Neamine Inhibits Oral Cancer Progression by Suppressing Angiogenin-mediated Angiogenesis and Cancer Cell Proliferation

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Abstract. Background: Angiogenin undergoes nuclear translocation and stimulates ribosomal RNA transcription in both endothelial and cancer cells. Consequently, angiogenin has a dual effect on cancer progression by inducing both angiogenesis and cancer cell proliferation. The aim of this study was to assess whether neamine, a blocker of nuclear translocation of angiogenin, possesses antitumor activity toward oral cancer. Materials and Methods: The antitumor effect of neamine on oral cancer cells was examined both in vitro and in vivo. Results: Neamine inhibited the proliferation of HSC-2, but not that of SAS oral cancer cells in vitro. Treatment with neamine effectively inhibited growth of HSC-2 and SAS cell xenografts in athymic mice. Neamine treatment resulted in a significant decrease in tumor angiogenesis, accompanied by a decrease in angiogenin- and proliferating cell nuclear antigen-positive cancer cells, especially of HSC-2 tumors. Conclusion: Neamine effectively inhibits oral cancer progression through inhibition of tumor angiogenesis. Neamine also directly inhibits proliferation of certain types of oral cancer cells. Therefore, neamine has potential as a lead compound for oral cancer therapy.

In 2014, 28,030 new cases and 5,850 deaths of oral cancer are projected to occur in the United States (1). The majority of oral carcinomas are oral squamous cell carcinomas (OSCCs). The three major modalities of treatment that have been applied as conventional treatment strategies for oral cancer include surgery, radiation, and chemotherapy. However, the overall cure rate for patients with oral cancer has remained at less than 50% (2). It is, therefore, necessary to develop new and effective therapeutic agents.

Angiogenin was originally identified and characterized as an angiogenic ribonuclease. It has a dual effect of inducing both tumor angiogenesis and cancer cell proliferation by stimulating ribosomal RNA (rRNA) transcription in endothelial cells and cancer cells (3-6). Angiogenin has also been shown to have an anti-apoptotic effect by targeting p53, thereby accelerating cancer progression (7). Thus, anti-angiogenin therapy is considered as a good strategy for cancer treatment.

Angiogenin antagonists, including its monoclonal antibody (8), soluble binding protein (9), antisense oligos (10), siRNA (4, 5), nuclear translocation blockers (11-13), and enzymatic inhibitors (14) have all been shown to inhibit growth of human tumor cell xenografts in athymic mice. Among these antagonists, agents that target nuclear translocation of angiogenin would be more advantageous and practical than those that target angiogenin directly because angiogenin normally circulates in plasma at a concentration of 250 to 350 ng/ml (15, 16) and would need an exceedingly high dose of direct inhibitor to neutralize it (13).

During a study on the mechanism by which angiogenin is translocated to the nucleus of endothelial cells, neomycin, approved by the Food and Drug Administration as an aminoglycoside antibiotic, was found to block nuclear translocation of angiogenin, thereby inhibiting angiogenin-induced cell proliferation and angiogenesis (17). Neomycin is also known to inhibit phospholipase C-γ, which is activated by angiogenin (18). However, nuclear translocation of angiogenin does not seem to be dependent on phospholipase C-γ as another phospholipase C-γ inhibitor, U-73122, has no effect (17). The mechanism by which neomycin blocks
nuclear translocation of angiogenin remains unknown (19, 20). Nevertheless, neomycin has been shown to inhibit growth of human prostate cancer cells in xenografts in athymic mice (5). Unfortunately, neomycin itself cannot be directly applied as a cancer therapeutic agent due to its nephrotoxicity and ototoxicity (21). In a search for less toxic analogs and derivatives, neamine (Figure 1), a non-toxic derivative of neomycin, was identified (22). Both neamine and neomycin effectively block nuclear translocation of angiogenin (11) and inhibit growth of human prostate cancer cell xenografts in athymic mice by inhibiting both tumor cell proliferation and angiogenesis (13). Neamine has also been shown to prevent and reverse prostate intra-epithelial neoplasia in protein kinase B (Akt)-transgenic mice (12).

