Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 181–183
Bioorganic & Medicinal Chemistry Letters

N-Hydroxy-2-(naphthalene-2-ylsulfanyl)-acetamide, a novel hydroxamic acid-based inhibitor of aminopeptidase N and its anti-angiogenic activity

Jiyong Lee,[a,†] Joong Sup Shim,[a,†] Sun-Ah Jung,[b] Seung-Taek Lee[b] and Ho Jeong Kwon[a,*]

[a]*Chemical Genomics National Research Laboratory, Department of Bioscience and Biotechnology, Institute of Bioscience, Sejong University, 98 Kunja-dong, Kwangjin-gu, Seoul 143-747, Korea*
[b]*Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Korea*

Received 31 August 2004; revised 30 September 2004; accepted 5 October 2004
Available online 22 October 2004

Abstract—In the course of our screening, *N*-hydroxy-2-(naphthalene-2-ylsulfanyl)-acetamide (**1**), which contains a metal-chelating hydroxamate group, has been identified as a potent inhibitor of aminopeptidase N (APN, EC 3.4.11.2). Compound **1** potently inhibited APN activity with a K_i value of 3.5 μM. It also inhibited the basic fibroblast growth-factor-induced invasion of bovine aortic endothelial cells at low micromolar concentrations.

Aminopeptidase N (APN/CD13, EC 3.4.11.2) is a member of M_1 family of zinc metallopeptidases, which is identical to cell surface molecule, CD13, a lineage marker for myeloid cell differentiation.[1] Functional studies on APN have revealed that the metallopeptidase mainly localizes at the cellular membrane and can cleave bioactive proteins on the cell surface including several cytokines and is involved in the down-regulation of signal peptides such as enkephalines.[2] In the brain and peripheral organs, APN participates in the enzymatic cascade of the renin–angiotensin system.[3] APN has been also known to play an important role in metastatic tumor cell invasion.[4–6] These multi-functionalities of APN seem to be derived from its wide spectrum of tissue distribution, and each function of the enzyme may be site dependent. APN is found in the surface of many cells and tissues, including myeloids, renal tubular epithelium, intestinal epithelium, kidney epithelial cells, and in synaptic membranes of central nervous system.[2,7,8]

Recently, APN was identified as an endothelial cell receptor for NGR (Asn-Gly-Arg) peptide motif, which is capable of homing selectively to tumor vasculatures.[9] The only vascular structures with detectable APN were tumor vessels and other types of vessels undergoing angiogenesis. In addition, several inhibitors of APN including antibodies capable of inhibiting the enzymatic activity of APN potently inhibited angiogenesis and tumor growth in mice injected subcutaneously with human breast carcinoma cells. These studies demonstrate that the ectoenzyme can be a new therapeutic target for tumor angiogenesis.[9,10]

To date, several inhibitors of APN, including bestatin, amastatin, actinonin, homothalamide, and aminophosphinic acid derivatives have been developed and some of them are currently investigated for clinical uses.[6,11–13] Our recent study aimed to develop new APN inhibitors with anti-angiogenic activity, through the extensive screening from chemical libraries and natural products. Experiments were performed through both APN enzyme assay and endothelial cell invasion assay in parallel. From the screening, we previously identified curcumin, a potent chemopreventive agent with strong anti-angiogenic activity, is an irreversible inhibitor of APN from the natural products.[14] In the present study, a new hydroxamate-containing inhibitor of APN was isolated from the chemical library and evaluated its biological activities.

The assay of APN activity was performed based on the detection of the enzymatic degradation products of the fluorogenic substrate of APN as described previously.[14]

Keywords: Aminopeptidase N; Angiogenesis; Metalloproteinase.
* Corresponding author. Tel.: +82 2 3408 3640; fax: +82 2 3408 3334; e-mail: kwonhj@sejong.ac.kr
† These authors contributed equally to this work.

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.10.010

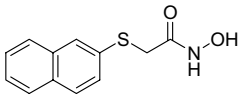

Figure 1. The chemical structure of compound **1**.

Total 3000 of natural products and chemical libraries were used to the high throughput screening. The high throughput screening identified *N*-hydroxy-2-(naphthalene-2-ylsulfanyl)-acetamide (**1**)[15] as an inhibitor of APN from the chemical library (Fig. 1). Compound **1** inhibited the activity of APN with an IC_{50} value of 3.4 μM (Fig. 2a). Bestatin, a transition state analog of the dipeptide substrate Phe-Leu also inhibited APN activity at similar concentration ranges to that of compound **1**.

