Neamine Inhibits Prostate Cancer Growth by Suppressing Angiogenin-Mediated rRNA Transcription

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Abstract

Purpose: Angiogenin (ANG) undergoes nuclear translocation and stimulates rRNA transcription in both prostate cancer cells and endothelial cells. The purpose of this study is to assess the antitumor activity of neamine, a nontoxic degradation product of neomycin that blocks nuclear translocation of ANG.

Experimental Design: The anti-prostate cancer activity of neamine was first evaluated in a xenograft animal model. It was then examined in the murine prostate-restricted AKT transgenic mice that develop prostate intraepithelial neoplasia (PIN) owing to AKT transgene overexpression.

Results: Neamine inhibits xenograft growth of PC-3 human prostate cancer cells in athymic mice. It blocks nuclear translocation of ANG and inhibits rRNA transcription, cell proliferation, and angiogenesis. Neamine also prevents AKT-induced PIN formation as well as reverses fully developed PIN in murine prostate-restricted AKT mice, accompanied by a decrease in rRNA synthesis, cell proliferation, and angiogenesis and an increase in prostate epithelial cell apoptosis.

Conclusion: We confirmed that ANG is a molecular target for cancer drug development and that blocking nuclear translocation of ANG is an effective means to inhibit its activity. Our results also suggested that neamine is a lead compound for further preclinical evaluation.

Increasing evidence points to an important role of angiogenin (ANG), a 14-kDa angiogenic ribonuclease, in the development and progression of prostate cancer (1–5). ANG has been shown to be up-regulated in human prostate cancer (5). The circulating level of ANG in plasma is significantly higher in prostate cancer patients, especially those with hormone-refractory diseases, compared with normal controls (4). Immunohistochemical studies indicated that ANG expression is the most significantly up-regulated gene in AKT-induced prostate intraepithelial neoplasia (PIN) in murine prostate-restricted AKT (MPAKT) mice (4).

ANG has been shown to undergo nuclear translocation in proliferating endothelial cells (6) where it stimulates rRNA transcription (7), a rate-limiting step in protein translation and cell proliferation (8). We have therefore proposed that ANG-stimulated rRNA transcription is a general requirement for endothelial cell proliferation and angiogenesis (9). ANG inhibitors abolish the angiogenic activity of ANG and that of other angiogenic factors including vascular endothelial growth factor and basic fibroblast growth factor (9). Moreover, ANG has been found to play a direct role in cancer cell proliferation (10). Nuclear translocation of ANG in endothelial cells is inversely dependent on cell density (11) and is stimulated by growth factors (9). However, ANG is constitutively translocated to the nucleus of cancer cells in a cell density-independent manner (10, 12). It is plausible that constitutive nuclear translocation of ANG is one of the reasons for sustained growth of cancer cells, a hallmark of malignancy (1).

The dual role of ANG in prostate cancer progression suggested that ANG is a molecular target for the development of cancer drugs (1). ANG inhibitors would combine the benefits of both antiangiogenesis and chemotherapy because both angiogenesis and cancer cell proliferation are targeted. Moreover, because ANG-mediated rRNA transcription is essential for other angiogenic factors to induce angiogenesis (9), ANG antagonists would also be more effective as angiogenesis inhibitors than others that target only one angiogenic factor.

The activity of ANG in both endothelial and cancer cells is related to its capacity to stimulate rRNA transcription; for that to occur, ANG needs to be in the nucleus physically (7). ANG has a typical signal peptide and is a secreted protein (13). The mechanism by which it undergoes nuclear translocation is not clear as yet (14), but it obviously is a target for anti-ANG.
Therapy. Targeting nuclear translocation of ANG would be more advantageous than targeting ANG directly because normally ANG circulates in the plasma (15) at a concentration of 250 to 350 ng/mL (16, 17) and would require a high dose of inhibitors to neutralize them.

Neomycin, an aminoglycoside antibiotic, has been shown to block nuclear translocation of ANG (18) and to inhibit xenograft growth of PC-3 cells in athymic mice. Because of the nephrotoxicity, neomycin itself cannot be directly applied as a prostate cancer therapeutic. We have now established that neamine, a nontoxic derivative of neomycin, effectively blocks nuclear translocation of ANG, inhibits xenograft growth of human prostate cancer cells in athymic mice, and prevents and reverses prostate intraepithelial neoplasia in AKT transgenic mice. Therefore, the current work identified neamine as a lead compound for further development of prostate cancer therapeutics.

