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Sub-chronic 90-day toxicity of neamine in SD rats and its anti-liver cancer activity *in vitro* and *in vivo*

Yanli Wu [a,1], Yongdong Feng [b,1], Yanling Li [a], Yiping Xu [c], Nian Shi [c], Guo-fu Hu [d], Yunxia Wu [a,*]

[a] Department of pharmacology, School of Pharmacy, Huazhong University of Science and Technology, Wuhan 400030, China
[b] Department of Gastrointestinal Surgery, Tongji Hospital, Huazhong University of Science and Technology, Wuhan 400030, China
[c] Department of hygienic toxicology, School of Public Health, Huazhong University of Science and Technology, Wuhan 400030, China
[d] Molecular Oncology Research Institute, Tufts Medical Center, Tufts University, Boston 02111, USA

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ABSTRACT

Neamine, an inhibitor of angiogenin (ANG), is a new investigative anticancer drug currently in preclinical stage. Here we report the 90-day sub-chronic toxicity of neamine in SD rats and its anti-liver cancer activity *in vitro* and *in vivo*. Neamine has a No Observed Adverse Effect Level (NOAEL) of 12 and 16 $mg \cdot kg^{-1} \cdot d^{-1}$ for female and male rats, respectively. No mortality was found. The adverse effects included increased organ coefficients of spleen and kidney, increased BUN in both female and male rats at high dose, increased CR and decreased organ coefficients of heart and liver in male rats at high dose. All of which, except the kidney coefficient and BUN in males, returned to normal levels after 28-day recovery. Histopathological examination revealed vacuolar degeneration of glomerulus, degeneration of renal tubules and cast in the kidneys, which were also recovered except in males of high-dosing group. These results indicate that kidney is the most susceptible organ for neamine toxicity. Tissue microarray analysis validated that ANG is up-regulated in hepatocellular carcinoma accompanied by increased nuclear translocation, suggesting that ANG is a possible target for drug development in liver cancer treatment. Neamine blocked nuclear translocation of ANG in HUVEC and HepG2 cells, and inhibited ANG-stimulated cell proliferation without affecting basal level cell proliferation. Neamine also inhibited progression of HepG2 xenografts in athymic mice accompanied by decreased angiogenesis and cancer cell proliferation. These results suggest that neamine is a specific ANG inhibitor with low toxicity and high anti-liver cancer efficacy.

1. Introduction

Angiogenin (ANG), a 14.4 kD angiogenic ribonuclease, was first isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells based on an *in vivo* angiogenesis assay (Fett et al., 1985), and has been shown to play an essential role in tumor angiogenesis as well as in cancer cell proliferation, migration and invasion (Hu et al., 1994; Shestenko et al., 2001). ANG undergoes nuclear translocation in endothelial cells where it accumulates in the nucleolus and stimulates ribosomal RNA (rRNA) transcription, a rate-limiting step in ribosome biogenesis. ANG has also been shown to trigger a number of biological processes, including cell migration, invasion, proliferation, and formation of tubular structures (Gao and Mechanisms, 2008). Although the mitogenic activity of ANG is not as strong as other angiogenic proteins such as acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), ANG is indispensable for cell proliferation and angiogenesis induced by these factors (Kishimoto et al., 2005).

It has been reported that ANG is up-regulated in a variety of cancer cells and its concentration in plasma is elevated in many types of human cancer patients than in normal subjects (Eberle et al., 2000; Bodner-Adler et al., 2001; Montero et al., 1998; Shimoyama et al., 2002; Campo et al., 2005; Ugurel and Rappl, 2001; Kushlinskii et al., 2000; Shimoyama et al., 1999). ANG antagonists including monoclonal antibodies (Olson et al., 2002), soluble binding proteins (Olson et al., 1995), and small molecules inhibitors (Kao et al., 2002) have been shown to inhibit growth of xenograft human tumor in athymic mice, accompanied by a marked decrease in tumor angiogenesis.

Neomycin has been reported to block nuclear translocation of ANG in endothelial cells, thereby inhibiting cell proliferation and angiogenesis induced by ANG (Neomycin, 1998) and other angiogenic factors (Hu, 2001). Neomycin may thus serve as an effective anti-tumor agent by targeting ANG. However, neomycin has not been considered as a favorable candidate drug for clinical development because of its known nephrotoxicity and ototoxicity (Koeda et al., 1982).

In an effort to search for low toxic derivatives and analogs of neomycin, neamine was found to have equivalent inhibitory effects on ANG as

* Corresponding author.
E-mail address: wuyunxia@hust.edu.cn (Y. Wu).
[1] Those authors have contributed equally to this paper.

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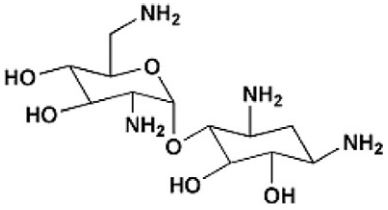
does neomycin (Saori et al., 2005). Neamine has been shown to inhibit ANG-mediated angiogenesis and cell proliferation in a variety of human xenograft cancers including HT-29 colon adenocarcinoma, MDA-MB-435 breast carcinoma, A431 epidermoid carcinoma, H7402 hepatoma (Zhao et al., 2010), HSC-2 and SAS oral cancer (Kishimoto et al., 2014), PC-3 prostate cancer, and HeLa cervical cancer cells (Ibaragi et al., 2009; Liu et al., 2015a; Liu et al., 2015b). Since the nephrotoxicity and ototoxicity of neamine were reported to be only 5% (Williams et al., 1987) and 6% (Au et al., 1986) of those of neomycin, respectively, neamine appears to be a promising low-toxic anti-cancer agent suitable for further development.