Next, we performed the kinetics analysis of the enzyme inhibition by compound **1**. The Lineweaver–Burk plot of the inhibition showed that compound **1** competitively inhibited the APN activity (Fig. 2b). The K_i value of the inhibition was determined as 3.5 μM from the Dixon plot of the inhibition (data not shown).

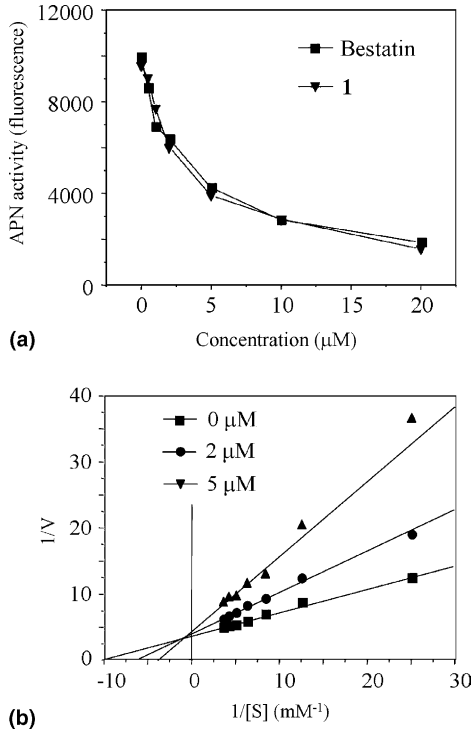

Figure 2. Effect of compound **1** on the enzymatic activity of APN. (a) Dose-dependent inhibition of APN activity by compound **1** and bestatin is shown. (b) The kinetics analysis of APN inhibition by compound **1** is shown using Lineweaver–Burk plot.

Compound **1** contains a metal-chelating hydroxamate functional group, which can interfere with certain metalloenzymes, including matrix metalloproteinases (MMPs). We thus examined the effect of compound **1** on the activities of both APN and several MMPs, including MMP2, MMP9, and MMP14 (MT1-MMP).[16] As shown in the Table 1, compound **1** did not inhibit any of MMPs analyzed in this study, even at 50 μM concentration. BB-94 (also known as batimastat) was used as a positive control compound for the specific MMP inhibitor.[17] We also tested the isotype specificity of compound **1** using adipocyte-derived leucine aminopeptidase (A-LAP), another member of M_1 family of zinc metallopeptidases.[18] The enzyme assay of A-LAP was conducted through same procedures as APN assay with a different fluorogenic substrate, leu-7-amido-4-methyl-coumarin.[19] Interestingly, compound **1** did not inhibit the activity of A-LAP at concentration ranges up to 50 μM. In contrast, a broad-range aminopeptidase inhibitor, bestatin, inhibited A-LAP activity with an IC_{50} of 11.2 μM (Table 1). These results demonstrate that compound **1** is an isotype-specific inhibitor of APN.

We, finally, examined the effect of compound **1** on in vitro angiogenesis of bovine aortic endothelial cells (BAECs).[23] In Figure 3a, control cells grown in serum-free media showed a basal level invasiveness. The stimulation of BAECs with basic fibroblast growth factor (bFGF) highly increased the invasion of BAECs. However, the compound **1** treatment caused dose-dependent inhibition of bFGF-induced invasion of BAECs. As shown in Figure 3b, compound **1** specifically inhibited the invasion of BAECs at the same magnitude of bFGF-stimulation and did not inhibit the basal level invasion of the endothelial cells. Compound **1** did not affect the viability of BAECs up to 10 μM treatment (data not shown). These data demonstrate that compound **1** is a new inhibitor of APN with potent anti-angiogenic activity.

