross-react with mouse angiogenin inhibited the growth of human tumor cells in mice (12, 44). A human angiogenin–specific antisense oligo has also been

Fig. 5. Inhibition of the growth of established A431 human tumors in athymic mice by neamine and neomycin. A431 xenografts were maintained by serial passage in athymic mice. A tumor suspension, 0.2 mL containing 2 × 10^6 cells, was injected s.c. into the left flank of the mice. All 24 mice used developed palpable tumors by day 8 and were then randomly grouped for experiments. A, tumor size as a function of time. The animals were treated i.v. daily with PBS, paromomycin (40 mg/kg), neomycin (40 mg/kg), or neamine (20 mg/kg). Tumor sizes were measured with a microcaliper every 3 days. B, the wet tumor weight determined at the end of the experiment. C, the excised tumors.

Fig. 6. Inhibition of angiogenesis and tumor cell proliferation by neamine and neomycin. MDA-MB-435 cell tumors were from the orthotopic tumor model described in Fig. 4D. Thin sections (4 μm) were cut and stained with antiangiogenin (A–C), anti–proliferating cell nuclear antigen (D–F) and anti-CD31 (G–I) antibodies. A, D, and G, tumor tissue from control animals treated with PBS. B, E, and H, neomycin-treated animals. C, F, and I, neamine-treated animals. Figures from a representative slide; data beneath the figures are means ± SE of four tumor-bearing animals from each group.
shown to inhibit human tumor growth in mice when the circulating level of mouse angiogenin was not affected (45). In the process of tumor angiogenesis in xenograft tumor models, human tumor–secreted angiogenin acts on mouse endothelial cells in a paracrine manner. Decreased tumor angiogenesis in neomycin- and neamine-treated tumors is likely a result of inhibition of this process.

The underlying mechanism of the antitumor activity of neomycin and neamine is unlikely to involve the sequestration of tumor-secreted angiogenin or inhibition of its ribonucleolytic activity. We have shown that neomycin does not affect the ribonucleolytic activity of angiogenin (21) and have not observed any appreciable physical interactions between angiogenin and neomycin (data not shown). In both the ecotopic and the orthotopic models, about half of the mice never developed tumors (Fig. 4A and D). The other half eventually developed tumors but with significantly lower tumor burden (Fig. 4B and E) and decreased tumor angiogenesis (Fig. 4C and F). These results suggest that angiogenin plays a role in both tumor establishment and progression and that neamine is effective in inhibiting both processes.

A clinically more relevant model for evaluating antiangiogenesis and antitumor activity would be the one with the established tumors (46, 47). In this study, we used A431 cells that had undergone three serial passages in athymic mice to obtain a more uniformed tumor burden among mice. Treatment with neomycin and neamine resulted in a significant decrease in tumor growth rate (Fig. 5A). It is noteworthy that not a single animal in neomycin- or neamine-treated group developed a tumor that is larger than any of the tumors in control animals treated with PBS or paromomycin.

Currently, a variety of antiangiogenesis inhibitors are being evaluated for the treatment of human cancers and many have entered clinical trials (48). The vascular endothelial growth factor–specific antibody bevacizumab (Avastin, Genentech, South San Francisco, CA) has recently been approved by the Food and Drug Administration for use in combination with 5-fluorouracil–based chemotherapy as a treatment for patients with metastatic colon and rectal cancer (49). Among the various angiogenesis inhibitors, neamine has some properties that may facilitate its clinical development. First, it inhibits both angiogenesis and tumor cell proliferation per se, as shown by both in vitro and in vivo experiments (Fig. 6). Treatment with neamine would potentially have the combined benefit of antiangiogenesis therapy and chemotherapy. Second, neamine is relatively nontoxic: indeed, its toxicity is lower than or similar to those of netinimicin, kanamycin, and streptomycin, all three of which are currently in clinical use as antibiotics. Moreover, the underlying mechanism of its antitumor activity has been revealed to be related to the blockade of nuclear translocation of angiogenin. Further studies aiming to understand the molecular details of this action will allow the development of inhibitors with enhanced antiangiogenesis and antitumor potency. Furthermore, because neamine only inhibits the angiogenic factor–induced cell proliferation without a significant effect on basal level and spontaneous proliferation, it might be more selective than nonspecific endothelial cell inhibitors and other cytotoxic agents that affect all proliferating cells.

References


