Cancer Chemother Pharmacol (2012) 69:1029–1038
DOI 10.1007/s00280-011-1799-1

ORIGINAL ARTICLE

CIP-13F, a novel aminopeptidase N (APN/CD13) inhibitor, inhibits Lewis lung carcinoma growth and metastasis in mice

Ke-Ling Pei · Yi Yuan · San-Hai Qin · Yan Wang · Ling Zhou · Hou-Li Zhang · Xian-Jun Qu · Shu-Xiang Cui

Received: 8 October 2011 / Accepted: 5 December 2011 / Published online: 21 December 2011

Abstract
Purpose Aminopeptidase N (APN/CD13) is highly expressed on the surface of cancer cells and is thought to be involved in cancer growth and metastasis. The research of APN/CD13 inhibitors is considered as a strategy of cancer treatment. We aimed to evaluate the efficacy of CIP-13F, a novel APN/CD13 inhibitor, using a Lewis lung carcinoma (LLC) implantation mouse model.
Methods C57BL/6 mice were subcutaneously inoculated with LLC cells in anterior flank. Then, 0, 50 and 100 mg/kg of CIP-13F were injected via vena caudalis. Bestatin was used as the positive control. Administration of CIP-13F or bestatin was performed daily for 3 consecutive weeks. Mice were killed, and the tumors in anterior flank and metastasis nodules in lungs were examined. The assays of immunohistochemical staining, immunofluorescent flow cytometry and western blotting were performed to estimate the expression of APN/CD13 in LLC cells. We carried out the experiments of Annexin-V/PI staining, DNA fragmentation analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining to determine apoptotic cells in LLC tissues. Using immunohistochemical staining with CD34, the antiangiogenesis of CIP-13F was evaluated in LLC tissue sections.
Results CIP-13F treatment resulted in a significant delay of LLC growth in anterior flank. Examination of lungs showed that the number of metastatic nodules of LLC was also markedly decreased. The inhibitory effect of CIP-13F on LLC growth was further evidenced by the induction of LLC apoptosis, showing the increases in Annexin-V/PI staining cells, DNA fragmentation and TUNEL staining cells. Molecular analyses of LLC tissues in CIP-13F-treated mice suggested that the decrease in APN/CD13 expression by CIP-13F might account for its actions of mechanism. Further, the inhibition of angiogenesis in LLC tissues was determined, showing the decreases in microvessel density (MVD) and angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-alpha (TGF-α).
Conclusion Our results showed that CIP-13F effectively inhibited LLC growth and pulmonary metastasis in mice and suggested that CIP-13F is a potential drug for the treatment for cancers with positive APN/CD13 expression.

Keywords Aminopeptidase N (APN/CD13) · Lewis lung carcinoma (LLC) · Lung metastasis · Apoptosis · Angiogenesis · Cyclic-imide peptidomimetics compound

Introduction

Aminopeptidase N (APN/CD13) is a type II membrane-bound metalloproteinase, which is expressed on several types of cells including intestinal epithelium, liver, placenta and lung [1, 2]. APN/CD13 has a variety of functions, such as inflammatory and immunological responses, signal transduction, antigen processing and cytokine degradation [3, 4].

Yi Yuan and Ke-Ling Pei contributed equally to this work.

K.-L. Pei · S.-H. Qin · Y. Wang · L. Zhou · H.-L. Zhang · S.-X. Cui (✉)
Key Laboratory for Rare Disease of Shandong Province, Department of Pharmacology, Institute of Materia Medica, Shandong Academy of Medical Sciences, Jinan 250062, China
e-mail: sxccui@sina.com

Y. Yuan · X.-J. Qu
Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China

High expression of APN/CD13 has been found in skin, ovary, thyroid, lung, stomach, colon, kidney and prostate tumors and is considered to correlate with increased malignancy of cancers [5, 6]. A number of reports showed that APN/CD13 displays a key role in cancer progression by the regulation of processes such as cell–cell contact, proliferation, invasion and metastasis, and angiogenesis [7–9]. So far, bestatin is the only APN/CD13 inhibitor used clinically as an adjuvant in the treatment for malignancies such as leukemia and ovarian carcinoma and lung cancers [10, 11]. However, bestatin is not a specific inhibitor of APN/CD13; hence, it has lower efficacy and significant toxicity for bone marrow and gastric and intestinal mucosa [12, 13]. Thus, attempts have been made to design new APN/CD13 inhibitors.

CIP-13F, a cyclic-imide peptidomimetics compound, was designed based on the X-ray structure of APN/CD13 and the reference drug bestatin [9, 14]. Our previous studies suggested that CIP-13F displayed a great inhibitory effect on activity and expression of APN/CD13 and ES-2 ovarian carcinoma growth [9]. CIP-13F has been considered as a potential candidate compound for the treatment for cancers with positive activity and expression of APN/CD13. However, the inhibitory effect of CIP-13F on cancer growth and metastasis has not been fully evaluated, and the mechanism underlying these actions has not been determined. We aimed to examine the inhibitory effect of CIP-13F on growth and pulmonary metastasis of Lewis lung carcinoma (LLC) using a mouse model.

Materials and methods

Chemicals

CIP-13F, (S)-2-amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide hydrochloride, was designed and synthesized as described previously [9, 14]. The purity of CIP-13F as measured by high-performance liquid chromatography (HPLC) was 99.8%. Bestatin, the reference drug, was purchased from Apeloa Kangyu Pharmaceutical Co. Ltd., China. CIP-13F and bestatin were dissolved in phosphate-buffered saline (PBS) before use.

Inhibition of tumor growth and metastasis in LLC implantation mouse model

C57BL/6 female mice, 5–6 weeks of age, were purchased from the Animal Center of China Academy of Medical Sciences (Beijing, China). The animals were housed under pathogen-free conditions. The procedures were complied with the protocols approved by the institutional guidelines of Animal Care and Use Committee at Shandong University.

To establish LLC implantation mouse model, LLC cells (1×10^7) were suspended in 0.2 ml of PBS and were injected subcutaneously into the left anterior flank of mice [15]. Next day, 0, 50 and 100 mg/kg of CIP-13F in 0.2 ml of PBS were injected via vena caudalis. Other mice injected with bestatin (100 mg/kg) served as positive control. Mice were weighed weekly and checked daily for any signs of illness. The tumor in anterior flank was determined every 3 days by measuring the diameter of the tumors using calipers. The volume was calculated using formula, $V = (ab^2)/2$, where a is the long axis and b is the short axis. Administration of CIP-13F or bestatin was performed daily for 3 consecutive weeks. The mice were killed, and the LLC tumors in anterior flank were removed for weighting and further analysis. The rates of tumor growth inhibition were defined as a percentage of control tumor weight [16]. The tumors were then performed with western blotting analysis, the assays of immunohistochemical staining and TUNEL staining. The lungs were removed, and the metastases nodules on the surface of pulmonary lobes were counted under a dissecting microscope after staining with Bouin's solution.

