Neamine inhibits cell proliferation, migration, and invasion in H7402 human hepatoma cells

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ABSTRACT

الأهداف: اكتشاف مدى تأثير النيامين (Neamine) على هجرة الخلايا السرطانية (H7 402) وغزوها وتكاثرها في خلايا الكبد البشرية.

الطريقة: أُجريت هذه الدراسة في معهد علم الأحياء والجينات بمدرسة العلوم التابعة للجامعة الشمالية الشرقية، شانغشون، الصين وذلك خلال الفترة من أكتوبر 2008م إلى فبراير 2010م. لقد قمنا أولاً باعتماد مقايسة إم تي تي (MTT assay) واختبار الأغار الطري (soft agar assay) من أجل تحديد مدى تأثير النيامين على تكاثر الخلايا السرطانية (H7 402)، فيما تم التعرف على هجرة الخلايا السرطانية وغزوها من خلال اختبار ترانزويل (transwell assay)، وبعد ذلك قمنا بتحديد مدى الاضطراب الصبغي الذري أو الإزفاء (translocation) في بروتين الأنجيوجينين (angiogenin) من خلال اختبار صبغة الفلورسين المناعي (immunofluorescene). وأخيراً تم إعادة الاستقرار للخلايا من خلال إدخال البلازميدات pCI–Ang (+) وبلازميدات pCI–Ang (–) داخل غشاء الخلايا، حيث تحتوي هذه البلازميدات على منطقة تشفير كاملة لبروتين الأنجيوجينين البشري، وقد قمنا بهذه الخطوة من أجل تحديد معدلات الأنجيوجينين الزائدة والناقصة وتقييم مدى تأثير النيامين على تثبيط نشاط الأنجيوجينين المسئول عن تحفيز تكاثر الخلايا السرطانية.

النتائج: أظهرت النتائج بأن النيامين قد قام بتثبيط تكاثر الخلايا السرطانية وغزوها وهجرتها، حيث أنه قام بإيقاف الاضطراب الصبغي في بروتين الأنجيوجينين وبالتالي تثبيط نشاط هذا البروتين الذي يقوم بتحفيز تكاثر الخلايا السرطانية.

خاتمة: أشارت الدراسة إلى فعالية النيامين في تثبيط نمو الخلايا السرطانية (H7 402)، وبما أن تأثيره السمي على الجسم يعد أقل بكثير من تأثير النيومايسين (neomycin)، كما أنه مشابه لفعالية وقلة سمية الستريبتومايسين (streptomycin) والكانامايسن (kanamycin) فإنه قد يكون عاملاً مهماً في تطوير علاج سرطان الخلايا الكبدية.

Objectives: To explore the effect of neamine on cell proliferation, migration, and invasion in H7402 human hepatoma cells.

Methods: This study was conducted at the Institute of Genetics and Cytology, School of Life Science, Northeast Normal University, Changchun, China between October 2008 and February 2010. First, we employed the MTT (thiazol blue tetrazolium bromide) and soft agar assay to detect the effect of neamine on cell proliferation, and investigated the migration and invasion by using a transwell assay in H7402 cells. We, then, investigated nuclear translocation of angiogenin by immunofluorescence staining. Finally, we stable transfected H7402 cells with the plasmids pCI-Ang (+) and pCI-Ang (-), which contain the entire coding region of human angiogenin in the sense and antisense orientations, to obtain angiogenin under-expressing/over-expressing transfectants, and investigated the effect of neamine on angiogenin induced cell proliferation.

Results: The results showed that neamine positively inhibited the proliferation, migration, and invasion of H7402 cells. Nuclear translocation of angiogenin was blocked by neamine, and angiogenin-induced cell proliferation was inhibited by neamine.

Conclusion: Neamine positively inhibited H7402 cells. Since the toxicity of neamine is much less than neomycin, and is close to that of streptomycin and kanamycin, it may serve as a lead agent for the development of hepatocellular carcinoma therapeutics.

