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Inhibition of Aminopeptidase N by Two Synthetic Tripeptides

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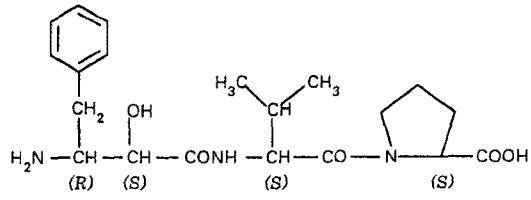
MR-387A1 (AHPA-Val-Pro) and A2 (AHPA-Val-Hyp) were prepared as aminopeptidase N inhibitors through the synthesis of peptide MR-387A and B analogues which contained 3-amino-2-hydroxy-4-phenyl butanoic acid (AHPA) as a zinc-chelating moiety. They are competitive inhibitors of aminopeptidase N with inhibition constants(K*i*) of 4.1×10^{-7} and 1.1×10^{-6} M, respectively. MR-387A1 also strongly inhibited aminopeptidase B of human myelogenous leukemia K-562 cell with IC_{50} of 0.35 μM. Inhibitions of aminopeptidase N activity by AHPA-bearing inhibitors of various peptide chain lengths also have been studied. IC_{50} values of AHPA-Val (bestatin), AHPA-Val-Pro (MR-387A1) and AHPA-Val-Pro-Leu (MR-387C) compared against porcine kidney aminopeptidase N were 20.1, 0.60 and 0.08 μM, respectively. These results support that a multiple interaction between the $S_1 \rightarrow S'_3$ sites of aminopeptidase N and the $P_1 \rightarrow P'_3$ of the inhibitor plays a crucial role in stabilizing strongly the enzyme-inhibitor complex.

Aminopeptidase N (AP-N, EC 3.4.11.2, or aminopeptidase M) is a zinc-containing proteolytic ectoenzyme which removes the N-terminal amino acid of protein and peptide substrates (24). It is a stalked integral membrane protease, mainly located in the small intestinal and kidney brush borders but is also found in brain, lung, liver and primary cultures of fibroblasts and has been shown to be identical to the myeloid leukemia marker CD 13 (7, 16).

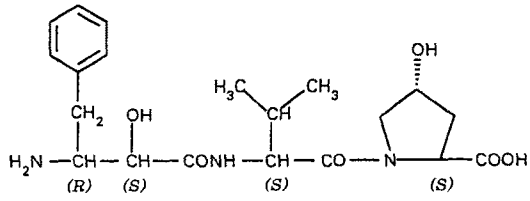
AP-N plays a functional role in human ovarian folliculogenesis or successful implantation (6, 11). In malignancy, Saiki *et al.* (22) and Menrad *et al.* (17) have found AP-N to play an important role in the invasion of metastatic tumors *in vitro*. Recently, Ino *et al.* (12) reported that bestatin and actinonin, AP-N inhibitors, suppress the growth or metastasis of cancer. Thus, the inhibition of the enzyme could provide a new avenue to development of anti-cancer drugs.

Most known aminopeptidase inhibitors are amino acid or peptide analogues. They include zinc-chelating agents such as amino acid hydroxamate (9) and aminothiol derivatives (19) and amino acid and peptide analogues capable of mimicking the tetrahedral adducts, such as, boronic acid derivatives (24), aminoaldehydes (2), phosphorus-containing peptide analogues (15), peptides bearing α-ketoamides (20) or ketomethylene peptide bond esters (8), and 3-amino-2-tetralone derivatives (23). Finally, among the most intriguing inhibitors of this class of enzyme are naturally occurring compounds such as bestatin (27) and probestin (3) that have been the most extensively studied and may have important medicinal applications.

In our laboratory, two new AP-N inhibitors MR-387A and B were isolated from the culture filtrate of *Strep-*



MR-387A1



MR-387A2

Fig. 1. Structures of MR-387A1 and A2.

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tomyces neyagawaensis SL-387 (4). From the basis of the structures of both inhibitors, we obtained the novel synthetic tetrapeptide MR-387C which had the same molecular weight (MW) as MR-387A, and inhibited AP-N more strongly than both MR-387A and B (5). We continued the synthesis of MR-387 derivatives and obtained two novel tripeptides, named MR-387A1 and A2 (Fig. 1), having lower MWs than MR-387A, B and C.