Immunofluorescent flow cytometry assay

An immunofluorescent flow cytometry assay was carried out to evaluate the inhibitory effect of CIP-13F on APN/CD13 expression in LLC cells. LLC tissues were dispersed mechanically and then placed in collagenase–dispase medium at 37°C for 90 min. Cancer cells were passed through a 100-μm strainer for analysis. Mouse monoclonal anti-APN/CD13 (sc-6995, Santa Cruz, USA) was added to 1×10^6 cells in 100 μl with an irrelevant IgG as a control. After washing, the cells were incubated with fluorescein isothiocyanate (FITC) (100 μl)-conjugated goat anti-mouse IgG (81-6511, Zymed, USA) for 30 min, washed and resuspended in 2% paraformaldehyde. LLC cells (10,000 per measurement) were analyzed by FACScan using Cell Quest software (Becton–Dickinson, USA). The expression of APN/CD13 on cell surface of LLC was determined by mean fluorescence intensity (MFI) after subtracting those of the control antibody-treated cells [17].

Western blotting assay

Western blotting assay was performed to evaluate the expressions of APN/CD13 and angiogenic molecules in LLC cells. LLC tissues were dispersed mechanically in PBS, the supernatants were collected and total protein was determined using the Bradford method [18]. Tumor lysates (30 μg of protein per lane) were separated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membranes, and the protein levels were determined

using the dilutions of the primary antibodies. The primary antibodies included anti-APN/CD13 (sc-6995), anti-vascular endothelial cell growth factor (VEGF) (sc-7269), anti-basic fibroblast growth factor (bFGF) (sc-136255), anti-transforming growth factor-alpha (TGF-α) (sc-27155) (Santa Cruz, USA) and anti-β-actin (AC-74, Sigma, USA). The bound antibodies were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore, USA) and quantified by densitometry using ChemiDoc XRS + image analyzer (Bio-Rad, USA). The data are expressed as the relative density of the protein normalized to β-actin [19].

Immunohistochemical staining analysis

We performed immunohistochemical staining analysis in LLC tissue sections to determine APN/CD13 expression and angiogenesis. For analysis of APN/CD13 expression, anti-APN/CD13 (sc-6995, Santa Cruz, USA) was used to detect cell surface of the antigen. For examination of angiogenesis, anti-CD34 (BA0532, Boster, China) was used to mark the proliferation of endothelial cells in microvessels in LLC tissues. After incubation at 4°C, LLC tissue sections were washed and treated with biotinylated anti-immunoglobulin, reacted with avidin-conjugated horseradish peroxidase H complex and incubated in diaminobenzidine and hydrogen peroxide. The sections were rinsed and stained with hematoxylin and mounted. For angiogenesis analysis, all morphological structures with a lumen surrounded by CD34-positive endothelial cells were considered as blood vessels [8]. Quantification was carried out at the level of endothelial cells lining the blood vessels by their brown cytoplasmic staining. Images were captured and quantified by means of computer-assisted image analyzer for mean vascular density (MVD) [20].

Annexin-V/PI staining assay

Cell surface of phosphatidylserine in apoptotic LLC cells was quantitatively determined by using Annexin-V/FITC and PI apoptosis detection kit according to manufacturer's instruction (Becton–Dickinson, USA). LLC tissues were washed and dispersed mechanically and then placed in collagenase–dispase medium at 37°C for 90 min. Cancer cells were passed through a 100-μm strainer for flow cytometry analysis. Apoptotic LLC cells were analyzed on a FACScan flow cytometry (Becton–Dickinson, USA).

DNA fragmentation detection

DNA ladder quantification was performed by apoptotic DNA ladder kit (Roche applied Science, Germany) according to manufacturer's instruction. LLC cells from tumor tissues were lysed on ice for 30 min using lysis buffer (10 mM Tris–HCl, 10 mM urea, 6 M guanidine-HCl, 20% Triton X-100), and the lysate was treated with RNase A (50 μg/ml) and proteinase K (100 μg/ml). DNA was precipitated and electrophoresed on 1% agarose gel for fragmentation detection. After staining with ethidium bromide, the gel was photographed under a UV transilluminator (Tanon, China).

Assessment of apoptotic cells by TUNEL staining

Apoptotic cells in LLC tissue sections were further identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using TACS TdT kit (R&D Systems, Minneapolis, MN, USA). Serial 5-μm sections were cut from formalin-fixed LLC tissues, and the staining was performed according to manufacturer's instruction. The proportion of the TUNEL-positive LLC cells were counted in four randomly selected visual fields for each treatment. The apoptotic index was calculated as a ratio of apoptotic nuclei to total nuclei.

Statistical analysis

Data were presented as the mean ± SD and analyzed by independent Student's *t* test. Multiple comparisons between the groups were performed using S-N-K method. The limit of statistical significance was $P < 0.05$. Statistical analysis was performed with SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois, USA).

Results

Inhibition of LLC growth in anterior flank and metastasis in lung

The inhibitory effect of CIP-13F on LLC growth in anterior flank was evaluated by weighing the tumor tissues after remove from mice. At 3 weeks after the injection of LLC cells, the weight of tumors in anterior flank developed to 2.72 g on average in control group. CIP-13F treatment resulted in a significant delay of LLC growth. As shown in Fig. 1a and Table 1, at doses of 50 and 100 mg/kg of CIP-13F, the weights of tumors were markedly reduced to 1.92 g (29.4% inhibition, $P = 0.019$ vs. control) and 1.47 g (45.8% inhibition, $P < 0.001$ vs. control), respectively. Bestatin (100 mg/kg) inhibited LLC growth by 33.2% ($P = 0.021$ vs. control). Periodic measurement of the tumor volume also indicated the inhibitory effect of CIP-13F on LLC growth. As shown in Fig. 1b, the volume of LLC in anterior flank was 1103 m^3 after 21-day growth on average in control mice. The volume of LLC tissue was reduced to