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Neomycin, an aminoglycoside antibiotic, has been shown to block the nuclear translocation of angiogenin in endothelial cells, thereby inhibiting its mitogenic and angiogenic activity.[1] However, the nephro- and oto-toxicity of neomycin preclude its use as a prolonged agent. Neamine, a nontoxic degradation product of neomycin, has an antiangiogenic activity comparable to neomycin. Moreover, it has been reported that the xenograft growth of MDA-MB-435 human breast cancer cells and HT-29 human colon adenocarcinoma in athymic mice was inhibited by neamine.[2] Angiogenin is a 14 kDa angiogenic protein, which is originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells.[3] It has been shown to play an important role in tumor angiogenesis.[4] Its expression is up-regulated in many types of cancers, including hepatocellular carcinoma.[5-9] The establishment, progression, and metastasis of xenographic human tumors in athymic mice might be inhibited or delayed when angiogenin activity was inhibited.[10] Angiogenin undergoes nuclear translocation in endothelial cells, and this process is necessary for angiogenesis. The angiogenic activity was abolished when inhibited nuclear translocation of angiogenin or mutated nuclear localization sequence of angiogenin.[11] Nuclear translocation of angiogenin is involved in ribosomal RNA transcription and cell proliferation in endothelial cells and tumor cells.[12,13] Hepatocellular carcinoma (HCC) is the most common hypervascular tumor and is one of the most common cancers worldwide.[14,15] Recent studies demonstrated that the expressions of many angiogenic factors are closely related to the growth and metastasis of HCC,[6,16] and HCC expressed a high level of angiogenin.[17] In the present study, we examined the effect of neamine on H7402 cells. Since the toxicity profile of neamine is close to that of streptomycin and kanamycin, which is ~20-fold less toxic than neomycin,[18] in the present study, we examined the effect of neamine on H7402 cells.

Methods. This study was conducted at the Institute of Genetics and Cytology, School of Life Science, Northeast Normal University, Changchun, China between October 2008 and February 2010. All tests were approved by the Ethical Committees of Northest Normal University.

Cell culture. The human hepatoma cell line H7402 was acquired from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences in Shanghai. The H7402 cells were cultured in Dulbecco's modifed Eagle's medium (DMEM) plus 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37^{0}C in a humidified atmosphere containing 5% CO_2.

Cell proliferation. An MTT (thiazol blue tetrazolium bromide) assay was used to analyze cell proliferation. Cells were seeded into 96-well plates at a density of 7.0x10^3 cells/well. After 48 hours of incubation, a 10µl MTT solution (5 mg/ml in phosphate buffer solution [PBS]) was added to each well. The tetrazolium/formazan reaction was allowed to proceed for 4 hours at 37^{0}C, and then 100µl of the solubilization buffer (10% sodium dodecyl sulfate in 0.1% HCl) was added to all the wells to stop the reaction. The absorbance was determined using a 96 well multiscanner autoreader at 540 nm.[19]

Anchorage-independent cell proliferation was determined by a soft agar assay. Cells were seeded at a density of 4×10^3 cells per 35-mm cell culture dish in 0.35% agar and cultured for 14 days at 37^{0}C.[12] Colonies were counted in the entire dish, and the colony size was determined by measuring the diameters of colonies in 10 microscope fields. The 2-tailed Student's t test was used to determine the differences between the groups.

Transwell migration and invasion assay. Cells were serum starved for 24 hours, harvested, and resuspended in medium containing 1% bovine serum albumin in the presence or absence of 200 µM neamine. Cells (5x10^4) were added to the top chambers of 24-well Transwell plates (invitrogen). For invasion assay, the top chambers were coated with 1mg/ml matrigel. Medium containing 10% fasting blood sugar was added to the bottom chambers. Cells were incubated for 48 hours at 37^{0}C. The cells were fixed with 0.1% glutaraldehyde-PBS for 20 minutes, and stained with 0.2% crystal violet for one hour. Nonmotile cells on top of each filter were removed. The number of migrating cells or invasive cells were counted with entire fields, and the results were calculated as migration/invasion rate in relation to parental control cells. Each experimental condition was carried out thrice.[20]

Immunofluorescence staining. Cells were washed with phosphate buffered saline (PBS), fixed with cold methanol, and washed again with PBS containing 5% bovine serum albumin (BSA). The fixed cells were then incubated with anti-angiogenin monoclonal antibody for one hour. The cells were then washed with PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for one hour. After washing with

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PBS, the cells were observed under laser scanning confocal microscopy (Olympus, FV1000, Tokyo, Japan).