Liver cancer is the second and sixth leading cause of cancer death in men in developing and developed countries, respectively (Msph et al., 2015). Most (70–90%) primary liver cancers occurring worldwide are hepatocellular carcinoma (HCC) (Msph et al., 2015). HCC is highly malignant, has poor prognosis with high morbidity and mortality (Rasmussen and Garden, 1996), and is one of the most resistant tumors to chemotherapy (Llovet and Bruix, 2000). A wide variety of chemotherapeutic agents have been tried and are currently in use, including fluorouracil, doxorubicin, mitoxantrone, and cisplatin, but no regimen has been proven to be curative (Alsowmely and Hodgson, 2015). ANG has been shown to be up-regulated in HCC (Hisai et al., 2003), having a 10–20 fold increase in HCC cells (Bárcena et al., 2015). Neomycin has displayed an effective inhibitory activity against HCC growth and angiogenesis through suppressing nuclear translocation of ANG (Bárcena et al., 2015). Neamine was also found to inhibit cell proliferation, migration, and invasion of H7402 human hepatoma cells (Zhao et al., 2010). The purpose of this study was to evaluate the anti-liver cancer activity of neamine, and document the nonclinical safety profile of neamine in SD rats during a 90-day intraperitoneal (ip.) administration and the reversibility, persistence or delayed occurrence of target organ toxicity after a 28-day recovery period.

2. Materials and methods

2.1. Reagents and cell culture

Neamine (molecular formula: $C_{12}H_{26}N_4O_6$, molecular weight: 322.36, purity: 98%, lot number: 20151227) was synthesized in house, structurally characterized by NMR, stored at 4 °C, and dissolved in saline when used.



Human umbilical vein endothelial cells (HUVECs) and HepG2 liver cancer cells were purchased from ATCC. The HUVECs and HepG2 cells were cultured in Endothelial Cell Medium (ECM) and Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Inc., Logan, UT, USA), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories), 200 U/mL penicillin, and 200 mg/mL streptomycin (Hyclone Laboratories) at 37 °C with 5% CO_2.

2.2. Experimental animals

Healthy post-weaning SD rats, 60 male and 60 female, were supplied by Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Laboratory animal facility license No. 00121912). The average body weight at the start of treatment was 135.65 ± 9.19 g for males and 167.93 ± 15.01 g for females. Basic feeding was provided by the Laboratory Animal Center. Animals were housed individually in sterilized stainless steel cage (30 × 24 × 20 cm) with ceramic food containers and polyethylene water bottles. The animals were acclimated to the environment for 10 day before drug administration.

Male balb/c nude mice (4 weeks old) were provided by Beijing HFK bioscience Co. Ltd. (No. 11401300039653). The mice were maintained under specific pathogen-free conditions (No. 00140238) in a temperature and humidity-controlled environment in the department of experimental animal center of Huazhong University of Science and Technology, and acclimatized for at least 1 week prior to experiments.

All animal experiments were approved by the IACUC of Huazhong University of Science and Technology, and were in accordance with the Chinese animal protection laws and guidelines for the use of live animals for scientific purposes.

2.3. Tissue microarray assay

Tissue microarray was purchased from Shanxi Chaoying Biotechnology Co., Ltd., China. This array contains 20 cases of hepatocellular carcinoma, four cases of hepatic normal tissues. Those were discarded pathological samples and coded in a way that patient identities cannot be revealed. The study was approved by IRB of Huazhong University of Science and Technology. The tissue microarray was immunohistochemically stained for ANG and observed under microscope at 400× magnification.

2.4. Sub-chronic toxicity study

2.4.1. Dose and treatment schedule. Rats (n = 15/sex/group) were grouped randomly. Neamine and saline were administrated i.p. for 5 consecutive days every week in the sub-chronic toxicity study. The doses of neamine were 60, 12, and 6 $mg \cdot kg^{-1}$ for female, and 80, 16, and 8 $mg \cdot kg^{-1}$ for male (Table 1), corresponding to 10, 2, and 1%, respectively, of the LD_{50} that was predetermined. Ten animals per group were sacrificed by cervical dislocation on day 90. The remaining 5 per group were continuously observed for another 4 weeks as the recovery period, and were sacrificed by cervical dislocation on day 118.

2.4.2. Clinical observation. Mortality and clinic signs were evaluated daily during the treatment and recovery period in the 118-day study. Any changes in behavior, reaction to treatment or illness were observed and recorded including time of occurrence and status. A complete necropsy was performed for every animal upon euthanization.

2.4.3. Body weight and food consumption. Body weights were recorded every week and cumulative weight gain was calculated. The amounts of food were recorded weekly before being supplied to each cage and the remnants were measured at the end of the week. Food consumption rate (weight gain / food consumption * 100%) was calculated every week.

2.4.4. Laboratory test. The parameters listed in Table 2 were measured on day 90 (n = 6) and 118 (n = 5) except for urinalysis.

(1) Urinalysis was carried out on the last two days of the two end points. Animals (fasted overnight but provided with water for

Table 1
Dosing regimen for male and female SD rats.

Group	Dose ($mg \cdot kg^{-1}$)		Remark
	Female	Male	
A	60	80	High dose
B	12	16	Medium dose
C	6	8	Low dose
D	0	0	The control