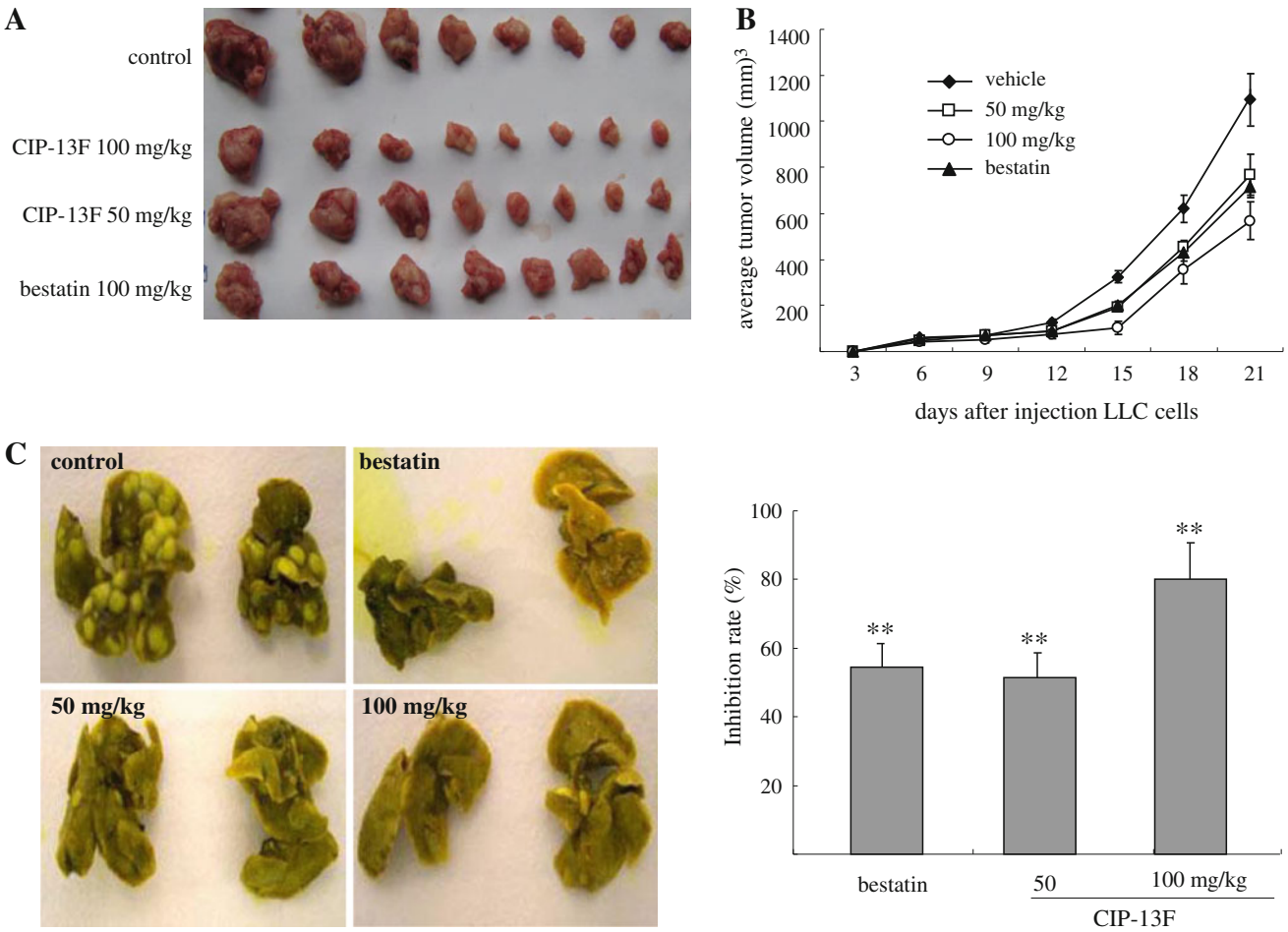

Fig. 1 Inhibitory effect of CIP-13F or bestatin on LLC implantation in mice. LLC cells were injected subcutaneously into anterior flank of mice and then treated daily via vena caudalis with CIP-13F or bestatin for 3 weeks. **a** The mice were killed, and the tumors in anterior flank were removed for weighting. The rates of tumor growth inhibition were defined as a percentage of control tumor weight. **b** Tumor volumes were determined every 3 days by measuring the diameter of tumors using calipers. **c** The lungs were removed, and the metastases nodules on the surface of pulmonary lobes were counted after staining with Bouin's solution. Data are mean ± SD. ($n = 8$) (*right*). $^{**}P < 0.001$ versus control group

Table 1 The inhibitory effect of CIP-13F on LLC tumor growth in anterior flank of C57BL mice

Dosage (mg/kg)	Mice survived (*n*)	Body weight (g) (initial/21 days)	Tumor weight (g; mean ± SD)	Tumor growth inhibition (%)
CIP-13F				
0	8	17.9 ± 1.2/21.7 ± 0.9	2.72 ± 0.67	–
50	8	18.9 ± 1.4/22.1 ± 1.4	1.92 ± 0.98*	29.4
100	8	18.8 ± 2.2/21.3 ± 1.2	1.47 ± 0.86**	45.8
Bestatin				
100	8	18.3 ± 0.8/20.6 ± 1.6	1.82 ± 1.05*	33.2

Established tumors were treated by vena caudalis injection with CIP-13F as described in "Materials and methods". Body weight was measured after drug administration. Tumor measurement was made after mice were killed

* $P < 0.05$, ** $P < 0.001$ versus control group

769 m^3 (30.3% inhibition, $P = 0.024$ vs. control) and 568 m^3 (48.5% inhibition, $P = 0.016$ vs. control), respectively, by 50 and 100 mg/kg of CIP-13F treatment. There was a significant difference between CIP-13F and bestatin at the same dosage (100 mg/kg, $P = 0.024$, between CIP-13F and bestatin, Fig. 1a and b). CIP-13F treatment was generally well tolerated by mice with no apparent loss of body weight (Table 1, $F = 0.48$, $P = 0.99$ vs. control) and other apparent specific toxic activity to mice (data not shown).

The inhibitory effect of CIP-13F on LLC growth was further evidenced by the decrease in metastasis in lung. As shown in Fig. 1c, the metastases nodules on the surface of pulmonary lobes were 58.5 on average each mouse in control group. The number of metastases nodules was markedly reduced to 27.9 (52.2% inhibition, $P < 0.001$ vs. control) and 10.9 (81.3% inhibition, $P < 0.001$ vs. control), respectively, by 50 and 100 mg/kg of CIP-13F treatment. Bestatin (100 mg/kg) inhibited the pulmonary metastasis by 53.6% ($P < 0.001$ vs. control, Fig. 1c, right). A significant difference existed between CIP-13F and bestatin at the same dosage (100 mg/kg, $P = 0.028$, between CIP-13F and bestatin).

Decrease in APN/CD13 expression in LLC cells

We evaluated the inhibitory effect of CIP-13F on APN/CD13 expression using various methods. LLC cells were incubated with anti-APN/CD13 and then were analyzed the mean fluorescence intensity (MFI) on a FAC-Scan flow cytometry. As shown in Fig. 2a, the MFI of APN/CD13 in LLC cells was markedly decreased by CIP-13F treatment, when compared to the MFI of control LLC cells. The rates of inhibition by 50 and 100 mg/kg of CIP-13F were 38.0 and 69.4% of control, respectively ($P < 0.001$ vs. control). Figure 2b showed the expressions of APN/CD13 in LLC cells as estimated by western blotting analysis. The levels of APN/CD13 were significantly decreased by 38.1 and 79.5% ($P < 0.001$ vs. control), respectively. Figure 2c showed APN/CD13 expression in LLC tissues as estimated by immunohistochemical staining method. APN/CD13 was chiefly observed in the membrane of LLC cells, and the levels of expression were evidently decreased by CIP-13F treatment (Fig. 2c). At doses of 50 and 100 mg/kg of CIP-13F, the rates of inhibition were 40.5 and 55.7% of control, respectively ($P < 0.001$ vs. control). Bestatin (100 mg/kg) decreased APN/CD13 expression by 45.8% (Fig. 2c, $P = 0.034$, between CIP-13F and bestatin).

