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Original article

Aminopeptidase N inhibitor 4cc synergizes antitumor effects of 5-fluorouracil on human liver cancer cells through ROS-dependent CD13 inhibition

Zhi-Peng Sun[a], Jian Zhang[b], Li-Hong Shi[a], Xiu-Rong Zhang[a], Yu Duan[c], Wen-Fang Xu[d], Gong Dai[a,*], Xue-Jian Wang[a,*]

[a] Key Laboratory of Applied Pharmacology in Universities of Shandong, Department of Pharmacology, School of Pharmacy, Weifang Medical University, Weifang 261053, Shandong, China
[b] Department of Medical Chemistry, School of Pharmacy, Weifang Medical University, Weifang 261053, Shandong, China
[c] Department of Medicinal Chemistry, School of Pharmacy, Weifang Medical University, Weifang 261053, Shandong, China
[d] Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, Shandong, China

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ABSTRACT

Aminopeptidase N (APN, also known as CD13) is involved in cellular processes of various types of tumors and a potential anti-cancer therapeutic target. Here, we report the effect of an APN inhibitor 4cc in enhancing sensitivity of hepatocellular carcinoma (HCC) cell lines and xenograft model in response to 5-fluorouracil (5-FU) *in vivo* and *in vitro*. The treatment of the combination of 4cc with 5-FU, compared to the combination of bestain with 5-FU, markedly suppressed cell growth and induced apoptosis of HCC cells, accompanying the increase in the level of reactive oxygen species (ROS) and followed by a decrease in the mitochondrial membrane potential (ΔΨM). Furthermore, the combination of 4cc and 5-FU showed a significant inhibitory effect on the growth of HCC xenograft tumors. In addition, following the treatment of 4cc, APN activity and clonogenic formation and the number of CD13-positive cells in PLC/PRF/5 cells were significantly decreased, suggesting that 4cc may also inhibit liver cancer stem cells by CD13 inhibition. These results showed that the APN inhibitor 4cc synergizes antitumor effects of 5-FU on human liver cancer cells *via* ROS-mediated drug resistance inhibition and concurrent activation of the mitochondrial pathways of apoptosis.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and global patients with HCC and deaths rate are increasing [1]. For patients with unresectable or metastatic HCC, conventional chemotherapy is of limited or no benefit due to the toxic, low efficiency and drug resistance [2–4]. However, the data obtained from clinical trials have suggested that drug combinations molecular targeted agents with chemotherapy are now being tested and may bring about a great help to overcome chemo-resistance in the clinical treatment of patients with advanced HCC [5–7].

APN/CD13 is a potential therapeutic target in tumor cells [8,9]. APN is a zinc-binding type 2 transmembrane ectopeptidase of 150 kDa that forms a noncovalently bound homodimer on the cellular membrane [10,11], which is involved in various cellular processes, including cell invasion, metastasis, motility, angiogenesis and cellular attachment of various malignancies [12–16]. In addition, APN is a therapeutic target in human liver cancer stem cells, which is correlated with the self-renewal, differentiation potential, signal transduction, drug resistance, recurrence and prognosis of liver cancer stem cells (LCSCs) [34,35]. APN inhibitors can inhibit the proliferation and differentiation of tumor cells through targeting APN. Bestain, the first reported APN inhibitor, has been shown to be cytotoxic to tumor cell lines *in vitro*, and inhibits dose-dependently the growth of lung cancer and leukemic cell lines [17–19]. Also, bestain combining conventional chemotherapy drugs not only showed synergistic and highly potent antitumor efficacy but also minimized the side effects of commonly used chemotherapy drugs [20].

* Corresponding authors at: School of Pharmacy, Weifang Medical University, Baotong west road 7166#, Weifang 261053, Shandong, China.
Fax: +86 536 8462465.
E-mail addresses: daigong@wfmc.edu.cn (G. Dai), wangxuejian@wfmc.edu.cn (X.-J. Wang).

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Recently, we designed and synthesized a novel APN inhibitor 4cc, (S)-4-methyl-2- (3-(naphthalen-1-yl-methyl)ureido)-pentanoic acid hydroxyamide, which showed a higher effect against some cancer cells growth compared to bestain. The compound 4cc has been shown to play a vital role in anti-tumor metastasis and anti-angiogenesis in several cancer cell types [21]. The purpose of the present study was to investigate whether 4cc can potentiate the antitumor effects of 5-FU against human liver cancer cells. We observed synergistic antitumor effects with the 4cc and 5-FU combination *in vitro* and *in vivo*. Furthermore, we demonstrated that 4cc interaction with 5-FU activated the intrinsic and extrinsic apoptotic pathways through ROS generation and depolarization of the mitochondrial membrane potential. Given the specific inhibition on CD13, we also speculated that 4cc may inhibit liver cancer stem cells to enhance the chemosensitivity of PLC/PRF/5 cells.

2. Methods and materials

2.1. Chemicals

Bestain (Fig. 1A) was purchased from Biochempartner (Shanghai, China). The compound 4cc (Fig. 1B) was synthesized based on the core skeleton of bestain. The purity of 4cc was 99.9% detected by high-performance liquid chromatography. Bestain and 4cc were dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, Missouri, USA) at 200 mM as stock solution. In the following study, DMSO and bestain were used as vehicle control and positive control, respectively.