In this paper, the synthesis, physico-chemical properties and biological activities of MR-387A1 and A2 are reported. Additionally, the relationship of the length of the peptide chain in MR-387 analogues to activity is also discussed herein.

MATERIALS AND METHODS

Instrumental Analysis

Mass spectra were recorded on a JEOL JMS-HX 110A/110A spectrometer. NMR spectra were recorded on a Brucker AMX-FT500MHz and a Varian UNITY 300MHz spectrometer. UV spectra and IR spectra were recorded on a Shimazu UV-260 spectrophotometer and a Laser Precision Analytical IFX-65S spectrophotometer, respectively. HPLC analysis used a Hitachi L-6200 integral pump with a Hitachi L-4000 UV detector system.

Cell Lines and Cultivation

The human fibrosarcoma cell line HT-1080 and the human myelogenous leukemia cell line K-562 were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco) at 37°C in a humidified atmosphere of 5% CO_2.

Assay for AP-N and Inhibitory Activities

The inhibitory activity of MR-387A1 and A2 against AP-N from the microsomal membrane of porcine kidney (Sigma L-0632) was determined as reported previously (4, 5). Percent inhibition was calculated by the formula (A-B)/A×100, where A is the measured value of the enzymatic reaction in the system without an inhibitor, and B is the value with an inhibitor. The IC_{50} value is the concentration of inhibitor which produced a 50% inhibition of enzyme activity.

Cell surface AP-N activity in each cell line was detected spectophotometrically as reported by Amoscato *et al* (1). In brief, after incubating 5×10^5 cells in the culture medium in a 96-well microplate for 48 h at 37°C, the cells were used for enzyme assay. After aspirating off the medium and washing with phosphate-buffered saline (PBS), prewarmed 200 μM leucine-*p*-nitroanilide and 20 μl of water or aqueous solution containing the test compound were added to each well as a substrate (final volume 200 μl). The plate was then incubated at 37°C for 1 h and the reaction terminated by centrifugation for 3 min. The optical density of the supernatant at 405 nm was measured with a microplate reader (Bio-Rad Model 3550). Cell surface AP-B activity in K562 cell line was detected by the same procedure as AP-N activity determination except using lysine-*p*-nitroanilide as a substrate.

Synthesis of MR-387A1 and A2

MR-387A1 and A2 were automatically synthesized by the solid phase method (24) using an Applied Biosystems (Model 431A) peptide synthesizer on HMP-resin(4-hydroxy-methylphenoxymethyl copolystyrene-1% divinylbenzene resin). The t-BOC-(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoic acid (t-BOC-AHPA) used in peptide synthesis was purchased from Sigma Peptides and Amino Acids (USA). The amino acid sequences of MR-387A1 and A2 were designed as AHPA-Val-Pro and AHPA-Val-Hyp, respectively. Purification of the inhibitor resulting from a cocktail of synthesis reactions was carried out by HPLC on a YMC-ODS-A column (ϕ 4.6×250 mm, 1.5 ml/min, 17% MeCN-0.1% TFA in H_2O). Active fractions were combined and concentrated under reduced pressure in a small volume of water, then lyophilized under a freeze dryer to give an amorphous white powder.

RESULTS AND DISCUSSION

Physico-chemical Properties

Physico-chemical properties of MR-387A1 and A2 are summarized in Table 1. The molecular weights and formula of both inhibitors were determined to be $C_{20}H_{29}N_3O_5$ (MW 374, M+H) and $C_{20}H_{29}N_3O_6$ (MW 390, M+H), respectively based on ESI-MS and NMR studies. Color reaction with ninhydrin showed positive, suggesting the presence of an amino group in both molecules. Rf values of MR-387A1 and A2 on silica gel TLC (Merck Art No.

Table 1. Physico-chemical properties of MR-387A1 and A2.

	MR-387A1	MR-387A2
Appearence	White powder	White powder
ESI-MS (m/z)	374 $(M+H)^+$	390 $(M+H)^+$
Molecular	$C_{20}H_{29}N_3O_5$	$C_{20}H_{29}N_3O_6$
UV $\lambda_{max}^{MeOH}(\varepsilon)$	264 (149), 258 (155), 252 (144), 220 (446)	264 (197), 258 (209), 252 (196), 220 (642)
IR ν_{max}^{KBr} cm^{-1}	3200, 2970, 1675, 1446, 1205, 1138, 840, 800, 723	3200, 2966, 1671, 1444, 1201, 1138, 839, 800, 723
Color reaction	ninhydrin	ninhydrin
Rf values*	0.65	0.53
Solubility		
soluble:	H_2O, MeOH, EtOH, DMSO,	H_2O, MeOH, EtOH, DMSO
insoluble:	$CHCl_3$, Hexane, EtOAc, Et_2O	$CHCl_3$, Hexane, EtOAc, Et_2O

*On silica gel TLC plate(Merck Art No. 5715) with BuOH-AcOH-H_2O (4 : 1 : 1).