Induction of apoptosis in LLC tissues

The inhibitory effect of CIP-13F on LLC growth was further evidenced by the induction of apoptosis in LLC tissues. LLC tissues were incubated in collagenase–dispase medium, and then the LLC cells were passed through a 100-μm strainer for flow cytometry analysis. As shown in Fig. 3a, the Annexin-V-positive cells were significantly increased in CIP-13F-treated tumors. In 50 and 100 mg/kg of CIP-13F, the percentages of apoptotic cells were 7.8% ($P = 0.041$ vs. control) and 18.4% of control ($P = 0.026$ vs. control), respectively. We then analyzed the chromosomal DNA fragmentation in LLC cells by agarose gel electrophoresis. The increase in DNA fragmentation was obviously detected in CIP-13F-treated tumors, showing the characteristic of DNA ladder (Fig. 3b). Further analysis with TUNEL staining in LLC tissues showed that the TUNEL-positive cells were markedly increased in CIP-13F-treated tumors, which recognized by their purple/black nuclear staining (Fig. 3c). The rates of apoptotic cells by 50 and 100 mg/kg of CIP-13F were 15.3% ($P = 0.038$ vs. control) and 32.8% of control ($P = 0.023$ vs. control), respectively. Bestatin (100 mg/kg) induced TUNEL-positive cells by 17.2% ($P = 0.016$, between CIP-13F and bestatin, Fig. 3c).

Inhibition of angiogenesis in LLC tissues

We evaluated the inhibitory effect of CIP-13 on angiogenesis in LLC tissues using immunohistochemical staining method. Anti-CD34 was used as a marker to indicate the proliferation of endothelial cells and represented as MVD (Fig. 4a). The number of MVD in LLC tissues of control was 25.8. At doses of 50 and 100 mg/kg of CIP-13F, MVD was reduced to 18.6 (27.9% inhibition, $P < 0.001$ vs. control) and 14.9 (41.9% inhibition, $P < 0.001$ vs. control), respectively. Bestatin reduced MVD by 17.6 (31.7% inhibition, $P = 0.027$, between CIP-13 and bestatin, Fig. 4a).

Further, we examined the expressions of angiogenic molecules in LLC tissues by western blotting analysis. As shown in Fig. 4b–d, the expressions of VEGF, bFGF and TGF-α were obviously inhibited in LLC tissues of CIP-13F-treated mice. The rates of decrease by 50 and 100 mg/kg of CIP-13F were 97.3 and 90.6%, respectively, for VEGF; 70.2 and 80.6%, respectively, for bFGF; and 43.5 and 70.2%, respectively, for TGF-α. The inhibitory effects of CIP-13F were stronger than those of bestatin. Bestatin (100 mg/kg) decreased the expressions of VEGF, bFGF and TGF-α by 34.2, 43.7 and 38.5%, respectively ($P = 0.019$, between CIP-13F and bestatin).

Discussion

In previous study, CIP-13F was found to display the inhibitory effect on APN/CD13 activity and cancer growth of human OVCA and hepatocellular carcinoma cell lines [9, 21]. The inhibitory effect of CIP-13F was greater than that of bestatin. In this study, a LLC cell line bearing in mice, which has positive APN/CD13 expression, was used to further evaluate the inhibitory effect of this novel compound. The results revealed that CIP-13F effectively inhibited LLC growth in anterior flank as well as pulmonary metastasis in mice. Further examination showed that the induction of apoptotic LLC cells might account for its inhibitory action because CIP-13F did not show apparent specific toxic activity to mice [9], showing the induction of