Angiogenin is up-regulated in almost all types of cancer, including breast, cervical, colonic, colorectal, endometrial, gastric, hepatic, renal, ovarian, pancreatic, prostatic, head and neck, and urothelial, as well as melanoma, lymphoma, leukemia, osteosarcoma (6). In breast and cervical cancer, and melanoma, angiogenin is thought to be up-regulated due to the hypoxic microenvironment (23-25). We have previously shown that angiogenin is up-regulated in OSCC tissue, at least partially, through the hypoxia-mediated increase of hypoxia-inducible factor-1α expression (26). We also showed that down-regulation of angiogenin expression in HSC-2 OSCC cells inhibited ribosome biogenesis and cell proliferation in vitro, as well as xenograft growth and tumor angiogenesis in vivo. This previous study established that in a hypoxic environment, angiogenin is related to oral cancer progression and can serve as a molecular target for oral cancer drug development (26).

The present study investigated the antitumor activity of neamine towards oral cancer both in vitro and in vivo and evaluated its potential as a lead compound for oral cancer therapy. We chose OSCC cell lines HSC-2 and SAS as the target tumor cell lines because HSC-2 cells secrete much higher levels of angiogenin under both normoxic and hypoxic conditions than do SAS cells (26).

Materials and Methods

Cell culture. Human OSCC cell lines HSC-2 and SAS were obtained from the Health Science Research Resources Bank (Osaka, Japan). All cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 nutrient mixture (DMEM/F-12) supplemented with 10% FBS for 24 hours. The cells were cultured in DMEM/F12 supplemented with 10% FBS for 24 hours, washed three times with serum-free DMEM/F-12, and incubated with 1 μg/ml angiogenin in the presence of 100 μM neomycin, neamine or paromomycin (Sigma-Aldrich, Saint Louis, MO, USA) at 37˚C for 30 min. As paromomycin differs from neomycin only at the C6 position of the D-glucopyranosyl ring, where -NH2 (shown in red, Figure 1) is replaced by -OH, and does not inhibit nuclear translocation of angiogenin in human umbilical vein endothelial cells (HUVECs), we used it as a control. At the end of the incubation period, the cells were washed with phosphate-buffered saline (PBS) three times and fixed with methanol at −20˚C for 10 min. The fixed cells were blocked with 30 mg/ml bovine serum albumin in PBS and incubated with 30 μg/ml of angiogenin monoclonal antibody 26-2F for 1 h, washed three times, and incubated with Alexa 488-labeled goat F(ab')2 anti-mouse IgG (Life Technologies, Eugene, OR, USA) at a 1:250 dilution for one hour. The cells were finally washed, mounted in 50% glycerol, and examined with a IX81 inverted fluorescence microscope (Olympus, Tokyo, Japan).

Cell proliferation. HSC-2 and SAS cells were seeded at a density of 2.5×10^4 cells per 35-mm dish and starved in serum-free DMEM/F12 for 24 h. They were then washed in PBS three times and cultured in serum-free DMEM/F12 in the presence of neamine or paromomycin for 48 h. Thereafter, the cells were detached by trypsinization and counted. The percentage of cell proliferation was calculated based on the cell number in the absence of inhibitors.

Growth of HSC-2 and SAS xenograft tumors in athymic mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of Okayama University (Approval No. OKU-2012191). Five-week-old male athymic mice (nu/nu) were obtained from CLEA Japan Inc. (Tokyo, Japan). HSC-2 or SAS cells, 5×10^5 per mouse, were inoculated subcutaneously into the right dorsal region of each mouse. The animals were treated with local subcutaneous injections of PBS or neamine (30 mg/kg) three times weekly. Five mice per group were used. Tumor sizes as well as body weights were measured weekly, and the former were recorded in cubic millimeters (length x width^2/2). The animals were sacrificed on day 35 for HSC-2 xenograft tumors and on day 21 for SAS xenograft tumors, and the tumor tissues were removed and weighed.