Stable transfection. Cells were transfected with the plasmids pCI-Ang (+) and pCI-Ang (-), which contain the entire coding region of human angiogenin in the sense and antisense orientations, or with the pCI-neo vector alone by using the electroporation method and selected by G418.[19]

Enzyme-linked immunosorbent assay. The angiogenin secretion levels in transfected cells were detected by enzyme-linked immunosorbent assay (ELISA) as described previously.[19] A standard curve was obtained by using human recombinant angiogenin.

Statistical analysis. Experimental results are expressed as means ± SD of at least 3 independent experiments. For statistical analysis, a Student's t test was used. A value of $p<0.05$ was considered significant. All analyses were carried out using the Statistical Product and Service Solutions 12.0 (SPSS Inc., Chicago, IL, USA).

Results. Neamine inhibits H7402 cell proliferation and colony formation. The effect of neamine and neomycin on H7402 cell proliferation is shown in **Figure 1a**. Neamine and neomycin inhibit H7402 cell proliferation in a dose-dependent manner. Cell proliferation was inhibited by 20.7% and 40.6% in the presence of 100μM and 400μM neamine. The colony formation of H7402 cells was also inhibited by neamine (**Figure 1b**). Compared with control cells, 200μM neamine decreased the colony number by 36.6%, from 1321±37 to 837±16 (p=0.00085), the colony size was also decreased by 41.1% from an average diameter of 90±5 to 53±7 (p=0.00092).

Neamine inhibits the migration and invasion ability of H7402 cells. We evaluated the migration of neamine-stimulated cells and their parental control cells on Transwell plates. **Figure 2a** is a representative photograph of the migration assay. There was a decrease of 51% in the number of migrating cells in the neamine stimulated cells compared with the parental control (p=0.0013, **Figure 2b**). The invasive ability of neamine stimulated cells was only 41% of parental control cells using a Matrigel invasion assay (p=0.0017, **Figure 2c**). We did the cell count after 24 hours of starvation to estimate the effect of the proliferation rate in similar conditions on migration and invasion assays. Neomine inhibits nuclear transloction of angiogenin in H7402 cells. Immunofluorescence was used to monitor nuclear translocation of angiogenin in H7402 cells. As shown in **Figure 3a**, after one hour of incubation in the absence of angiogenin, the angiogenin accumulated in the nucleolus. In the presence of 400 μM neamine (**Figure 3b**), the amount of nuclear angogenin was decreased markedly,