**Materials and Methods**

**Cells and animals.** PC-3 cells were cultured in DMEM + 10% fetal bovine serum. Outbred male athymic mice (nu/nu) were from Charles River Laboratories. A breeding pair of MPAKT mice was provided by Translational Relevance

Angiogenin (ANG) plays a dual role in prostate cancer progression by stimulating rRNA transcription in both endothelial and cancer cells. ANG inhibitors would have the benefit of combining chemotherapy and antiangiogenesis therapy. Neomycin, a Food and Drug Administration-approved aminoglycoside antibiotic, has been shown to block nuclear translocation of ANG and to inhibit xenograft growth of PC-3 cells in athymic mice. Because of the nephrotoxicity, neomycin itself cannot be directly applied as a prostate cancer therapeutic. We have now established that neamine, a nontoxic derivative of neomycin, effectively blocks nuclear translocation of ANG, inhibits xenograft growth of human prostate cancer cells in athymic mice, and prevents and reverses prostate intraepithelial neoplasia in AKT transgenic mice. Therefore, the current work identified neamine as a lead compound for further development of prostate cancer therapeutics.

**Fig. 1.** Neamine inhibited xenograft growth of PC-3 human prostate cancer cells in athymic mice. Male athymic mice were inoculated with 5 x 10^5 PC-3 cells and treated subcutaneously with PBS or neamine at a dose of 30 mg/kg body weight twice weekly for 8 wk. Twelve mice were used per group. A, mice were examined by palpation for tumor appearance. B, tumor sizes were measured with a caliper and expressed as length x width^2. At day 56, mice were sacrificed and tumor tissues were removed, photographed (C), and weighed (D).

Neamine, an aminoglycoside antibiotic, has been shown to block nuclear translocation of ANG (18) and to inhibit xenograft growth of human prostate cancer cells in athymic mice (1). However, the nephrotoxicity and ototoxicity of neomycin (19) would seem to preclude its prolonged use as an anticancer agent. We have now established that neamine (20), a nontoxic degradation product of neomycin, effectively inhibits nuclear translocation of ANG (12). It has also been shown to inhibit angiogenesis induced both by ANG and by basic fibroblast growth factor and vascular endothelial growth factor (9). Moreover, it inhibits xenograft growth of HT-29 human colon adenocarcinoma and MDA-MB-435 human breast cancer cells in athymic mice (12). Because the toxicity profile of neamine is close to that of streptomycin and kanamycin, which is ~20-fold less toxic than neomycin (21, 22), it may serve as a lead agent for the development of prostate cancer therapeutics. Therefore, we examined its capacity to prevent the establishment and to inhibit the growth of PC-3 human prostate cancer cells in mice as well as its capacity to prevent and to reverse AKT-induced PIN in MPAKT mice.

**Materials and Methods**

**Cells and animals.** PC-3 cells were cultured in DMEM + 10% fetal bovine serum. Outbred male athymic mice (nu/nu) were from Charles River Laboratories. A breeding pair of MPAKT mice was provided by
Dr. W.R. Sellers (Dana-Farber Cancer Institute). All animal experiments were approved by Institutional Animal Care and Use Committee of Harvard Medical School.

**Xenograft growth of PC-3 cell tumors.** Five-week-old male athymic mice were inoculated subcutaneously with 100 μL of a mixture containing 5 x 10^5 PC-3 cells and 33 μL Matrigel. The mice were treated subcutaneously with PBS or neamine (30 mg/kg) twice weekly for 8 weeks. Tumor sizes were measured every 3 days and recorded in mm³ (length x width²; the longer side of the tumor was designated as the length). Mice were sacrificed at day 56 and the tumors were removed and the wet weights of the PC-3 tumors were recorded.

**Treatment of MPAKT mice with neamine.** For PIN prevention experiments, 4-week-old MPAKT mice were treated with daily intraperitoneal injection of PBS or neamine at a dose of 10 mg/kg body weight for 4 weeks. To examine the effect of neamine on established PIN, 12-week-old MPAKT mice with fully developed PIN were treated with daily intraperitoneal injection of PBS or neamine at a dose of 10 mg/kg body weight for 4 weeks. The animals were sacrificed and the entire genitourinary tract was removed, fixed with 4% paraformaldehyde, and embedded in paraffin.