Immunohistochemistry (IHC) for HSC-2 and SAS xenograft tumor specimens. Paraffin blocks of specimens were cut at a 4-μm thickness. IHC was performed with polyclonal antibody to angiogenin used at a 1:100 dilution (sc-9044; Santa Cruz Biotechnology, Paso Robles, CA, USA) and monoclonal antibody to proliferating cell nuclear antigen (PCNA) at a 1:200 dilution (PC10; Dako, Carpenteria, CA, USA). Neovessels were stained with polyclonal antibody against cluster of differentiation 31 (CD31) at a 1:100 dilution (ab28364; Abcam, Cambridge, UK). Sections were incubated with the primary antibodies at 4°C for 16 h, and visualized with the Envision system (Dako). The sections were counterstained with Mayer’s hematoxylin.
Because nuclear angiogenin is known to stimulate cells, neamine inhibits the proliferation of HSC-2 but not SAS translocation of angiogenin in human OSCC cells. This indicates that neomycin and neamine inhibited nuclear angiogenin in the presence of neomycin or neamine. These results still bound to the cell surface and was probably trapped there. In the presence of 100 μM paromomycin (control), the amount of nuclear angiogenin decreases in tumor cells derived from neamine-treated animals (Figure 5A, right panel). Nuclear angiogenin staining was decreased in tumor cells derived from neamine-treated animals (Figure 5A, right panel), indicating that neamine inhibited nuclear translocation of angiogenin in HSC-2 cells in vivo. IHC staining with antibody against PCNA showed that the percentage of PCNA-positive cells decreased from 82.8±3.8% in PBS-treated HSC-2 tumors to 56.3±3.6% in neamine-treated HSC-2 tumors, representing a 32% decrease in cell proliferation (Figure 5B). Vessel density in the control and neamine-treated HSC-2 tumor groups, as shown by CD31-positive vessels, was 52.3±6.1 and 34.2±9.5 per microscopic area, respectively, representing a 35% decrease in tumor angiogenesis (Figure 5C). These results indicate that neamine reduced the growth of HSC-2 cells in xenografts in athymic mice and that this inhibition correlated with a decrease in both proliferation at a concentration up to 3 mM. These results suggest that the inhibitory activity of neamine against OSCC cells varies with cell type, and probably depends on the amount of angiogenin secreted by these cells. Higher angiogenin secretion may predict a more sensitive response to inhibition with neamine. Consistent with this hypothesis, HSC-2 cells secrete a much higher level of angiogenin than SAS cells do (26).

Neamine inhibits the growth of HSC-2 and SAS xenografted cells in athymic mice. During the xenograft experiment, no difference was noted in body weight, grooming behavior or food and fluid intake between PBS- (control) and neamine-treated groups. Treatment with neamine significantly reduced the growth rate of HSC-2 xenograft tumors as compared to those treated with PBS (Figure 4A and B). The average tumor weight at the end of the experiment (day 35) from the animals treated with PBS and from those treated with neamine was 1,380±487 and 480±311 mg, respectively, indicating a 65% decrease in tumor growth after neamine treatment (Figure 4C). Interestingly, treatment with neamine also significantly reduced the growth rate of SAS xenograft tumors (Figure 4A and B). The final tumor weight on day 21 from the groups of animals treated with PBS and from those treated with neamine was 1,814±154 and 696±361 mg, respectively, indicating a 62% decrease in tumor growth (Figure 4C). These results suggest that neamine effectively inhibited the xenograft tumor growth of both types of human OSCC cells in athymic mice. It should be noted that neamine does not effectively inhibit SAS cancer cell proliferation in vitro (Figure 3). Therefore, the tumor-inhibitory activity observed in vivo with SAS xenograft is most likely attributed to the effect of neamine on tumor angiogenesis, as shown below.