5715) developing with BuOH-AcOH-H_2O (4 : 1 : 1) were 0.65 and 0.53, respectively.

Structure Identification

The ^1H NMR data on MR-387A1 and A2 are presented in Table 2. The assignments of protons were determined by the ^1H-^1H COSY spectra. These data supported the presence of the amino acids which were designed in the synthesis procedure. The amino acid sequences were determined by the NOESY data in DMSO-d_6. In DMSO-d_6, the protons of NH_2 (7.90 ppm) and OH (6.7 ppm) of AHPA and NH (8.10 ppm) of Val were detected in both molecules. As shown in Fig. 2, NOEs of between 2-CH of AHPA and HN of valine and between α-CH of valine and δ-CH_2 of proline or hydroxyproline were detected. These results supported that the amino acid sequences of MR-387A1 and A2 are AHAP-Val-Pro and AHPA-Val-Hyp, respectively.

Biological Activities

Table 2. ^1H-NMR data of MR-387A1 and A2 in D_2O at 300 MHz.

Assignment		MR-387A1	MR-387A2
AHPA	2-CH	4.28 (d, 4.8)	4.26 (d, 5.4)
	3-CH	3.80 (m)	3.83 (m)
	4-CH_2	3.09 (dd, 6.6, 14.4)	3.08 (dd, 4.2, 14.4)
		2.93 (dd, 8.1, 14.4)	2.95 (dd, 8.4, 14.4)
	Ph-*o,m,p*	7.29-7.42 (m)	7.28-7.41 (m)
Val	α-CH	4.41 (d, 7.5)	4.38 (d, 7.5)
	β-CH	ca. 2.10 (m)	2.12 (m)
	γ-CH_3	0.98 (d, 6.6)	0.99 (d, 6.9)
	CH_3	0.94 (d, 6.8)	0.94 (d, 6.9)
Pro	α-CH	4.37 (m)	-
	β-CH_2	2.30 (m), 2.01 (m)	-
	γ-CH_2	ca. 2.05-1.95 (m)	-
	δ-CH_2	3.70 (m), 3.18 (m)	-
Hyp	α-CH	-	4.48 (t, 8.7)
	β-CH_2	-	2.38 (br. dd, 7.8, 13.8), 2.15 (m)
	γ-CH	-	4.60 (m)
	δ-CH_2	-	3.93 (d, 11.7), 3.81 (d, <1.0)

The compounds were tested for inhibition of cell surface aminopeptidases. As shown in Table 3, MR-387A1 and A2 inhibited not only AP-N of porcine kidney microsome (Sigma, L-0632) with IC_{50} of 0.60 and 0.87 μM, but also intact AP-N of human fibrosarcoma HT-1080 with IC_{50} of 1.21 and 1.82 μM, respectively. MR-387A1 also inhibited strongly AP-B of human myelogenous leukemia K-562 with IC_{50} of 0.35 μM. As shown in Fig. 3, both synthetic tripeptides are competitive with the substrate. The *Ki* values of MR-387A1 and A2 are 4.1×10^{-7} M and 1.1×10^{-6} M, respectively. The *Km* value of porcine kidney AP-N used in these assays was 1.1×10^{-4} M.

Inhibition of AP-N by Inhibitors of Various Peptide Chain Lengths

Inhibition of AP-N activity was carried out by AHPA-bearing inhibitors of varous peptide chain lengths. As shown in Table 4, tripeptides MR-387A1 and A2 are better inhibitors of AP-N than dipeptide bestatin. Recently, AHPA-Val, a synthetic derivative of bestatin, was purified from the culture broth of *Streptomyces neyagawaensis* SL-387 which was cultured in a chemically defined medium by supplementing 3-amino-3-phenylpropionic acid. Inhibitory activity of AHPA-Val against AP-N is similar to that of bestatin. The production, purification, physico-chemical and biological activities of AHPA-Val will be reported in a later paper. Tripeptides MR-387A1 and A2, which lack one proline found in MR-387B and A, respectively, were found to be weaker inhibitors than tetrapeptides MR-387A and B. These results