**Immunohistochemistry.** Tissue sections (4 μm) were hydrated, incubated for 30 min with 3% H₂O₂ in methanol at room temperature, washed with H₂O and PBS, and microwaved in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Sections were blocked in 5% nonfat dry milk in PBS for 30 min and incubated with antibodies against human ANG (30 μg/mL; 26-2F), mouse ANG (10 μg/mL; R163), proliferating cell nuclear antigen (1:200; DAKO), von Willebrand factor (1:200; DAKO), and pAKT-S473 (1:100; Cell Signaling) in 1% bovine serum albumin in PBS at 4°C for 16 h. For detection of Ki-67, the sections were blocked in the M.O.M. mouse immunoglobulin blocking reagent for 60 min and incubated with anti-Ki-67 antibody (1:100; Vector Laboratories) in the M.O.M. diluent at 25°C for 1 h. The slides were washed with PBS, incubated with horseradish peroxidase-labeled second antibody, and visualized with the DAKO Cytomation EnVision System.

**In situ hybridization for 47S rRNA.** Riboprobes for human and mouse 47S rRNA were prepared and labeled with digoxigenin as described by Qian et al. (23). The templates for the sense riboprobes were prepared by PCR from mouse genomic DNA with sense primer containing a T7 promoter (5'-GGGTAATACGACTCACTATAGGGCGA). The primers for the initiation site of the 47S rRNA precursor were as follows: human forward 5'-GCTGACACGCTGTCCTCTGG-3' and reverse 5'-GAGAACGCCTGACACGCACG-3' and mouse forward 5'-GCCGAAATAAGGTGGCCCTC-3' and reverse 5'-GCCGAATAAGGTGGCCCTC-3'. PCR conditions were 5 min at 94°C, 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 7 min). Digoxigenin-labeled probes were generated by in vitro transcription from the above PCR templates using Digoxigenin RNA Labeling Kit (Roche Diagnostics). The control probe was the digoxigenin-labeled "antisense" Neo transcripts of 760 bases in length, which was transcribed by T7 RNA polymerase according to the standard protocol using pSPT18-Neo as the template. Tissue sections were deparaffinized with xylene and rehydrated with ethanol. After proteinase K treatment (1.5 μg/mL for 10 min at room temperature) and acetylation reaction.

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**Fig. 2.** Effect of neamine treatment on cell proliferation, angiogenesis, and 47S rRNA synthesis. PBS- and neamine-treated tumor tissues were fixed in formalin and embedded in paraffin, and sections of 5 μm were cut. A, localization of ANG was determined by immunohistochemistry with anti-human ANG monoclonal antibody 26-2F. B, ISH with a probe specific for the initiation site of the 47S rRNA. C, proliferating cells were stained with an anti-proliferating cell nuclear antigen monoclonal antibody. Proliferating cell nuclear antigen-positive and total numbers of cells were counted in five randomly selected areas at x200 magnification. D, blood vessels were stained with an anti-von Willebrand factor antibody and counted in five most vascularized areas at x200 magnification.
(0.25% acetic anhydride in 0.1 mmol/L triethanolamine at room temperature for 20 min), the sections were washed with 4× SSC, prehybridized at 45°C for 1 h in 5× SSC containing 50% formamide, 0.5 mg/mL heparin, and 0.1 mg/mL salmon sperm DNA. Hybridization was carried out in the same buffer as prehybridization but containing 800 ng/mL digoxigenin-labeled probe at 45°C for 16 h. After successive washing in 4× SSC (1 min at room temperature) and 50% formamide in 2× SSC (1 h at 45°C), 0.1× SSC (2 h at 45°C), and Tween 20-TBS (5 min at room temperature), the hybridization signal was visualized using an alkaline phosphatase-conjugated anti-digoxigenin antibody with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Silver staining of nucleolar organizer region. Formalin-fixed tissue sections were deparaffinized in xylene, rehydrated in ethanol and H2O, immersed in 10 mmol/L sodium citrate buffer (pH 6.0), and autoclaved at 120°C for 20 min. After an extensive wash with H2O, the slides were incubated at 37°C for 13 min in a solution containing 1 volume of 2% gelatin in 1% aqueous formic acid and 2 volumes of 50% silver nitrate.