Neamine inhibits HSC-2 and SAS xenografted tumor growth with different mechanisms. IHC staining with an angiogenin-specific antibody showed strong angiogenin staining in the nucleus of HSC-2 tumor cells grown in PBS-treated animals (Figure 5A, left panel). Nuclear angiogenin staining was decreased in tumor cells derived from neamine-treated animals (Figure 5A, right panel), indicating that neamine inhibited nuclear translocation of angiogenin in HSC-2 cells in vivo. IHC staining with antibody against PCNA showed that the percentage of PCNA-positive cells decreased from 82.8±3.8% in PBS-treated HSC-2 tumors to 56.3±3.6% in neamine-treated HSC-2 tumors, representing a 32% decrease in cell proliferation (Figure 5B). Vessel density in the control and neamine-treated HSC-2 tumor groups, as shown by CD31-positive vessels, was 52.3±6.1 and 34.2±9.5 per microscopic area, respectively, representing a 35% decrease in tumor angiogenesis (Figure 5C). These results indicate that neamine reduced the growth of HSC-2 cells in xenografts in athymic mice and that this inhibition correlated with a decrease in both

Silver staining of nucleolar organizer region (NOR). Tissue sections (4-μm thickness) were de-paraffinized with xylene and rehydrated in graded alcohols. The sections were then autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 20 min for protein retrieval. Silver staining was performed by immersing sections in a freshly prepared solution containing one part by volume of 2% gelatin in 1% formic acid and two parts of 50% aqueous silver nitrate (Sigma-Aldrich) solution for 25 min at room temperature, as previously described (28). After thorough rinsing in distilled water, the sections were dehydrated and mounted. Silver-stained NOR dots were counted in 60 randomly selected nuclei at x1,000 magnification.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. Tissue sections (4-μm thickness) were de-paraffinized 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 by using an in situ cell death detection-POD Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Nuclear staining with 1.0 μg/ml solution of 4',6-diamidino-2-phenylindole (DAPI) was also carried out to quantify cell numbers. The apoptotic index was calculated as follows: 100% × TUNEL-positive nuclei/DAPI-positive nuclei.

Statistical analysis. Statistical analyses were conducted with the SPSS statistical software (Dr. SPSS II for Windows; Nankodo, Tokyo, Japan). The unpaired t-test was used. Results were expressed as the means±SD. A p-value of less than 0.05 was considered statistically significant.

Results

Neamine inhibits nuclear translocation of angiogenin in HSC-2 and SAS cells in vitro. Firstly, we examined whether exogenous angiogenin undergoes nuclear translocation in HSC-2 and SAS cells. Nuclear translocation of angiogenin was monitored by immunofluorescence staining. As shown in Figure 2, after 30-min incubation with 1 μg/ml angiogenin in the presence of 100 μM paromomycin (control), the majority of cell-associated angiogenin was detected in the nucleus of both types of cells. In the presence of 100 μM neomycin or neamine, the amount of nuclear angiogenin decreased markedly. Instead, strong staining on plasma membranes was observed, suggesting that angiogenin was still bound to the cell surface and was probably trapped there in the presence of neomycin or neamine. These results indicate that neomycin and neamine inhibited nuclear translocation of angiogenin in human OSCC cells.

Neamine inhibits the proliferation of HSC-2 but not SAS cells. Because nuclear angiogenin is known to stimulate rRNA transcription and induce proliferation of endothelial cells and some cancer cells, we next examined whether neamine would reduce the proliferation of HSC-2 and SAS cells in vitro. At a concentration of 2 mM, neamine completely inhibited the proliferation of HSC-2 cells (Figure 3A). However, it inhibited SAS cell proliferation by only 30% (Figure 3B). Paromomycin had no effect on cell proliferation at a concentration up to 3 mM. These results suggest that the inhibitory activity of neamine against OSCC cells varies with cell type, and probably depends on the amount of angiogenin secreted by these cells. Higher angiogenin secretion may predict a more sensitive response to inhibition with neamine. Consistent with this hypothesis, HSC-2 cells secrete a much higher level of angiogenin than SAS cells do (26).
tumor cell proliferation and angiogenesis. In contrast, neamine-inhibited in vivo growth of SAS xenografts was only accompanied by reduced angiogenesis (Figure 5F). Vessel density in the PBS- and neamine-treated SAS tumors was 48.9±10.3 and 22.7±5.8 per microscopic area, respectively, representing a 54% decrease in tumor angiogenesis after neamine treatment (Figure 5F). No difference was observed in nuclear staining of angiogenin and in PCNA-positive cells between PBS- and neamine-treated SAS xenografts (Figure 5D and E). These results confirmed that the antitumor activity of neamine against SAS xenograft tumors was mainly due to inhibition of tumor angiogenesis.

