Brief Report

Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis

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Summary **Aminopeptidase N (APN)/CD13 is a widely expressed transmembrane protein and its altered expression has been detected in various cancer cells. Several APN inhibitors have been developed and some of them have been found to have effectiveness as anti-cancer agents. This article reports anti-cancer effects of a hydroxamic acid derivative 24F that was newly-synthesized as an APN inhibitor. 24F had the ability to inhibit the invasion of hepatocellular carcinoma (HCC) cell line HuH-7, although the growth of HuH-7 was not significantly inhibited at the analyzed concentrations of 24F and incubation times used. Furthermore, incubation of vascular endothelial cells with 24F was found to be effective for the suppression of the angiogenic phenomenon. These results suggest that the novel APN inhibitor 24F may work as an anti-cancer agent for HCC *via* inhibition of HCC cell invasion and angiogenesis.**

Keywords: Aminopeptidase N (APN), CD13, hepatocellular carcinoma (HCC), cancer growth, invasion, angiogenesis

1. Introduction

Aminopeptidase N (APN), which is also known as CD13, is a membranous glycoprotein expressed in a variety of cells and tissues (*1,2*). Several studies have suggested that APN plays important roles in several biological events during cancer progression such as cell proliferation and invasion. For example, overexpression of APN is detected in solid tumors and the expression level is likely to correlate with tumor malignancy (*3-6*). In addition, Yoneda *et al.* reported that APN functions to degrade extracellular matrix and thereby promotes cancer cell invasion and metastasis (*7*). Therefore, inhibition of APN function would have a significant role in the development of cancer chemotherapeutic agents.

Various natural or artificially-synthesized compounds with an ability to work as an inhibitor of APN have been developed (*8*). One well investigated APN inhibitor *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl]-L-leucine is named bestatin. Many researchers have analyzed the anti-cancer effects of bestatin and suggested that bestatin induced both the apoptotic effect in chronic myelogenous leukemia cells and the anti-angionic effect (*9,10*). In a recent study, Cui SX *et al.* developed a novel APN inhibitor named CIP13F that is a cyclic-imide peptidomimetic derivative and clarified the usefulness of CIP13F as an anti-proliferative agent of human ovarian carcinoma cells (*11,12*). In parallel with the progression of that study, the research group of Xu WF also synthesized a new compound named 24F that is an hydroxamic acid derivative with a free amino group ((*S*)-2-amino-*N*-((*S*)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide, Figure 1). 24F was found to react strongly with the APN molecule *in vitro*, inhibit its enzyme activity in the preliminary study and therefore it is expected that 24F can contribute to suppression of cancer progression *via* the inhibition of APN function.

Hepatocellular carcinoma (HCC) is a common malignant disease, especially in eastern Asia. Various therapeutic strategies of HCC treatment including

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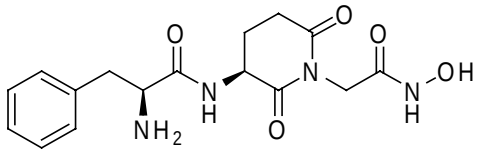

Figure 1. Chemical structure of 24F.

surgical techniques and some noninvasive treatment methods have been developed to improve the outcome of HCC patients. Recently, a novel molecular targeted agent named sorafenib, a multikinase inhibitor, has been developed as a chemotherapeutic agent for HCC (*13*), but discovery of a chemotherapeutic agent for the treatment of HCC has not progressed quickly. This study analyzed the effectiveness of 24F on suppression of HCC progression.

2. Materials and Methods

2.1. *Compound*

The hydroxamic acid derivative 24F was synthesized as one of a series of cyclic-imide peptidomimetics with a free amino group using a 3D-QSAR model (*11,12*). In the present study, this compound was provided by Prof. Xu in China-Japan Cooperation Center for Drug Discovery & Screen, Shandong University (Shandong, China), and dissolved in phosphate-buffered saline (PBS) for *in vitro* studies.