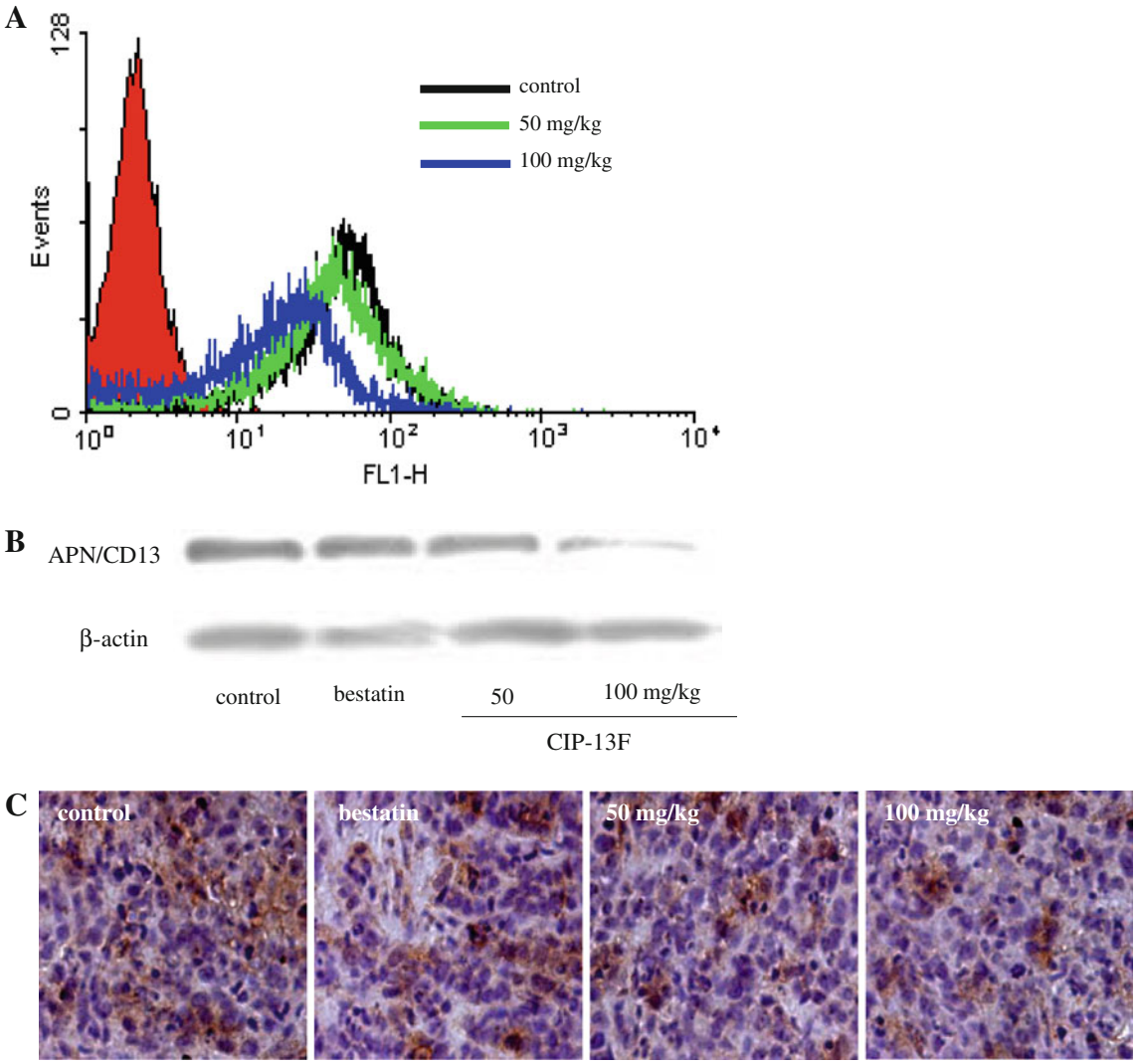

Fig. 2 Inhibition of APN/CD13 expression on the surface of LLC cells by CIP-13F or bestatin. **a** LLC cells were incubated with anti-APN/CD13, and the MFI was estimated by flow cytometric analysis. **b** LLC cells were incubated with anti-APN/CD13, and the levels were estimated by western blotting assay. **c** LLC tissue sections were incubated with anti-APN/CD13, and the expressions were determined by immunohistochemical staining (magnification ×200)

Annexin-V-positive cells, DNA fragmentation and TUNEL staining in LLC tissues. The molecular analysis suggested that the mechanism behind action of CIP-13F might associate with the decrease in APN/CD13 expression on the surface of LLC cells. In addition, our results showed that CIP-13F effectively inhibited angiogenesis in LLC tissues and the decreases in angiogenic molecules were also observed. These results suggested that CIP-13F hold a high efficacy on growth and metastasis of LLC tissues, indicating that CIP-13F might be an effective agent in the treatment for cancers with positive expression of APN/CD13.

Aminopeptidase N (APN/CD13), a Zn^{2+}-dependent ectopeptidase localized on cell surface, is a transmembrane protein that cleaves N-terminal neutral amino acids of various peptides and proteins [1, 2]. In a classic report, a significant correlation between APN/CD13 expression and metastasis of lung carcinoma was observed in patients with squamous cell carcinoma and ovarian cancer. High expression of APN/CD13 was showed to play an important role in tumor invasion and extracellular matrix degradation [10]. APN/CD13 degrades the extracellular matrix, which is associated with tumor cell motility and metastasis, and improves angiogenesis. Pasqualini et al. reported APN/CD13 to be a receptor for NGR peptides in tumor vasculature, while it is also involved in angiogenesis, thus suggesting that APN/CD13 can serve as a target for delivering drugs into tumors and for inhibiting angiogenesis [22]. Lewis lung carcinoma (LLC) implantation mouse has been recognized as a standard experimental model for study cancer growth and metastasis. Recent reports suggested that

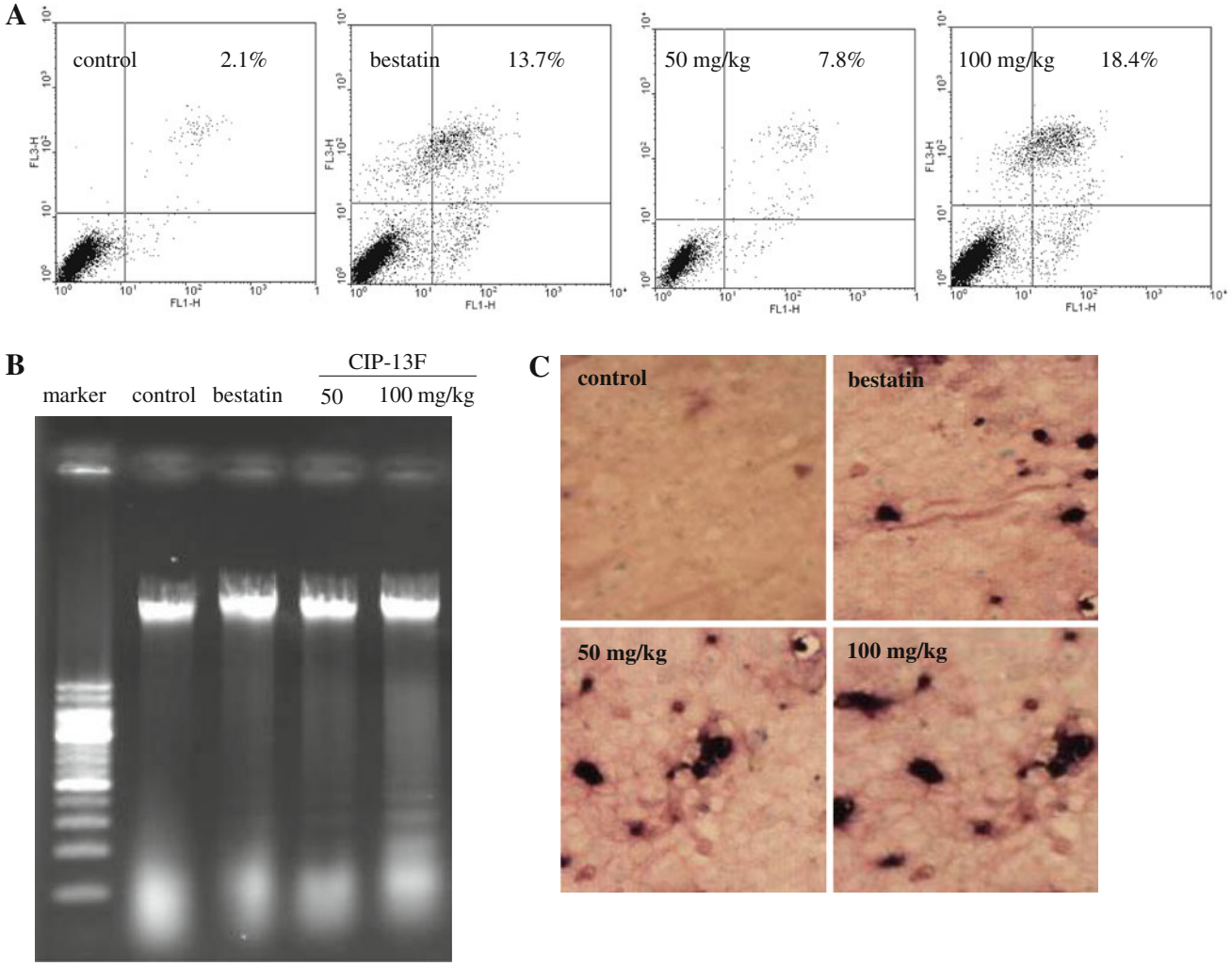

Fig. 3 Induction of apoptosis in LLC cells by CIP-13F or bestatin. **a** LLC cells were stained with Annexin-V/PI, and then the apoptotic cells were detected by flow cytometry analysis. **b** Fragmentation of DNA in LLC cells was measured by DNA ladder kit. **c** The apoptotic cells were detected by TUNEL staining, which was recognized by their *purple/black* nuclear staining (magnification ×200)

positive APN/CD13 expression in LLC cells correlated with malignant behavior, such as proliferation, angiogenesis, invasive ability and metastasis. Inhibition of APN/CD13 expression in LLC cells could effectively inhibit migration, invasion and metastasis of LLC cells in lungs [23–25]. Thus, LLC implantation mouse model was used to evaluate the efficacy of CIP-13F targeting APN/CD13. These results suggested that CIP-13F effectively suppressed APN/CD13 activity and expression in LLC cells. Consequently, the number and growth of metastases nodules in lungs was decreased in mice.