In summary, a novel hydroxamate inhibitor of APN was developed and evaluated its biological activities. Compound **1** competitively inhibited APN activity with a K_i value of 3.5 μM. The specificity of the compound on APN and other metalloproteinases was also demonstrated. Finally, compound **1** potently inhibited bFGF-induced in vitro angiogenesis of BAECs without cytotoxicity. These excellent biological activities of compound **1** open the possibility to develop more potent

Table 1. Target specificity of compound **1** on aminopeptidases and several MMPs

Compds	APN inhibition IC_{50} (μM)[a]	MMP-2 inhibition IC_{50} (μM)	MMP-9 inhibition IC_{50} (μM)	MMP-14 inhibition IC_{50} (μM)	A-LAP inhibition IC_{50} (μM)
1	3.4 (±0.6)	na	na	na	na
BB-94	nd	0.013 (±0.003)	0.025 (±0.005)	0.047 (±0.004)	nd
Bestatin	3.9 (±0.4)	nd	nd	nd	11.2 (±0.9)

[a] Values are means of three experiments, standard deviation is given in parentheses (na = not active, nd = not determined).

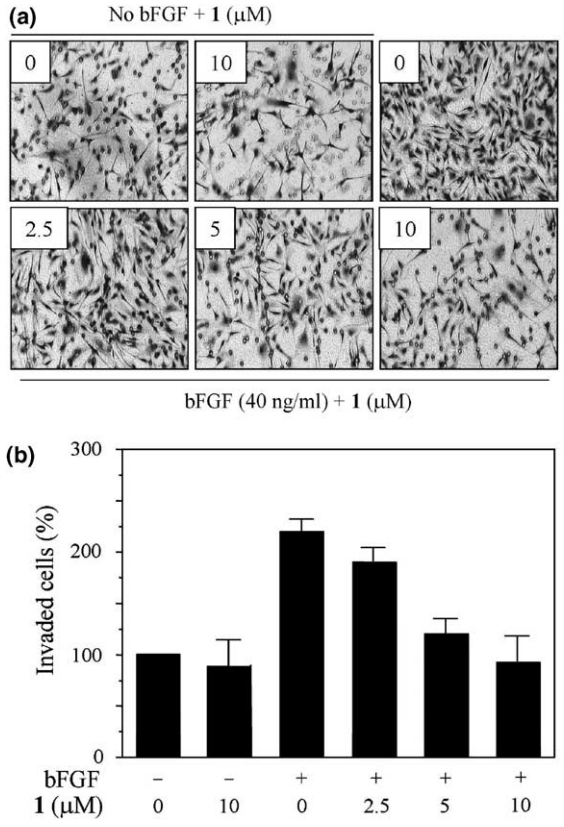

Figure 3. Effect of compound **1** on bFGF-induced invasion of BAECs. (a) BAECs starved with serum-free media for 12 h were treated with various concentrations of compound **1** in the presence or absence of bFGF and invasion assay was performed. Figures were selected as representative results from three independent experiments. (b) Quantitative data for the invasion assay is shown. Data represents mean ± SE from three independent experiments.

derivatives of the compound. In addition, in vivo efficacy validation of compound **1** is under investigation for development of new anti-angiogenic agent.

Acknowledgements

This study was supported by the National Research Laboratory Grant from the Ministry of Science and Technology, Republic of Korea and the Brain Korea 21 Project.