2.2. *Cell lines*

HCC cell line HuH-7 and human promyelocytic leukemia cell line HL-60 were obtained from Health Science Research Resources Bank (HSRRB; Osaka, Japan). HuH-7 and HL-60 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10% fetal bovine serum (FBS), respectively. These media and reagents were purchased from Invitrogen, Carlsbad, CA, USA. Human umbilical vein endothelial cells (HUVEC) were maintained in EGM-2 medium. These cells and media were purchased from Sanko Junyaku Co., Ltd., Tokyo, Japan.

2.3. *Enzyme activity assay*

APN activity was measured using a spectrophotometric method with L-leucine-*p*-nitroanilide (Peptide Institute Inc., Osaka, Japan) as a substrate of APN (*14*). Continuously-cultivated HL-60 cells were collected in test tubes and washed with PBS. Cells (5×10^5) were resuspended in 200 µL of PBS with 0-2.7 mM of 24F and incubated at 37°C. APN enzyme activity was analyzed by measuring the absorbance at 405 nm using a micro-plate reader (Bio-Rad Laboratories) at 0, 15, 30, and 60 min after 1.6 mM substrate was added.

2.4. *Cell growth assay*

Continuously-cultivated HuH-7 cells were harvested in tubes and resuspended in DMEM containing 10% FBS after washing with PBS. Cells were seeded in triplicate in 96-well plates at a density of 2×10^3 cells in 100 µL with 0-200 µg/mL of 24F and incubated for 3 to 5 days at 37°C in a 5% CO_2 atmosphere. Cell viability was evaluated using a methylthiazole tetrazolium (MTT) cell proliferation assay kit in accordance with the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

2.5. *Invasion assay*

The cell invasion assay was performed using a BIOCOAT Matrigel invasion chamber (Becton-Dickinson, NJ, USA) according to the manufacturer's instructions. Continuously-cultivated HuH-7 cells were harvested and resuspended in serum-free medium, and then incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were harvested and resuspended at a density of 1×10^5 cells in 500 µL serum-free medium with 0-200 µg/mL of 24F. The cells were added to each chamber and allowed to invade the Matrigel for 48 h at 37°C in a 5% CO_2 atmosphere. After Matrigel, cells that had not penetrated the filter were removed with cotton swabs, and cells that had migrated to the lower surface of the filter were stained with a Diff-Quick stain kit (Sysmex International Reagents, Hyogo, Japan). After washing with water, the chambers were allowed to air-dry. The number of invading cells were counted under a light microscope.

2.6. *Tube formation assay*

Cultured HUVECs were harvested and resuspended in EGM-2 medium with 0-200 µg/mL of 24F. Cells were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. After Matrigel-coated 24-well plates (Becton-Dickinson) were incubated for 30 min at 37°C, the harvested HUVECs were seeded at a density of 5×10^4 cells in 500 µL cultured medium with 0-200 µg/mL of 24F. After 15 h-incubation at 37°C in a 5% CO_2 atmosphere, the morphology of capillary-like structures was visualized using an inverted microscope (Olympus, Tokyo, Japan) and photographed.

3. Results and Discussion

The enzyme reaction assay was performed to confirm whether newly-synthesized compound 24F can inhibit the activity of aminopeptidase that is expressed on the surface of cell membranes. HL-60 cells are positive for APN expression (*15*), and therefore this

cell line is available to use as a positive control for detecting aminopeptidase activity. As a result, the aminopeptidase activity was inhibited in the presence of 24F in a dose-dependent manner (Figure 2A) and the inhibition rate of ΔA/min under the condition of 0.27 mM (100 μg/mL) 24F was around 25% compared to the condition without 24F. In this analysis, IC_{50} of 24F (the volume of 24F that displayed 50% inhibition of enzyme activity) was calculated to be 1.88 mM. Therefore, it was shown that the newly-synthesized compound 24F can be used as an aminopeptidase inhibitor.