TUNEL assay. Formalin-fixed tissue sections were deparaffinized in xylene, rehydrated in ethanol, and incubated with proteinase K (0.02 mg/mL) for 20 min at room temperature. TUNEL staining was carried out using the Fluorescein-FragEL DNA Fragmentation Detection kit (Calbiochem) per the manufacturer’s instructions. TUNEL-positive luminal epithelial cells were counted in all ducts of the ventral prostate.

Results

Neamine inhibits xenograft growth of PC-3 human prostate cancer cells in athymic mice. The anti-prostate cancer activity of neamine was examined first in a xenograft tumor model in which PC-3 human prostate cancer cells were injected into athymic mice. Figure 1 shows that subcutaneous treatment with neamine at 30 mg/kg, a nontoxic dose that is 2.4% of the reported LD50 of 1,250 mg/kg (19), prevented tumor establishment in 50% of the athymic mice. At day 20, all of the untreated mice (n = 12) had tumors, whereas only 5 of the 12 neamine-treated animals had palpable tumors. Fifty percent of the animals never developed ectopic PC-3 tumors as a consequence of neamine treatment (Fig. 1A). In the animals that did develop tumors, their growth rate was decreased significantly (Fig. 1B). At day 56, when all animals were sacrificed, the average tumor weight in the control and neamine-treated groups was 620 ± 310 and 170 ± 50 mg, respectively (Fig. 1C and D), representing a 72.5% inhibition of tumor growth by neamine.

Neamine blocks nuclear translocation of ANG, suppresses rRNA transcription, and inhibits cell proliferation and angiogenesis. In efforts to understand how neamine inhibits PC-3 cell tumor growth in athymic mice, we next examined the status of nuclear human ANG in the tumor tissues grown in untreated and neamine-treated mice. Immunohistochemical staining with a human ANG-specific monoclonal antibody (26-2F) shows that ANG is stained predominantly in the nucleus of tumor cells grown in untreated animals (Fig. 2A, left), whereas most of the ANG is extracellular in neamine-treated tumors (Fig. 2A, right). These results indicate that, in PC-3 cells, neamine blocks nuclear translocation of ANG. Because the function of nuclear ANG is known to be related to rRNA transcription, we used in situ hybridization (ISH) with a probe specific for the initiation site of the 47S rRNA precursor to clarify the effect of neamine on rRNA transcription. Figure 2B shows that, in neamine-treated tumor tissues, the 47S rRNA level is decreased significantly when compared with that of control tumor tissues. Immunohistochemistry with antibodies against proliferating cell nuclear antigen (Fig. 2C) and von Willebrand factor (Fig. 2D) was used to determine cell proliferation and angiogenesis status, respectively. Neamine treatment decreased proliferating cell nuclear antigen-positive cells from 75.4 ± 6% to 25.6 ± 6.4% (Fig. 2C), representing a 66% decrease in cell proliferation. Vessel density decreased from 82 ± 3.2 to 22.3 ± 9.6 vessels/mm2, representing a 72.8% decrease in tumor angiogenesis (Fig. 2D). Jointly, all data suggest that neamine

![Image](image_url)

Fig. 3. Neamine prevented AKT-induced PIN formation in MPAKT mice. Four-week-old MPAKT mice were treated with daily intraperitoneal injection of PBS control or neamine at a dose of 10 mg/kg body weight, respectively, for 4 wk. Mice were sacrificed at week 8 and ventral prostates were processed for histologic examinations. A and B. H&E staining of the ventral prostates. Arrows, PIN lesions. C and D. Immunohistochemical examinations of nuclear translocation of ANG. Arrows, staining of nuclear ANG. E and F. Immunohistochemical examinations of phosphorylation status of AKT. Arrows, positive signals. G and H. ISH analysis for rRNA transcription. Arrows, positive signals. I and J. silver-stained NOR of the ventral prostate epithelial cells. Average numbers of NOR of 60 cells.
decreases nuclear accumulation of ANG, thereby suppressing rRNA transcription, cell proliferation, and angiogenesis, consistent with the previous report that ANG plays a dual role in prostate cancer progression by stimulating both angiogenesis and cancer cell proliferation (1). They also concur with the reports that nuclear function of ANG is related to rRNA transcription (7) and that the neomycin family of aminoglycoside antibiotics blocks nuclear translocation of ANG (18).