Many studies suggested that high expression of APN/CD13 could protect cancer cells from induction of apoptosis [12, 26]. The mechanism of apoptosis may relate to the phosphorylation of mitogen-activated protein kinases, such as ERK1/2, JNK and p38 [27–29]. Previous reports showed that bestatin could induce apoptosis or increase sensitivity to anticancer agents [30, 31]. The increase in IL-8 in vascular endothelial cells by bestatin resulted in the enhancement of apoptosis in leukemic cells [32]. Many reports also suggested that APN/CD13 contributes to the chemoresistance through proteolytically modifying peptides involved in the anti-apoptotic signaling. Therefore, APN/CD13 inhibitors could trigger apoptotic pathways in cancer cells with positive APN/CD13 expression. Bestatin was found to induce apoptosis through the inhibition of APN/CD13 in leukemic cell lines and increase the radiation-induced apoptosis in HeLa cells [12]. Accordingly, in this study, the possible explanation for these results is that CIP-13F may inhibit APN/CD13 expression, and thereby trigger the apoptotic signaling pathway in LLC cells.

A growing number of reports suggested that APN/CD13 is an important regulator of angiogenesis where its expression on cancer cells and the activated blood vessels is induced by angiogenic signals [33, 34]. High expressions of angiogenic molecules could induce the transcription of

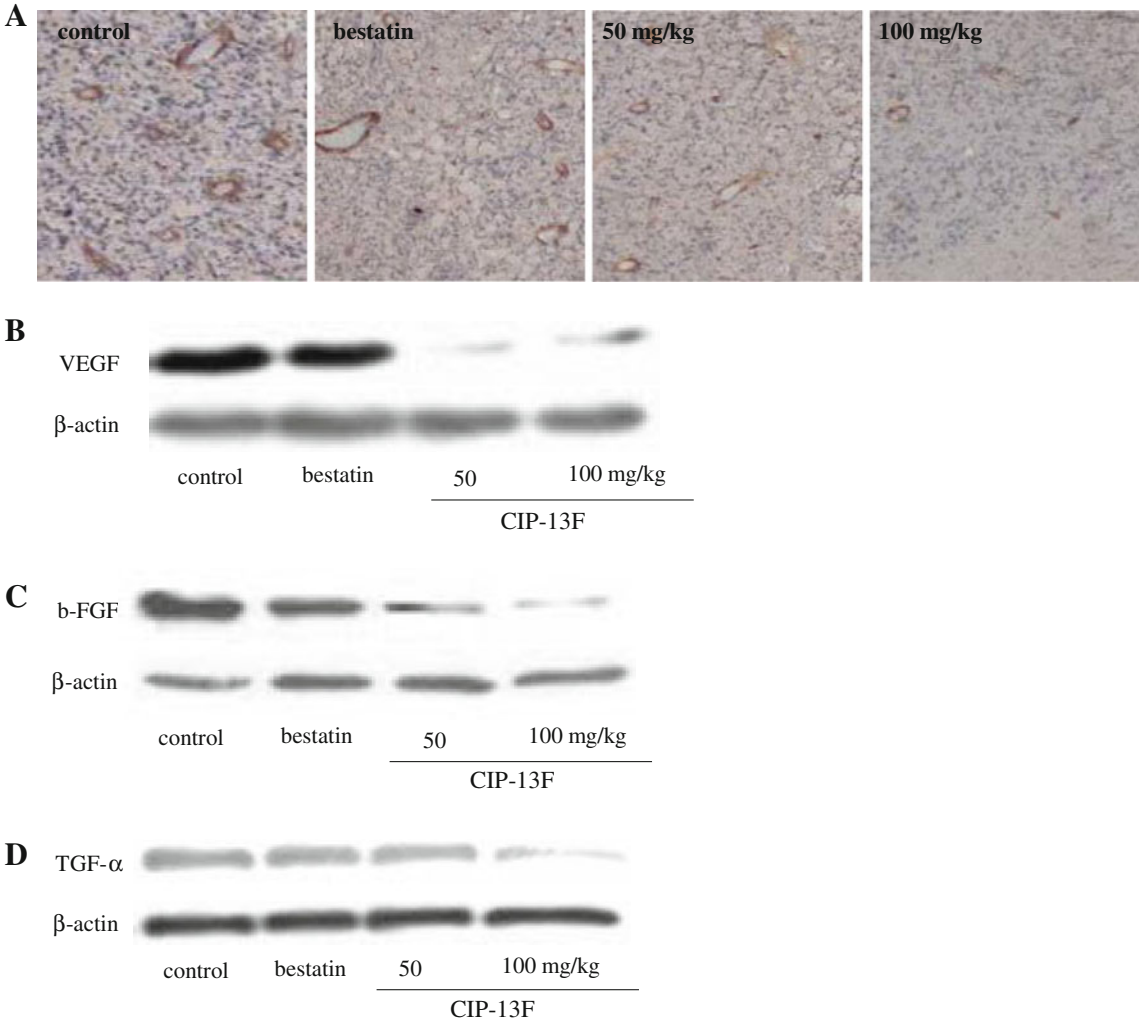

Fig. 4 Inhibition of angiogenesis and decreases in angiogenic molecules in LLC tissues. **a** LLC tissue sections were incubated with anti-CD34, and angiogenesis was estimated by measurement of CD34-positive endothelial cells. Angiogenesis was quantified by means of computer-assisted image analyzer for mean vascular density (MVD) (magnification ×200). **b–d** Decreases in VEGF, b-FGF and TGF-α in LLC tissues treatment with CIP-13F. Total proteins from LLC tissues were fractionated and incubated with anti-VEGF, anti-b-FGF and anti-TGF-α, respectively. The bound antibodies were then incubated with second antibody and visualized using an ECL system and quantified by densitometry using an electrophoresis image analysis system. Data are mean ± SD ($n = 3$)