Next, the effect of 24F on HCC cell growth was analyzed using HuH-7 cells that were confirmed to have positive expression of APN by flowcytometric analysis (data not shown). HuH-7 cell growth was inhibited by incubation with 24F, but there was no significant difference in the inhibition rate between 1-200 μg/mL of 24F. The inhibition rate of cell growth was 8.7% at a maximum which was detected in the sample incubated 120 h with 200 μg/mL of 24F. No acute cytotoxic effect was confirmed using microscopic observation in those analyzed concentrations of 24F. This result indicated that 24F might be workable as an anti-proliferative agent of HCC without acute cytotoxic effects, although a higher concentration of 24F than the inhibition of APN enzyme activity is required. Further analyses should be performed to clarify whether 24F can work as an anti-proliferative agent of other HCC cell lines using a shorter incubation period.

Cell invasion is the essential event for cancer progression and metastasis (*16*). Therefore, for cancer therapy, inhibition of cancer cell invasion is an important strategy, along with inhibition of cancer cell growth. This study analyzed the effect of 24F on HuH-7 cell invasion by means of a Matrigel invasion chamber assay. Figure 2B displays typical photographs of stained cells that invaded Matrigel. The number of invading cells was significantly decreased in the presence of 100 μg/mL 24F (*right panel*) compared with that in non-treated cells (*left panel*). This result suggests that 24F has an ability to inhibit the invasion of HuH-7 cells, which displayed a 56% inhibition rate in the sample incubated with 100 μg/mL 24F. Previous studies showed that APN had an important role in the degradation of extracellular matrix and induced cancer cell invasion (*7,17*). Thus, 24F is suggested to suppress HCC cell invasion *via* inhibition of APN enzymatic reaction. Further studies should be performed to clarify the importance of APN in HCC cell invasion and the mechanism of the inhibitory effect of 24F.

Moreover, we also examined the inhibitory effect of 24F on angiogenesis by using the *in vitro* method. Several studies clarified that inhibition of APN seemed to contribute to suppression of angiogenesis