Neamine prevents AKT-induced PIN in MPAKT mice. The anti-prostate cancer activity of neamine was examined further in AKT transgenic mice known to develop PIN spontaneously, the precursor of prostate cancer. ANG is the highest up-regulated gene in the PIN lesion of MPAKT mice (4). However, the role of ANG in AKT-induced proliferation of prostate epithelial cells has been uncertain. To understand whether ANG is involved in AKT-induced prostate epithelial cell proliferation and PIN formation, we have treated 4-week-old MPAKT mice with neamine at a daily intraperitoneal dose of 10 mg/kg for 4 wk. The mice were sacrificed at week 8 and the ventral prostates were examined histologically for PIN formation. H&E staining shows that neamine inhibited PIN formation (Fig. 3A and B). The percentage of PIN in the ventral prostate decreased from 55.1 ± 4.3% to 9.6 ± 1.4% after neamine treatment as determined by the use of established criteria for PIN such as intraglandular cell expansion and lumen formation, nuclear atypia, and loss of cell polarity (4). Immunohistochemistry with an anti-mouse ANG antibody shows strong nuclear staining of ANG in the prostate epithelial cells from the ventral prostate of untreated animals (Fig. 3C). In neamine-treated ones, ANG was predominantly cytoplasmic and extracellular (Fig. 3D), indicating blockage of nuclear translocation of ANG in the prostate epithelial cells. To exclude the possibility that neamine might have affected AKT transgene expression or phosphorylation, we performed immunohistochemistry with an anti-pAKT antibody and showed that AKT phosphorylation in neamine-treated samples (Fig. 3F) did not differ from those of controls (Fig. 3E), showing that nuclear ANG is not involved in the AKT phosphorylation pathway and that neamine does not affect AKT transgene expression and phosphorylation. ISH with a probe specific for the initiation site of the mouse 47S rRNA shows that the level of 47S rRNA in the ventral prostate epithelial cells decreased dramatically after neamine treatment (Fig. 3G and H), thereby confirming the activity of nuclear ANG in rRNA transcription. We did not notice any adverse reactions of the animals after neamine treatment. There were no difference in body weight, grooming behaviors, and food and fluid intakes between PBS-treated and neamine-treated groups.

To obtain more quantitative assessment of the changes in rRNA transcription, we examined the effect of neamine on nucleolar organizer region (NOR) of the prostate epithelial cells. NOR are loops of rDNA that are actively being transcribed (24). NOR are associated with argyrophilic proteins and can be visualized by silver staining. Both the numbers and the size of the NOR reflect the degree of ribosome biogenesis (25). Treatment of neamine decreased the average number of NOR per cell from 2.9 ± 0.8 (Fig. 3I) to 1.9 ± 0.9 (Fig. 3J), indicating a significant decrease in ribosome biogenesis (P < 10⁻⁸).

ANG plays a dual role in prostate cancer progression by stimulating rRNA transcription in both endothelial and cancer cells (1). It undergoes nuclear translocation in both cell types and can be inhibited by neomycin (1, 18). We therefore examined the effect of neamine treatment on both angiogenesis and AKT-induced prostate luminal cell proliferation.
Immunohistochemistry with an anti-CD31 antibody shows that neamine treatment decreased interluminal angiogenesis (Fig. 4A). Vessel density in the control and treated ventral prostate was 11.5 ± 4.5 and 4 ± 0.5 per mm², respectively. Neamine treatment also decreases cell proliferation in the ventral prostate (Fig. 4B). Ki-67-positive cells decreased from 61.1 ± 9.3% in untreated PIN to 24.9 ± 8.4% in neamine-treated samples. Thus, neamine inhibits both angiogenesis and cell proliferation.

**Neamine treatment reverses established PIN in MPAKT mice.** We next examined the effect of neamine on established PIN. For this purpose, 12-week-old MPAKT mice were treated by daily intraperitoneal injection of neamine (10 mg/kg) for a period of 4 weeks. The animals were sacrificed at week 16 and ventral prostates were examined. H&E staining. Arrows, PIN lesions. C and D, immunohistochemical detection of pAKT. Arrows, positive signals. E and F, ISH for rRNA transcription. Arrows, positive signals. G and H, apoptosis of luminal epithelial cells were examined by TUNEL staining. Arrows, apoptotic cells.