2.2. Cells, reagents and animals

Human liver cancer cells PLC/PRF/5, HepG2 and H7402, were obtained from the Cell Bank of Shanghai (Shanghai, China) and maintained in DMEM (Thermo Fisher Scientific, Inc., Beijing, China) supplemented with 10% fetal calf serum (FCS; Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China). L-leucine-*p*-nitroan ilide (cat. no. L9125) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The APN/CD13 mouse-anti-human antibody (CD13mAb clone WM15) was purchased from BD Pharmingen (San Diego, CA, USA). Anti-GCLM antibody was purchased from Abcam. MTT, DMSO and 2′,7′-dichlorofluorescein diacetate were purchased from Sigma Chemical Co. The Annexin V and PI assay kits were purchased from Invitrogen. The JC-1 and mitochondrial incubation buffers were purchased from KeyGEN Biotech (Jiangshu, China). Four to five-week-old female Kunming mice were purchased from the Experimental Animal Center of Weifang Medical University (Weifang, China).

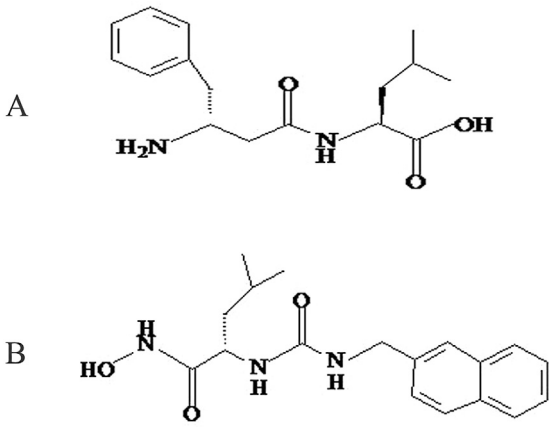

Fig. 1. Chemical structures of bestain (A) and the compound 4cc (B).

2.3. Enzyme activity assay

The APN activity was detected spectrophotometrically by using L-leucine-*p*-nitro anilide as an APN substrate. PLC/PRF/5 cells (5×10^5) were resuspended in 200 μl of PBS in each well of a 96-well plate containing increasing concentrations of bestain (positive control drug) or 4cc (0.1–200 μM), and L-leucine-*p*-nitroanilide was added to each well (final 2 mM). After 30 min at 37 °C, the APN enzyme activity was estimated by measuring the absorbance at 405 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The APN activity inhibition rates of the compounds were calculated using the following formula: (ODcontrol − ODtested)/ODcontrol × 100%.

2.4. Cell viability assay

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoli-um bromide assays. Briefly, PLC/PRF/5, HepG2 and H7402 cells were seeded into 96-well plates (5×10^3 cells/well). After 24 h at 37 °C and 5% CO_2, cells were treated with bestain, 4cc, 5-FU or a combination of 5-FU and bsestain or 4cc. After 48 h, 20 μl MTT solutions (5 mg/ml) were added to each well, incubation was continued for an additional 4 h at 37 °C. Then the supernatants were removed and DMSO was added to each well (200 μl). Fifteen minutes later, the absorbance (OD) of each well was measured at 570 nm using an Emax Microplate reader (EL800, Bio-Tek Instrument Inc., USA). Concentrations that induced a 50% reduction in cell viability (IC_{50}) were determined from dose–response curves.

2.5. Cellular ROS detection

2×10^5 cells were seeded into 6-well plates overnight and exposed to different drug samples. After 24 h incubation,cells were isolated and incubated for 30 min with 2′,7′-dichlorofluorescein diacetate at 37 °C in the dark. Then the samples were analyzed using Cyflogic v.1.2.1 software with an FITC-A band pass filter on a FACSCan Flow Cytometer. Each determination was based on the mean fluorescence intensity of 10,000 events and analyzed using FlowJo 7.6 software.

2.6. Apoptosis assay

To quantify the apoptotic death of PLC/PRF/5 cells, annexin V and PI (propidium iodide) staining was performed, followed by flow cytometry. Briefly, 2×10^5 cells were seeded into 6-well plates overnight and exposed to different drug samples. After 24 h incubation, cells were washed twice with PBS, and a total of 400 μl cell suspension was incubated in presence of 5 μl Annexin V-FITC and 10 μl PI for 15 min at 4 °C in the dark and flow cytometry performed on a BD FACScan flow cytometer (BD Bioscience). The data were analyzed using FlowJo 7.6 software (Tree Star Inc., Ashland, OR, USA). Cells that were Annexin V-positive and PI-negative were considered early apoptotic cells. Each determination was based on the mean fluorescence intensity of 10,000 events.

2.7. JC-1 staining assay

To determine $\Delta\Psi M$, PLC/PRF/5 cells were treated with single or combined drug for 24 h. Then cells were washed twice with PBS, and JC-1 was added to the cells for 30 min. After the removal of JC-1, the cells were harvested by trypsinization, and resuspended in PBS. The amount of JC-1 retained by 10,000 cells per sample was