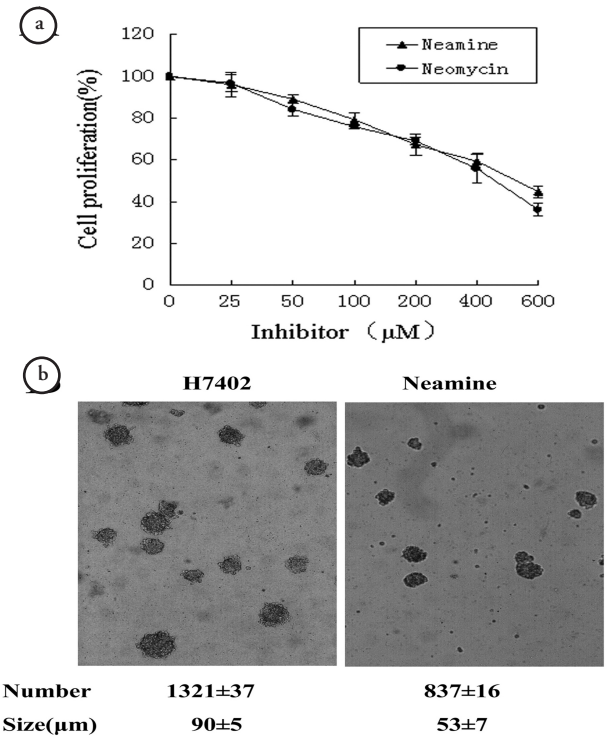

Figure 1 - Neamine inhibits H7402 cell proliferation and colony formation. **a)** MTT assay was carried out to detect cell proliferation. The result showed that neamine and neomycin both inhibit H7402 cell proliferation in a dose-dependent manner. **b)** Colony formation of H7402 cells was detected by Soft agar assay. The result showed that colony number and colony size were decreased in the presence of 200μM neamine.

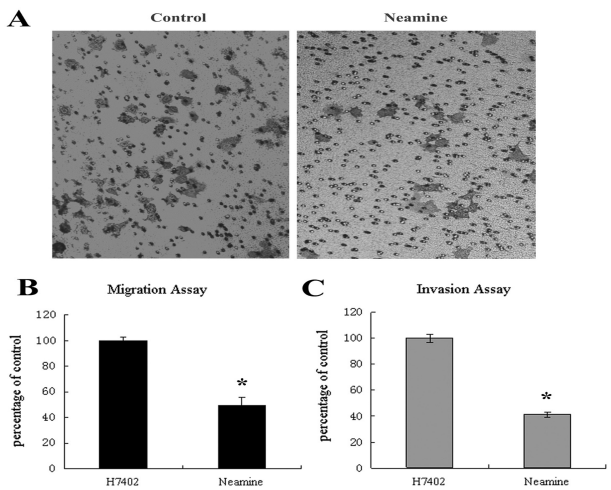

Figure 2 - The migration and invasion ability of H7402 cells is inhibited by neamine. **a)** Representative photos of migration assay. Magnification, 100x. **b)** Neamine decreased migration in comparison with their parental control cells. **c)** Neamine decreased invasion compared with control cells. Results are expressed as percentage of parental control cells. (Columns, mean of three independent experiments, *p<0.002).

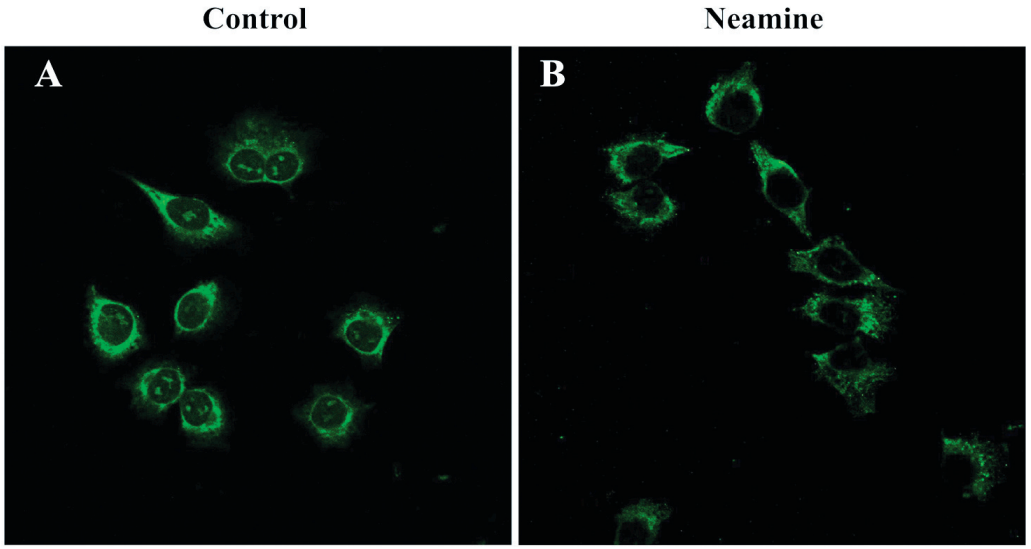

Figure 3 - Neamine inhibits the nuclear translocation of angiogenin in H7402 cells. H7402 cells were cultured in Dulbecco's modifed Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C for 24 hours and then stimulated with **a**) 1μg/ml angiogenin **b**) 1μg/ml angiogenin and 400μM neamine for one hour. Immunofluorescence staining was used to detect the nuclear translocation of angiogenin.