Fig. 5. Neamine treatment reversed established PIN in MPAKT mice. Twelve-week-old MPAKT mice with fully developed PIN were treated with daily intraperitoneal injection of PBS control or neamine at a dose of 10 mg/kg body weight, respectively, for 4 wk. Mice were sacrificed at week 16 and ventral prostates were examined. A and B, H&E staining. Arrows, PIN lesions. C and D, immunohistochemical detection of pAKT. Arrows, positive signals. E and F, ISH for rRNA transcription. Arrows, positive signals. G and H, apoptosis of luminal epithelial cells were examined by TUNEL staining. Arrows, apoptotic cells.

rRNA transcription in endothelial cells is a general requirement for angiogenesis (9). Earlier work has shown that ANG is essential for angiogenesis induced by a variety of other angiogenic factors including acidic and basic fibroblast growth factor, epidermal growth factor, and vascular endothelial growth factor (9). Targeting ANG would therefore be more effective than targeting other individual angiogenic factors. Moreover, recent work has shown ANG to play a direct role in prostate cancer proliferation (1, 10), making inhibition of ANG an even more attractive target for cancer drug development. It is conceivable that ANG inhibitors would provide the benefits of both antiangiogenic and traditional chemotherapy.

To develop anti-ANG therapy, both ANG and its receptor could serve as targets. The cell surface receptor of ANG has not been identified as yet and the signal transduction pathways are not yet fully characterized. Past efforts have focused on targeting ANG itself. A variety of approaches have been explored and proofs-of-principle have been established that ANG inhibitors are possible anticancer agents. Thus, ANG inhibitors including specific antisense (3) and small interfering RNA (26), monoclonal antibodies (26), or soluble binding protein (27) as well as a small-molecule enzymatic inhibitors (28) have all been shown to inhibit xenograft growth of human cancer. The relatively high concentration of ANG (~250-350 ng/mL) that circulates in plasma (16, 17) is a caveat of these strategies. The majority of the circulating ANG is produced by the liver (29). Moreover, with a seeming fast turnover rate and a half-life of 2 h (30), a large quantity of ANG inhibitors would be needed to neutralize the circulating ANG.

An alternative approach to inhibit the function of ANG would be blockage of its nuclear translocation. The biological function of ANG is related to rRNA transcription (31), which requires ANG to be in the nucleus physically (7). Nuclear translocation of ANG seems to be essential for its biological function (6). Targeting nuclear translocation of ANG would avoid potential problems caused by its high plasma concentration.

**Discussion**

ANG is a proven target for prostate cancer therapy, owing to its dual role in prostate cancer progression (1). ANG-stimulated
A distinct advantage of targeting nuclear translocation of ANG would be that it might not have serious side effects because nuclear translocation of ANG occurs only in proliferating endothelial and cancer cells.

In efforts to understand the mechanism by which ANG is translocated to the nucleus of endothelial cells, neomycin was discovered to block nuclear translocation of ANG and to inhibit ANG-induced cell proliferation and angiogenesis (18). Moreover, neomycin has been shown to inhibit xenograft growth of PC-3 cells in athymic mice (1). Neomycin is an aminoglycoside antibiotic isolated originally from Streptomyces fradiae (32). Similar to other aminoglycosides, neomycin has high activity against Gram-negative bacteria and has partial activity against Gram-positive bacteria. However, neomycin is nephrotoxic and ototoxic to humans and its clinical use has been restricted to topical preparation and oral administration as a preventive measure for hepatic encephalopathy and hypercholesterolemia by killing bacteria in the small intestinal tract and keeping ammonia levels low (19). The nephrotoxicity of neomycin is associated with selective accumulation in the kidney where the cortical levels may reach as high as 20 times those of circulating levels in serum. The mechanism underlying selective renal accumulation has been shown to be tubular reabsorption, extraction from the circulation at the basolateral surface, and brush border uptake (21). The antibiotic activity and the renal toxicity of neomycin seem to be separable from its capacity to inhibit nuclear translocation of ANG. This led our search for less toxic derivatives and analogues of neomycin and led to the finding that neamine (33), a virtually nontoxic derivative of neomycin, has comparable activity in blocking nuclear translocation of ANG (12). Neamine is equally effective in inhibiting angiogenesis induced by ANG as well as by other angiogenic factors (9). Other aminoglycoside antibiotics including streptomycin, gentamicin, kanamycin, amikacin, and paromomycin do not block nuclear translocation of ANG and are not antiangiogenic (18).