APN/CD13 in vascular endothelial and cancer cells. APN/CD13 is transcriptionally activated by angiogenic signals, mediated by the rennin-ang system/MAPK/Ets-2 signaling pathway and is essential for endothelial morphogenesis and capillary tube formation [9]. Studies also suggested that the suppression of APN/CD13 interfered with endothelial cell morphogenesis, thus resulted in decrease in capillary tube formation [8]. These findings implicate APN/CD13 as a molecular target for the suppression of angiogenesis. In this study, the activity and expression of APN/CD13 on cell surface of LLC were inhibited, and the angiogenic signal pathway was consequently inhibited by CIP-13F. Thus, we observed a decrease in the mean vascular density (MVD) in LLC tissues following CIP-13F treatment. We suggested that the suppression of APN/CD13 activity contributed to the delay of LLC growth via the inhibition of angiogenesis. However, current results did not mean that the inhibition of APN/CD13 activity was solely responsible for the anti-angiogenic property of CIP-13F treatment. CIP-13F might also inhibit angiogenesis by a decrease in angiogenic signals in addition to the inhibition of APN/CD13 activity. Anyway, we suggested that antiangiogenesis of CIP-13F was considered to be involved in the inhibition of LLC growth and metastasis in lungs.

The mechanism of CIP-13F targeting APN/CD13 and high activity of inhibitory effects on cancer growth and metastasis has not been fully determined. CIP-13F, (S)-2-amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin -3-yl)-3-phenylpropanamide hydrochloride, was designed based on the X-ray structure of APN/CD13 and

the reference drug bestatin. Using ES-2 cells, we examined the activity of APN/CD13 by quantitating the enzymatic cleavage of substrate L-leucine-p-nitroanilide. The results suggested that CIP-13F might directly inhibit APN/CD13 activity and expression on cell surface of ovarian carcinoma [9]. The possible explanation for these observations is its structure. We then analyzed the structure of CIP-13F and compared it to bestatin. As indicated in these reports, the chemical structure of CIP-13F is composed of three parts, the backbone of cyclic-imide peptidomimetics, side chain of amino acid and NHOH group [9]. The structure–activity analysis suggested that cyclic-imide peptidomimetics which linked with zinc-binding group (ZBG) coordinate with zinc ion in enzyme active site [35]. The side chain of amino acid which correspond to phenyl group of bestatin can interact with the anion-binding site of enzyme S1 that contain Glu264 and Glu121 residues, as these residues are of great importance during the recognition of substrates or inhibitors by enzyme. The NHOH group in structure can form hydrogen bond interaction with His297 in enzyme S'1, which are the essential amino acids of the conserved sequence (HEXXHX18E) in catalytic domain that is well conserved in peptidase M1 family, and Asp327 and Arg293 as well [9, 36, 37]. These structures in CIP-13F might accommodate the active pockets S1 and S'1 of APN/CD13 and improve the selectivity with respect to APN/CD13. Therefore, the activity and expression of APN/CD13 on the surface of LLC cells were effectively decreased exposure to CIP-13F. CIP-13F might hold more potently than those of bestatin [9]. Further works on other cancer cell lines as well as in-depth studies of the mechanism are currently underway.

In conclusion, CIP-13F is a novel cyclic-imide peptidomimetics compound with a great inhibitory effect on APN/CD13 expression. Administration of CIP-13F in mice delayed the growth of LLC and metastasis in lung without apparent toxicity. These results suggest that CIP-13F is a potential APN/CD13 inhibitor for the treatment for cancers with positive activity and expression of APN/CD13.

Acknowledgments This project was supported by Natural Science Foundation of China (81072665, 30973550), Shandong Provincial Foundation for Natural Science (Y2008C07) and the project for development of science and technology in Shandong (2011GSF21810).

References

1. Firla B, Arndt M, Frank K, Thiel U, Ansorge S, Täger M, Lendeckel U (2002) Extracellular cysteines define ectopeptidase (APN, CD13) expression and function. Free Radic Biol Med 32:584–595
2. Murakami H, Yokoyama A, Kondo K, Nakanishi S, Kohno N, Miyake M (2005) Circulating aminopeptidase N/CD13 is an independent prognostic factor in patients with non-small cell lung cancer. Clin Cancer Res 11:8674–8679
3. Bank U, Bohr UR, Reinhold D, Lendeckel U, Ansorge S, Malfertheiner P, Tager M (2008) Inflammatory bowel diseases: multiple benefits from therapy with dipeptidyl and alanyl-aminopeptidase inhibitors. Front Biosci 13:3699–3713
4. Reinhold D, Bank U, Täger M, Ansorge S, Wrenger S, Thielitz A, Lendeckel U, Faust J, Neubert K, Brocke S (2008) DP IV/CD26, APN/CD13 and related enzymes as regulators of T cell immunity: implications for experimental encephalomyelitis. Front Biosci 13:2356–2363
5. Aozuka Y, Koizumi K, Saitoh Y, Ueda Y, Sakurai H, Saiki I (2004) Antitumor angiogenesis effect of aminopeptidase inhibitor bestatin against B16-BL6 melanoma cells orthotopically implanted into syngeneic mice. Cancer Lett 216:35–42
6. Pérez I, Varona A, Blanco L, Gil J, Santaolalla F, Zabala A, Ibarguen AM, Irazusta J, Larrinaga G (2009) Increased APN/CD13 and acid aminopeptidase activities in head and neck squamous cell carcinoma. Head Neck 31:1335–1340
7. Gao JJ, Gao ZH, Zhao CR, Yuan Y, Cui SX, Zhang XF, Cheng YN, Xu WF, Tang W, Qu XJ (2011) LYP, a novel bestatin derivative, inhibits cell growth and suppresses APN/CD13 activity in human ovarian carcinoma cells more potently than bestatin. Invest New Drugs 29:574–582
8. Gao JJ, Xue X, Gao ZH, Cui SX, Cheng YN, Xu WF, Tang W, Qu XJ (2011) LYP, a bestatin dimethylaminoethyl ester, inhibited cancer angiogenesis both in vitro and in vivo. Microvasc Res 82:122–130
9. Cui SX, Qu XJ, Gao ZH, Zhang YS, Zhang XF, Zhao CR, Xu WF, Li QB, Han JX (2010) Targeting aminopeptidase N (APN/CD13) with cyclic-imide peptidomimetics derivative CIP-13F inhibits the growth of human ovarian carcinoma cells. Cancer Lett 292:153–162
10. Tokuhara T, Hattori N, Ishida H, Hirai T, Higashiyama M, Kodama K, Miyake M (2006) Clinical significance of aminopeptidase N in non-small cell lung cancer. Clin Cancer Res 12:3971–3978
11. Piedfer M, Dauzonne D, Tang R, N'Guyen J, Billard C, Bauvois B (2010) Aminopeptidase-N/CD13 is a potential proapoptotic target in human myeloid tumor cells. FASEB J 25:2831–2842
12. Tsukamoto H, Shibata K, Kajiyama H, Terauchi M, Nawa A, Kikkawa F (2008) Aminopeptidase N (APN)/CD13 inhibitor, Ubenimex, enhances radiation sensitivity in human cervical cancer. BMC Cancer 8:74
13. Sakuraya M, Tamura J, Itoh K, Kubota K, Naruse T (2000) Aminopeptidase inhibitor ubenimex inhibits the growth of leukaemic cell lines and myeloma cells through its cytotoxicity. J Int Med Res 28:214–221
14. Li Q, Fang H, Wang X, Hu L, Xu W (2009) Novel cyclic-imide peptidomimetics as aminopeptidase N inhibitors. Design, chemistry and activity evaluation Part I. Eur J Med Chem 44:4819–4825
15. Wang X, Xiong XP, Lu J, Zhu GP, He SQ, Hu CS, Ying HM (2010) The in vivo study on the radiobiologic effect of prolonged delivery time to tumor control in C57BL mice implanted with Lewis lung cancer. Radiat Oncol 6:4
16. Liang CZ, Zhang JK, Shi Z, Liu B, Shen CQ, Tao HM (2011) Matrine induces caspase-dependent apoptosis in human osteosarcoma cells in vitro and in vivo through the upregulation of Bax and Fas/FasL and downregulation of Bcl-2. Cancer Chemother Pharmacol. doi:10.1007/s00280-011-1699-4
17. Qu XJ, Yang JL, Russell PJ, Goldstein D (2004) Changes in epidermal growth factor receptor expression in human bladder cancer cell lines following interferon-alpha treatment. J Urol 172:733–738
18. Okutucu B, Dincer A, Habib O, Zihnioglu F (2007) Comparison of five methods for determination of total plasma protein concentration. J Biochem Biophys Methods 70:709–711
19. Qi FH, Li AY, Lv H, Zhao L, Li JJ, Gao B, Tang W (2008) Apoptosis-inducing effect of cinobufacini, Bufo bufo gargarizans