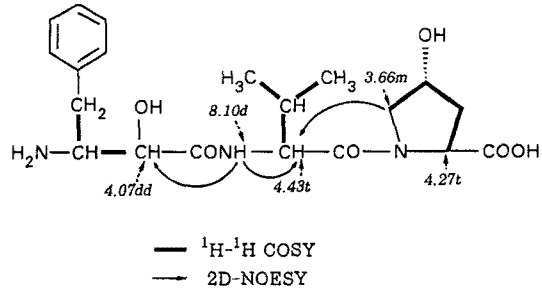

Fig. 2. The selected ^1H-^1H COSY (D_2O) and 2-D NOESY (DMSO-d_6) data of MR-387A2.

Table 3. Inhibition of aminopeptidases by synthetic peptides MR-387A1 and A2.

Target enzyme	Substrate	IC_{50} (μM)	
		MR-387A1	MR-387A2
Porcine kidney AP-N	Leu-*p*NA	0.60	0.87
Human fibrosarcoma HT1080 AP-N	Leu-*p*NA	1.21	1.82
Human myelogenous leukemia K562 AP-N	Leu-*p*NA	17.7	19.3
Human myelogenous leukemia K562 AP-B	Lys-*p*NA	0.35	1.83

AP-N activities of porcine kidney, fibrosarcoma HT1080 and leukemia K562 for determination of IC_{50} value were 104.2 nmole/h/test, 37.6 and 36.8 nmole/h/2×10^6 cells, respectively. AP-B activity of K562 cell was 21.8 nmole/h/2×10^6 cells. Leucine-*p*-nitroanilide or lysine-*p*-nitroanilide was used as a substrate of AP-N or AP-B, respectively. Aminopeptidase activity was detected spectrophotometrically by monitoring the increase in optical density at 405 nm.

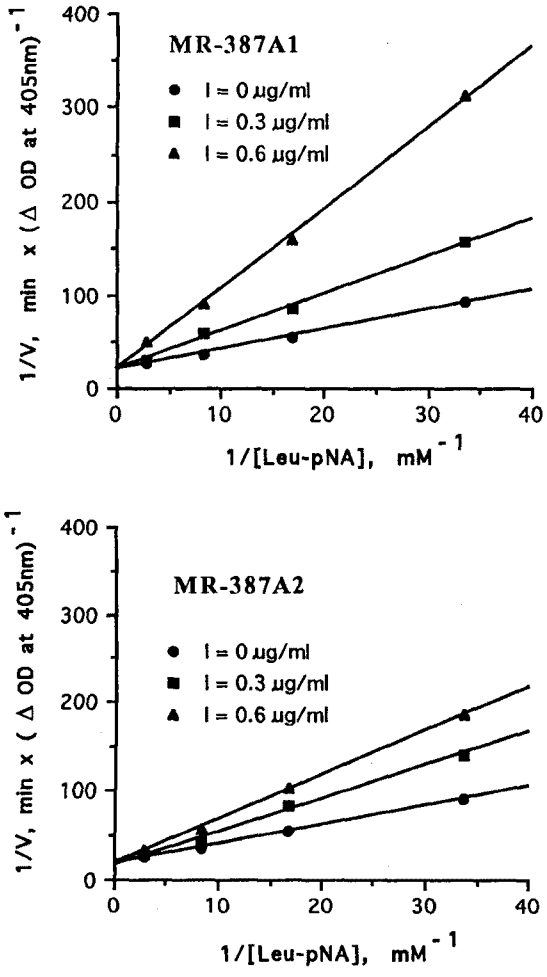

Fig. 3. Lineweaver-Burk plots of inhibition of porcine kidney aminopeptidase N by MR-387A1 and A2.

Table 4. Inhibitory activities of various inhibitors against microsomal AP-N of porcine kidney.