Neamine is a degradation product of neomycin, although there is some evidence that it is also produced in small amounts by S. fradiae (33). Cell and organ culture experiments have shown that the nephrotoxicity and ototoxicity of neamine is ~5% and 6%, respectively, of that of neomycin (21, 22). Thus, the toxicity of neamine is similar to that of streptomycin, an antibiotic that is in clinical use. Neamine is also less neuro-muscularly toxic than neomycin. The acute LD10 (subcutaneous) in mice for neamine, neomycin, and streptomycin is 1.25, 0.22, and 0.60 g/kg, respectively (19). The recommended dosage for intramuscular injection of streptomycin in humans is 25 to 30 mg/kg twice weekly (34). Because neamine appears to be less toxic than streptomycin, the dose we used in this study (30 mg/kg subcutaneously and 10 mg/kg intraperitoneally) might be tolerated well. Indeed, we did not see any acute or chronic adverse side effects in experiments with these mice.

Neamine is effective in inhibiting prostate cancer growth in both xenograft and spontaneous mouse models. With the xenograft animal model, neamine prevented the establishment of PC-3 cell tumors in 50% of the animals with an overall inhibition of 72.5% in the growth rate (Fig. 1). Histology and immunohistochemical evaluation showed that neamine inhibited both angiogenesis and cancer cell proliferation (Fig. 2A and B). These results closely resemble those that we observed with neomycin, confirming the dual role of ANG and suggesting a similar mechanism of inhibition mediated by neamine and neomycin. Indeed, neamine treatment blocked nuclear translocation of ANG and suppressed rRNA transcription in cancer cells (Fig. 2C and D).

Neamine is effective in preventing AKT-induced PIN in MPAKT mice (Fig. 3), providing a strong rationale for its further development as an anti-prostate cancer agent. AKT kinase activity is frequently elevated in prostate cancers (35). Activated AKT promotes both cell growth and survival. Mouse ANG is the most significantly up-regulated gene in the prostate during PIN development in AKT transgenic mice (4). In these mice, expression of AKT in the ventral prostate results in activation of the p70S6K pathway and induction of PIN is similar in character to that observed in PTEN+/− mice (36). PTEN has been shown to regulate cell size in association with its ability to regulate ribosome biogenesis (37). Inactivating somatic mutation of PTEN or loss of the PTEN protein is common in prostate cancer cell lines and in primary and metastatic tumor specimens (38). Mutation of PTEN leads to deregulated phosphatidylinositol 3-kinase signaling, resulting in constitutive activation of downstream targets including the AKT kinase family. Transformation by phosphatidylinositol 3-kinase or AKT correlates directly with activation of mammalian target of rapamycin and its downstream target S6K (39). S6 phosphorylation has been associated with translation of a specific class of mRNA termed TOP (a terminal oligopyrimidine track in the 5’ untranslated region) mRNA (40). This class of mRNAs includes ribosomal proteins, elongation factors 1A1 and 1A2, and several other proteins involved in ribosome biogenesis or translation control (41). Thus, AKT activation will enhance ribosomal protein production. However, it is unknown how transcription of rRNA, which needs to be incorporated in an equimolar ratio, is elevated proportionally. It could very well be that ANG is up-regulated by AKT, so that rRNA transcription can be increased to fulfill the enhanced growth requirement resulting from AKT activation. The findings that neamine treatment decreases rRNA transcription in the luminal epithelial cells of the ventral prostate of the mice support such a hypothesis.

The capacity of neamine to reverse established PIN is a clinically more relevant finding (Fig. 5). Neamine treatment of the MPAKT mice that have fully developed PIN inhibited rRNA transcription and induced cell proliferation, resulting in a reversal of PIN phenotype and normalization of the luminal architecture. These results indicate that ANG is important not only for the initial cell expansion during PIN formation but also for cell survival and maintenance of established PIN. Given the nontoxic nature of neamine and its potent activity against ANG-mediated rRNA transcription, which is essential for prostate cancer progression, neamine is a promising candidate for further development as a therapeutic agent in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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