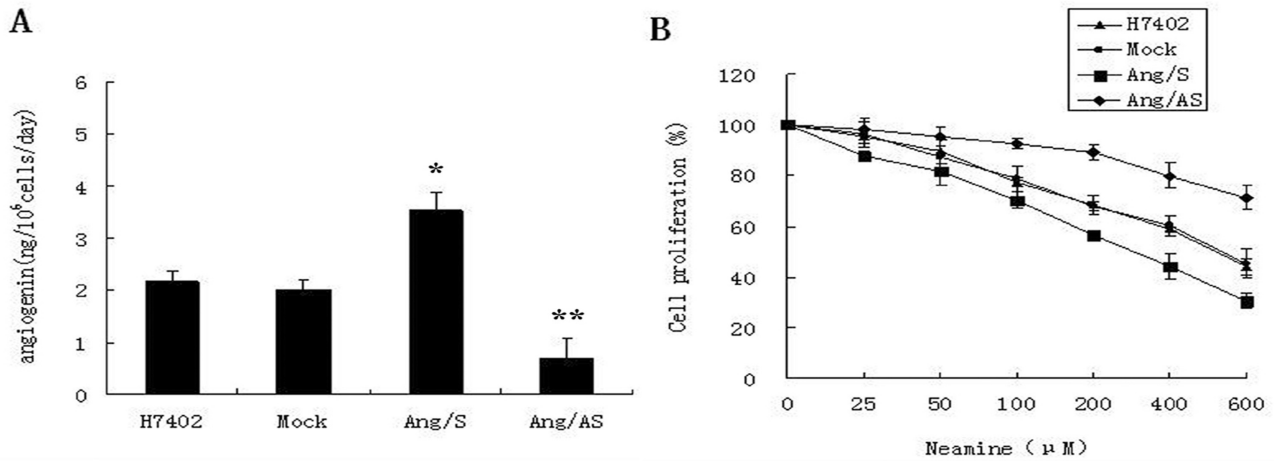

Figure 4 - Neamine inhibits angiogenin induced cell proliferation. **a**) Expression of angiogenin in transfectants was determined by enzyme-linked immunosorbent assay)(*p<0.002, †p<0.001). **b**) Neamine inhibits angiogenin induced cell proliferation. Transfectants and control cells (7.0×10^3) were seeded into a 96-well plate and cultured in Dulbecco's modifed Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) and neamine at 37°C for 48 hour. MTT assay was carried out to examine the cell proliferation. The data shown are means ± SD of 6 independent experiments.

suggesting that neamine blocked the nuclear transloction of angiogenin. Neamine inhibits angiogenin-induced cell proliferation. Since neamine prevents the nuclear translocation of angiogenin, here, we examined its effect on angiogenin-induced cell proliferation. We transfected H7402 cells with the plasmids pCI-Ang (+) and pCI-Ang (-) to obtain angiogenin under-expressing/over-expressing transfectants. As shown in **Figure 4a**, angiogenin was decreased in antisense transfectants (Ang/AS) and increased in sense transfectants (Ang/S) of H7402 cells. To understand whether or not the effect of neamine depended on angiogenin, we examined the cellular response of angiogenin under-expressing/over-expressing transfectants toward neamine. **Figure 4b** showed that the proliferation of angiogenin under-expressing transfectants was also inhibited by neamine, but to a much lesser extent. A 100μM concentration of neamine inhibited the cell proliferation by only 7.35%, and a 400μM concentration inhibited the proliferation by only 20.1%. On the contrary, the inhibitory effect of neamine in angiogenin over-expressing transfectants was much greater. A 100μM concentration already inhibited the proliferation by 29.6%, and a 400μM concentration inhibited the proliferation by 56%. These

results indicate that the inhibitory effect of neamine on cell proliferation was related to the angiogenin expression level.