Neamine reduces ribosome biogenesis and induces apoptosis of HSC-2 cells, but not SAS cells, in vivo. To assess neamine-induced changes in ribosome biogenesis, we examined the number of NOR in the tumor samples derived from PBS- and neamine-treated animals. Treatment with neamine reduced the average number of NOR dots per cell from 8.0±3.4 to 3.1±1.4 in HSC-2 xenograft tumor cells, indicating a 61% decrease in ribosome biogenesis (Figure 6A, left panel). Consistent with the observation that neamine does not directly affect SAS tumor cells, we did not observe any changes in NOR numbers in SAS xenograft tumor cells after neamine treatment (Figure 6A, right panel). TUNEL staining showed that neamine treatment induced robust apoptosis of HSC-2 xenograft tumor cells (Figure 6B, left panels). The apoptotic index in PBS- and neamine-treated HSC-2 tumors was 5.0±1.2% and 13.2±6.1%, respectively. Consistent with a lack of direct effect of angiogenin on SAS

![Chemical structure of neomycin and neamine](image)

**Figure 1.** Chemical structure of neomycin and neamine. The amino group in neomycin and neamine shown in red is essential for blocking nuclear translocation of angiogenin.

![Inhibition of nuclear translocation of angiogenin in HSC-2 and SAS cells by neamine](image)

**Figure 2.** Inhibition of nuclear translocation of angiogenin in HSC-2 and SAS cells by neamine. Cells were incubated with 1 μg/ml angiogenin in the presence of 100 μM neomycin, neamine or paromomycin at 37°C for 30 min. Angiogenin was visualized by monoclonal antibody and Alexa 488-labeled goat anti-mouse IgG.
cells, neamine treatment did not induce apoptosis of cells of SAS xenograft tumors (Figure 6B, right panels).

**Discussion**

Angiogenin is up-regulated in various types of human cancers (6). However, the pattern and intensity of angiogenin expression in the extracellular matrix, cytoplasm, and nucleus vary with the tumor type (5), suggesting that the ability of tumor cells to produce angiogenin and to translocate it into the nucleus varies according to tumor type. Increased nuclear translocation of angiogenin has so far been reported in cervical (4, 5), prostate (5), breast (11), and lung (29) cancer and is associated with tumorigenesis and cancer cell proliferation. In the nucleolus, angiogenin mediates rRNA transcription by binding to CT repeats in the promoter region of the rRNA of genes (30). Angiogenin also exerts its ribonucleolytic activity, which is essential for angiogenesis, by catalyzing the generation of 18S and 28S rRNA in endothelial cells (31). Both neomycin and neamine have been reported to inhibit nuclear translocation of angiogenin and to suppress cancer cell proliferation and angiogenesis in human PC-3 prostate (5), MDA-MB-435 breast (11), and A549 lung (29) cancer. It is, thus, considered that besides suppressing tumor angiogenesis, neomycin and neamine also directly inhibit proliferation of cancer cells that abundantly produce angiogenin and actively translocate it into the nucleus. In a previous report, levels of angiogenin secreted into the culture medium of HSC-2 cells were found to be 1.56 and 4.06 pg/10^3 cells/day under normoxic and hypoxic conditions, respectively (26). In contrast, levels of angiogenin secreted into the culture medium of SAS cells under these conditions were only 0.24 and 0.42 pg/10^3 cells/day, respectively (26). Therefore, HSC-2 cells secrete a much higher level of angiogenin under both normoxic and hypoxic conditions than SAS cells do. From these findings, we hypothesized that neamine would have a more effective anti-proliferative activity on HSC-2 than on SAS cells.