Cantor skin extract, on human hepatoma cell line BEL-7402. Drug Discov Ther 2:339–343
20. Sun CC, Zhang YS, Xue X, Cheng YN, Liu HP, Zhao CR, Lou HX, Qu XJ (2011) Inhibition of angiogenesis involves in anticancer activity of riccardin D, a macrocyclic bisbibenzyl, in human lung carcinoma. Eur J Pharmacol 667:136–143
21. Inagaki Y, Tang W, Zhang L, Du G, Xu W, Kokudo N (2010) Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends 4:56–60
22. Yamashita M, Kajiyama H, Terauchi M, Shibata K, Ino K, Nawa A, Mizutani S, Kikkawa F (2007) Involvement of aminopeptidase N in enhanced chemosensitivity to paclitaxel in ovarian carcinoma in vitro and in vivo. Int J Cancer 120:2243–2250
23. Jullienne B, Vigant F, Muth E, Chaligné R, Bouquet C, Giraudier S, Perricaudet M, Benihoud K (2009) Efficient delivery of angiostatin K1–5 into tumors following insertion of an NGR peptide into adenovirus capsid. Gene Ther 16:1405–1415
24. Hausheer FH, Parker AR, Petluru PN, Jair KW, Chen S, Huang Q, Chen X, Ayala PY, Shanmugarajah D, Kochat H (2011) Mechanistic study of BNP7787-mediated cisplatin nephroprotection: Modulation of human aminopeptidase N. Cancer Chemother Pharmacol 67:381–391
25. Terauchi M, Kajiyama H, Shibata K, Ino K, Nawa A, Mizutani S, Kikkawa F (2007) Inhibition of APN/CD13 leads to suppressed progressive potential in ovarian carcinoma cells. BMC Cancer 7:140
26. Mishima Y, Matsumoto-Mishima Y, Terui Y, Katsuyama M, Yamada M, Mori M, Ishizaka Y, Ikeda K, Watanbe J, Mizunuma N, Hayasawa H, Hatake K (2002) Leukemic cell-surface CD13/aminopeptidase N and resistance to apoptosis mediated by endothelial cells. J Natl Cancer Inst 94:1020–1028
27. Sekine K, Fujii H, Abe F, Nishikawa K (2001) Augmentation of death ligand-induced apoptosis by aminopeptidase inhibitors in human solid tumor cell lines. Int J Cancer 94:485–491
28. Santos AN, Langner J, Herrmann M, Riemann D (2000) Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. Cell Immunol 201:22–32
29. Wickström M, Johnsen JI, Ponthan F, Segerström L, Sveinbjörnsson B, Lindskog M, Lövborg H, Viktorsson K, Lewensohn R, Kogner P, Larsson R, Gullbo J (2007) The novel melphalan prodrug J1 inhibits neuroblastoma growth in vitro and in vivo. Mol Cancer Ther 6:2409–2417
30. Piedfer M, Dauzonne D, Tang R, N'Guyen J, Billard C, Bauvois B (2011) Aminopeptidase-N/CD13 is a potential proapoptotic target in human myeloid tumor cells. FASEB J 25:2831–2842
31. Cowburn AS, Sobolewski A, Reed BJ, Deighton J, Murray J, Cadwallader KA, Bradley JR, Chilvers ER (2006) Aminopeptidase N (CD13) regulates tumor necrosis factor-alpha-induced apoptosis in human neutrophils. Biol Chem 281:12458–12467
32. Mishima Y, Matsumoto-Mishima Y, Terui Y, Katsuyama M, Yamada M, Mori M, Ishizaka Y, Ikeda K, Watanabe J, Mizunuma N, Hayasawa H, Hatake K (2002) Leukemic cell-surface CD13/aminopeptidase N and resistance to apoptosis mediated by endothelial cells. J Natl Cancer Inst 94:1020–1028
33. Tokuhara T, Hattori N, Ishida H, Hirai T, Higashiyama M, Kodama K, Miyake M (2006) Clinical significance of aminopeptidase N in non-small cell lung cancer. Clin Cancer Res 12:3971–3978
34. Fukasawa K, Fujii H, Saitoh Y, Koizumi K, Aozuka Y, Sekine K, Yamada M, Saiki I, Nishikawa K (2006) Aminopeptidase N (APN/CD13) is selectively expressed in vascular endothelial cells and plays multiple roles in angiogenesis. Cancer Lett 243:135–143
35. Wang Q, Chen M, Zhu H, Zhang J, Fang H, Wang B, Xu W (2008) Design, synthesis, and QSAR studies of novel lysine derives as aminopeptidase N/CD13 inhibitors. Bioorg Med Chem 16:5473–5481
36. Shang L, Wang Q, Fang H, Mu J, Wang X, Yuan Y, Wang B, Xu W (2008) Novel 3-phenylpropane-1, 2-diamine derivates as inhibitors of aminopeptidase N (APN). Bioorg Med Chem 16:9984–9990
37. Luan Y, Xu W (2007) The structure and main functions of aminopeptidase N. Curr Med Chem 14:639–647