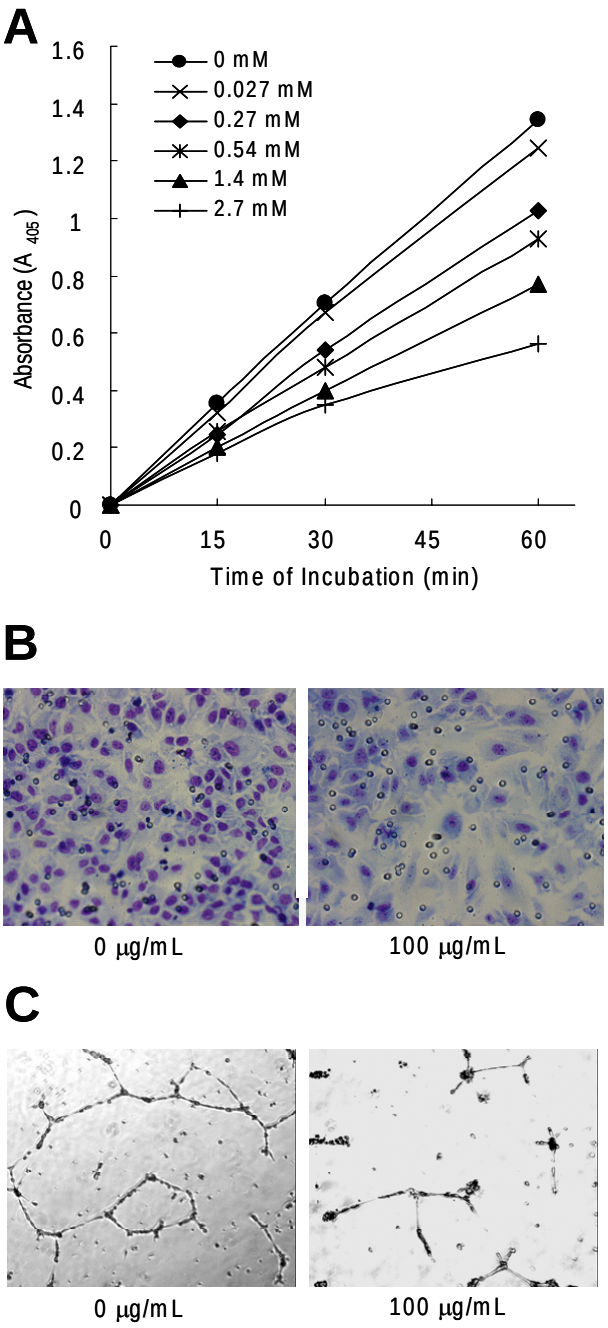

Figure 2. *In vitro* analyses of 24F. (**A**) Effect of 24F on the inhibition of APN enzyme activity. The absorbance, the level of enzyme reaction of APN, was decreased in samples with 24F in a dose-dependent manner. (**B**) Staining of HuH-7 cells that invaded Matrigel. The number of cells stained was decreased when incubating cells with 100 μg/mL of 24F (*right*) compared with incubating without 24F (*left*). Original magnification, ×200. (**C**) Typical example of tube-forming HUVECs on Matrigel. Tube formation was suppressed when incubating cells with 100 μg/mL of 24F (*right*) compared with incubating without 24F (*left*). Original magnification, ×40.

(*10,18*). Thus, our newly-synthesized compound 24F was expected to suppress migration and tube formation of vascular endothelial cells *via* inhibition of APN activity. The result of the tube formation assay showed that the number of tube-like structures of HUVECs on the surface of Matrigel was decreased

by incubating HUVECs with 100 μg/mL 24F (Figure 2C). Therefore, this result indicated that migration and tube formation of vascular endothelial cell can be inhibited by incubation with 24F. Additionally, in this analysis, HUVECs were incubated with 24F for 72 h before examination of the tube formation assay and there was no significant effect in the analysis without this incubation. Thus, it was suggested that persistent incubation with 24F has an ability to suppress angiogenesis by inhibiting the molecular mechanism of migration and tube formation of vascular endothelial cells. The mechanism of this inhibitory effect, however, seemed to be complex because the exact role of APN in angiogenesis is still unclear despite development of APN inhibitors which have an inhibitory effect on angiogenesis. Shim JS *et al.* clarified using DNA microarray analysis that their developed APN inhibitor affected the regulation of several angiogenesis-related genes in human fibrosarcoma cells (*19*). In subsequent study, alteration of expression of angiogenic factors in vascular endothelial cells should be examined in order to estimate the influence of 24F on the angiogenic pathway.

This study was performed with the aim of evaluating the effectiveness of the novel APN inhibitor 24F as an anti-cancer chemotherapeutic agent. The results clarified that 24F had an ability to inhibit HCC cell growth. Furthermore, several researchers showed that inhibition of APN activity can induce apoptosis in cancer cells (*9*). Thus, it is suggested that suppression of HCC cell growth by incubation with 24F is the result of the induction of apoptosis. Further study is required to clarify the mechanism of inhibition of HCC cell growth by 24F and its relation with apoptosis.

In conclusion, our newly-developed compound 24F can inhibit the activity of the targeted enzyme APN and suppress the invasive capacity of HCC cells. Furthermore, it was also suggested that 24F functions to suppress the angiogenic phenomenon of vascular endothelial cells, which are essential events for cancer progression. Novel APN inhibitor 24F is expected to work as a multi-functional anti-cancer chemotherapeutic agent.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and by JSPS and CAMS under the Japan-China Medical Exchange Program.