Discussion. Neomycin is a new angiogenesis inhibitor identified through mechanistic studies of angiogenin, one of the potent angiogenic factors. It inhibits both angiogenin-stimulated proliferation of endothelial cells and angiogenin-induced angiogenesis.[1] The antiangiogenesis activity is at least partially attributable to its inhibition of nuclear translocation of angiogenin in endothelial cells, a necessary step for angiogenesis. However, among the common aminoglycoside antibiotics, neomycin has the most severe nephrotoxicity and ototoxicity.[2] Parenteral use of neomycin has largely been abandoned for this reason. The nephro- and oto-toxicity of neomycin would seem to preclude its prolonged use as an anti-cancer agent. Neamine is a degradation product of neomycin, and it is also produced in small amounts by Strotomyces fradiae.[21] The toxicity profile of neamine is ~20-fold less than neomycin and is similar to that of streptomycin and kanamycin.[18] It has been shown to have an antiangiogenic activity comparable to that of neomycin and it can inhibit xenograft growth of HT-29 human colon adenocarcinoma and MDA-MB-435 human breast cancer cells in athymic mice.[2] Therefore, we investigated the effect of neamine on H7402 cells. The results showed that both neamine and neomycin effectively inhibited the growth of H7402 cells in a dose-dependent manner with an apparent IC50 of ~500μmol/L. The colony formation, migration, and invasion abilities were inhibited by neamine.

The function of angiogenin as a potent inducer of neovascularization has been described. It can induce cell proliferation, activate cell-associated proteases, and stimulate migration and invasion in endothelial cells.[22] Angiogenin-stimulated rRNA transcription is a general requirement for angiogenesis.[23] Kishimoto et al reported that angiogenin is essential for a variety of other angiogenic factors including acidic fibroblast growth factor, basic fibroblast growth factor, endothelial growth factor, and vascular endothelial growth factor induced angiogenesis.[23] It has been shown that endogenous angiogenin regulated rRNA transcription and ribosome biogenesis to increase HeLa cell proliferation.[12] In our previous work, we found that angiogenin plays an important role in melanoma cell proliferation.[19] Human angiogenin can be rapidly translocated to the nucleus of human umbilical vein endothelial cells, and this process is microtubule and lysosome independent.[13] Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity and the nuclear function of angiogenin is related to rRNA production.[24] A recent study[12] showed that angiogenin is translocated to the nucleus of HeLa cells and is involved in cell proliferation. The anti-tumor activity of neomycin and neamine is related to the blockade of nuclear translocation of angiogenin. Neomycin was discovered to block nuclear translocation of angiogenin and to inhibit angiogenin-induced cell proliferation in endothelial cells and PC-3 cells.[2]

Hepatocellular carcinoma is one of the most common hypervascular cancers. The expression of angiogenin increased in HCC.[6] In this study, we found that neamine inhibited the nuclear translocation of angiogenin in H7402 human hepatoma cells. The effect of neamine on angiogenin-induced cell proliferation was examined in angiogenin over-expressed/down-expressed cells. The results showed that the inhibition of neamine is related to the angiogenin level. Neamine has a much more effective inhibition on angiogenin over-expressed cells than down-expressed cells. These results suggest that the underlying mechanism of its anti-tumor activity of neamine is related to the blockade of angiogenin nuclear translocation in H7402 cells. However, the definite mechanism of the anti-tumor activity of neamine is still unknown, and the effect of neamine on tumor in vivo is not demonstrated in this study, and these will be researched in future work. Angiogenin plays an important role in both tumor establishment and progression. Neamine has an effective inhibition on proliferation, migration, invasion, and angiogenin nuclear translocation in H7402 cells. Further studies aiming to understand the molecular details of this action will allow the development of inhibitors with enhanced antiangiogenesis and antitumor potency, and neamine might serve as a promising candidate for further development as a therapeutic agent in hepatocellular carcinoma.

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