References and notes

1. Look, A. T.; Ashmun, R. A.; Shapiro, L. H.; Peiper, S. C. *J. Clin. Invest.* **1989**, *83*, 1299.
2. Matsas, R.; Stephenson, S. L.; Hryszko, J.; Kenny, A. J.; Turner, A. J. *Biochem. J.* **1985**, *231*, 445.
3. Zini, S.; Fournie-Zaluski, M. C.; Chauvel, E.; Roques, B. P.; Corvol, P.; Llorens-Cortes, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11968.
4. Fujii, H.; Nakajima, M.; Saiki, I.; Yoneda, J.; Azuma, I.; Tsuruo, T. *Clin. Exp. Metast.* **1995**, *13*, 337.
5. Saiki, I.; Fujii, H.; Yoneda, J.; Abe, F.; Nakajima, M.; Tsuruo, T.; Azuma, I. *Int. J. Cancer* **1993**, *54*, 137.
6. Menrad, A.; Speicher, D.; Wacker, J.; Herlyn, M. *Cancer Res.* **1993**, *53*, 1450.
7. Semenza, G. *Annu. Rev. Cell Biol.* **1986**, *2*, 255.
8. Kenny, A. J.; Maroux, S. *Physiol. Rev.* **1982**, *62*, 91.
9. Pasqualini, R.; Koivunen, E.; Kain, R.; Lahdenranta, J.; Sakamoto, M.; Stryhn, A.; Ashmun, R. A.; Shapiro, L. H.; Arap, W.; Ruoslahti, E. *Cancer Res.* **2000**, *60*, 722.
10. Bhagwat, S. V.; Lahdenranta, J.; Giordano, R.; Arap, W.; Pasqualini, R.; Shapiro, L. H. *Blood* **2001**, *97*, 652.
11. Yoneda, J.; Saiki, I.; Fujii, H.; Abe, F.; Kojima, Y.; Azuma, I. *Clin. Exp. Metast.* **1992**, *10*, 49.
12. Kagechika, H.; Komoda, M.; Fujimoto, Y.; Koiso, Y.; Takayama, H.; Kadoya, S.; Miyata, K.; Kato, F.; Kato, M.; Hashimoto, Y. *Biol. Pharm. Bull.* **1999**, *22*, 1010.
13. Chen, H.; Roques, B. P.; Fournie-Zaluski, M. C. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1511.
14. Shim, J. S.; Kim, J. H.; Cho, H. Y.; Yum, Y. N.; Kim, S. H.; Park, H. J.; Shim, B. S.; Choi, S. H.; Kwon, H. J. *Chem. Biol.* **2003**, *10*, 695.
15. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.74 (br s, 1H), 9.02 (br s, 1H), 7.88–7.82 (m, 4H), 7.55–7.45 (m, 3H), 3.67 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): 165.5, 134.3, 134.0, 131.9, 129.0, 128.3, 127.6, 127.4, 127.1, 126.5, 126.2, 34.5; ESI MS *m/z* 234.1 $[M+H]^+$.
16. MMP assays: the recombinant human proMMP-2 and proMMP-9 were expressed in Sf9 insect cells with infection of MMP-2 and MMP-9 baculoviruses, respectively. They were purified with gelatin–agarose column chromatography as described previously.[20] The human MMP-14 catalytic domain was expressed as inclusion body in *E. coli* and refolded as described.[21] The purified proMMP-2 and proMMP-9 were activated in the presence of 1 mM APMA at 37 °C for 30 min. The activated MMP-2, MMP-9, or MMP-14 catalytic domain (10 nM each) was incubated in 100 μL of the MMP assay buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 500 μM $ZnCl_2$) containing 1 μM of a quenched fluorescent peptide as a substrate and various concentrations of compounds at 37 °C for 30 min.[22] The quenched fluorescent peptide was Mca-Pro-Leu-Gly-Leu-Dap (Dnp)-Ala-Arg-NH_2 for MMP-2 and MMP-14 assays or Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Tyr-Met-Lys (Dnp)-NH_2 for MMP-9 assay. Reaction was stopped by addition of 0.1 M sodium acetate (pH 4.0) at final concentration. The fluorescence was measured at excitation wavelength 328 nm and emission 393 nm.
17. Wojtowicz-Praga, S. M.; Dickson, R. B.; Hawkins, M. *J. Invest. New Drugs* **1997**, *15*, 61.
18. Schomburg, L.; Kollmus, H.; Friedrichsen, S.; Bauer, K. *Eur. J. Biochem.* **2000**, *267*, 3198.
19. Miyashita, H.; Yamazaki, T.; Akada, T.; Niizeki, O.; Ogawa, M.; Nishikawa, S.; Sato, Y. *Blood* **2002**, *99*, 3241.
20. Jo, Y.; Yoon, D.-W.; Kim, M.-Y.; Lee, Y.-J.; Kim, H.-J.; Lee, S.-T. *J. Biochem. Mol. Biol.* **1999**, *32*, 60.
21. Koo, H. M.; Kim, J.-H.; Hwang, I. K.; Lee, S.-J.; Kim, T.-H.; Rhee, K.-H.; Lee, S.-T. *Mol. Cells* **2002**, *13*, 114.
22. Jo, Y.; Yeon, J.; Kim, H.-J.; Lee, S.-T. *Biochem. J.* **2000**, *345*, 511.
23. Invasion assay: the invasiveness of endothelial cells was examined in vitro using a Transwell chamber system with 8.0-μm pore-sized polycarbonate filter inserts, as described previously.[14] The total number of invaded cells on the lower side of the filter was counted using an optical microscope at a ×100 magnification.