In the present study, we first confirmed that angiogenin undergoes nuclear translocation in OSCC cells as it does in HUVECs. We found that exogenous angiogenin underwent nuclear translocation in both HSC-2 and SAS cells in vitro and that neamine clearly blocked this translocation in both cell lines. Because nuclear angiogenin stimulates rRNA transcription and leads to cell proliferation in both HUVECs and cancer cells (3-6), we next examined the effect of neamine on the proliferation of HSC-2 and SAS cells in vitro. Neamine was previously shown to inhibit angiogenin-stimulated HUVEC proliferation in a dose-dependent manner with an apparent half-maximal inhibitory concentration (IC_{50}) of ~5 μM (11). In the present study, we found that neamine also completely inhibited proliferation of HSC-2...
Figure 4. Effect of neamine on xenograft growth of HSC-2 and SAS cells in athymic mice. HSC-2 or SAS cells, $5 \times 10^5$ per mouse, were inoculated subcutaneously into the right dorsal region of each mouse. The animals were treated with local subcutaneous injections of PBS or neamine (30 mg/kg) three-times weekly. Five mice per group were used. A: Tumor size (as volume) was measured weekly and recorded in cubic millimeters ($\text{length} \times \text{width}^2/2$). B: Mice were sacrificed at day 35 for HSC-2 and day 21 for SAS cells. Tumor tissues were dissected, weighed, and photographed. C: Final tumor weights are shown as the means±SD. *p<0.05, **p<0.01.
cells. However, a relatively high dose of neamine, with an apparent IC₅₀ of 1 mM, was needed for the inhibition of HSC-2 proliferation. In contrast, neamine barely inhibited the proliferation of SAS cells. Maximum inhibition of only ~30% was achieved. The reason for the differential effect of neamine towards HSC-2 and SAS cells may be related to the different amount of angiogenin these cells secrete and the different level of nuclear translocation of angiogenin.

Consistently, angiogenin was found both in the nucleus and in the cytoplasm of HSC-2 xenograft tumors grown in nude mice. In contrast, very weak angiogenin signals were revealed in SAS xenograft tumors. Treatment with neamine reduced nuclear angiogenin and the number of NOR dots, as well as PCNA-positive tumor cells, in HSC-2 xenograft tumors but not in SAS xenograft tumors. Neamine treatment also increased tumor cell apoptosis in the xenograft tumors derived from HSC-2 but not in those derived from SAS cells. These results suggest that angiogenin has a more profound role in OSCC tumors that produce a high amount of angiogenin than in those that produce a low amount of angiogenin. Contrary to our prediction, subcutaneous treatment with neamine at a dose of 30 mg/kg of body weight effectively reduced the growth of both HSC-2 and SAS cell xenografts in athymic mice. The percentage of inhibition of xenograft growth of the two cell lines was 65% and 62%, respectively. Tumor angiogenesis decreased in both types of xenografts. However, tumor cell proliferation and apoptosis were altered only in neamine-treated HSC-2 xenografts. One plausible interpretation for a similar inhibitory activity of neamine towards in vivo growth of HSC-2 and SAS cells would be that inhibition of tumor angiogenesis overrides that of direct tumor cell proliferation, which will actually increase the clinical utility of this class of compound as the expression level of angiogenin in OSCCs seems to be heterogeneous (26).

In conclusion, our results suggest that neamine effectively inhibits oral cancer progression through inhibition of tumor angiogenesis. Neamine also directly inhibits proliferation of certain types of oral cancer cells. Taken together, neamine is a promising candidate as a therapeutic agent in oral cancer.

Figure 5. IHC analyses of HSC-2 and SAS xenograft tumor specimens. IHC staining for angiogenin, PCNA, and neovessels in the tumors from control (PBS-treated) and neamine-treated groups. CD31-positive neovessels in each tumor were counted in five most vascularized areas at ×200 magnification and averaged. PCNA-positive and total numbers of cells were counted in five randomly selected areas at ×200 magnification. Images shown were from a representative animal of each group. Vessel density (vessels per field) and percentage of PCNA-positive cells are shown as the means±SD for each group. Bars, 100 μm. **p<0.01.
Conflicts of Interest
None declared.

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