References

1. Stange T, Kettmann U, Holzhausen HJ. Immunoelectron microscopic single and double labelling of aminopeptidase N (CD13) and dipeptidyl peptidase IV (CD26). Acta Histochem. 1996; 98:323-331.
2. Dixon J, Kaklamanis L, Turley H, Hickson ID, Leek RD, Harris AL, Gatter KC. Expression of aminopeptidase-n (CD13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin. J Clin Pathol. 1994; 47:43-47.
3. Menrad A, Speicher D, Wacker J, Herlyn M. Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. Cancer Res. 1993; 53:1450-1455.
4. Ikeda N, Nakajima Y, Tokuhara T, Hattori N, Sho M, Kanehiro H, Miyake M. Clinical significance of aminopeptidase N/CD13 expression in human pancreatic carcinoma. Clin Cancer Res. 2003; 9:1503-1508.
5. Hashida H, Takabayashi A, Kanai M, Adachi M, Kondo K, Kohno N, Yamaoka Y, Miyake M. Aminopeptidase N is involved in cell motility and angiogenesis: Its clinical significance in human colon cancer. Gastroenterology. 2002; 122:376-386.
6. Kehlen A, Lendeckel U, Dralle H, Langner J, Hoang-Vu C. Biological significance of aminopeptidase N/CD13 in thyroid carcinomas. Cancer Res. 2003; 63:8500-8506.
7. Yoneda J, Saiki I, Fujii H, Abe F, Kojima Y, Azuma I. Inhibition of tumor invasion and extracellular matrix degradation by ubenimex (bestatin). Clin Exp Metastasis. 1992; 10:49-59.
8. Bauvois B, Dauzonne D. Aminopeptidase-N/CD13 (EC 3.4.11.2) inhibitors: chemistry, biological evaluations, and therapeutic prospects. Med Res Rev. 2006; 26:88-130.
9. Sawafuji K, Miyakawa Y, Weisberg E, Griffin JD, Ikeda Y, Kizaki M. Aminopeptidase inhibitors inhibit proliferation and induce apoptosis of K562 and STI571-resistant K562 cell lines through the MAPK and GSK-3beta pathways. Leuk Lymphoma. 2003; 44:1987-1996.
10. Mishima Y, Terui Y, Sugimura N, Matsumoto-Mishima Y, Rokudai A, Kuniyoshi R, Hatake K. Continuous treatment of bestatin induces anti-angiogenic property in endothelial cells. Cancer Sci. 2007; 98:364-372.
11. Cui SX, Qu XJ, Gao ZH, Zhang YS, Zhang XF, Zhao CR, Xu WF, Li QB, Han JX. Targeting aminopeptidase N (APN/CD13) with cyclic-imide peptidomimetics derivative CIP-13F inhibits the growth of human ovarian carcinoma cells. Cancer Lett. 2010; 292:153-162.
12 Zhang J, Li X, Zhu HW, Wang Q, Feng JH, Mou JJ, Li YG, Fang H, Xu WF. Design, synthesis, and primary activity evaluation of pyrrolidine derivatives as matrix metalloproteinase inhibitors. Drug Discov Ther. 2010; 4:5-12.
13 Llovet JM, Ricci S, Mazzaferro V, *et al*. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008; 359:378-390.
14 Terauchi M, Kajiyama H, Shibata K, Ino K, Nawa A, Mizutani S, Kikkawa F. Inhibition of APN/CD13 leads to suppressed progressive potential in ovarian carcinoma cells. BMC Cancer. 2007; 7:140.
15 Hatanaka Y, Ashida H, Hashizume K, Fukuda I, Sano T, Yamaguchi Y, Endo T, Tani Y, Suzukia K, Danno G. Up-regulation of CD13/aminopeptidase N induced by phorbol ester is involved in redox regulation and tumor necrosis factor alpha production in HL-60 cells. Inflammation. 2002; 26:175-181.
16 Inagaki Y, Xu HL, Nakata M, Seyama Y, Hasegawa K, Sugawara Y, Tang W, Kokudo N. Clinicopatholgy

of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers. Biosci Trends. 2009; 3:220-232.
17 Saiki I, Fujii H, Yoneda J, Abe F, Nakajima M, Tsuruo T, Azuma I. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. Int J Cancer. 1993; 54:137-143.
18 Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res. 2000; 60:722-727.
19 Shim JS, Park HM, Lee J, Kwon HJ. Global and focused transcriptional profiling of small molecule aminopeptidase N inhibitor reveals its mechanism of angiogenesis inhibition. Biochem Biophys Res Commun. 2008; 371:99-103.

(*Received February 14, 2010; Revised February 27, 2010; Accepted March 11, 2010****)***