Inhibitor	Structure	IC_{50} (μM)
Bestatin	AHPA-Leu	20.1
MR-387A1	AHPA-Val-Pro	0.60
MR-387A3	AHPA-Val-Hyp	0.87
Valistatin	AHPA-Val-Val	1.27
MR-387A	AHPA-Val-Pro-Hyp	0.20
MR-387B	AHPA-Val-Pro-Pro	0.16
MR-387C	AHPA-Val-Pro-Leu	0.08

Bestatin was purchased from Sigma Co. (USA), and valistatin was purified from the culture broth of *Streptomyces* sp. SL20209 in our laboratory (14).

coincided with the data of Tobe *et al.* (26) and Rich *et al.* (21). They studied the relationship of the length of the peptide chain in amastatin analogues and their activity, and suggested that a tetrapeptide had the strongest ac-

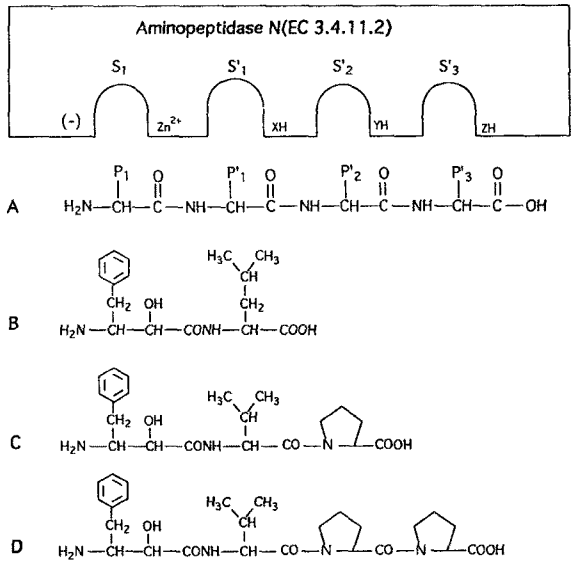

Fig. 4. Schematic models for the binding of putative substrate (A), bestatin(B), MR-387A1(C) and MR-387B(D) to the active site of aminopeptidase N.
(A) and (B), models proposed by Nishizawa *et al.* (18), Rich *et al.* (21) and Helene *et al.* (10) for binding of inhibitor to the active site of aminopeptidase N; (C) and (D), possible binding of MR-387 derivatives to aminopeptidase N.

tivitiy towards AP-A and AP-N. The number of amino acids in the inhibitor affects the tightness of binding between the inhibitor and AP-N. In addition, inhibition of AP-N has been postulated to proceed by chelation of the 2(*S*)-hydroxyl group and the 3-amino group in the APHA moiety in bestatin to zinc ion in the enzyme active site (18). IC_{50} values of AHPA-Val (bestatin), AHPA-Val-Pro (MR-387A1) and AHPA-Val-Pro-Leu (MR-387C) compared against porcine kidney aminopeptidase N were 20.1, 0.60 and 0.08 μM, respectively (Table 4). These results support that a multiple interaction between the $S_1 \rightarrow S'_3$ sites of aminopeptidase N and $P_1 \rightarrow P'_3$ of the inhibitor plays a crucial role in stabilizing strongly the enzyme-inhibitor complex as shown in Fig. 4 (21).

Additionally, replacement of proline in MR-387A1 by hydroxyproline reduced the inhibition of AP-N. MR-387A bearing hydroxyproline in the C-terminal also reduced the inhibition of AP-N compared to MR-387B. These results suggest that in order to inhibit AP-N strongly, hydrophobic amino acid proline as the third or fourth residue from the amino end is better than hydrophilic amino acid hydroxyproline.

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REFERENCES

1. Amoscato, A. A., J. W. Alexander, and G. F. Babcock. 1989. Surface aminopeptidase activity of human lymphocytes. *J. Immunol.* **142**: 1245-1252.
2. Anderson, L., T. C. Isley, and R. Wolfenden. 1982. α-Aminoaldehydes: transition state analogue inhibitors of leucine aminopeptidase. *Biochemistry* **21**: 4177-4180.
3. Aoyagi. T., Yoshida, S. Y. Nakamura, Y. Shigihara, M. Hamada, and T. Takeuchi. 1990. Probestin, a new inhibitor of aminopeptidase M, produced by *Streptomyces azureus* MH 663-2F6. I. Taxonomy, production, isolation, physicochemical properties and biological activities. *J. Antibiotics* **43**: 143-148.
4. Chung, M. C., H. K. Chun, H. J. Lee, and Y. H. Kho. 1994. Taxonomic characteristics of strain producing MR-387A abd B, new inhibitors of aminopeptidase M, and their production. *Kor. J. Appl. Microbiol. Biotechnol.* **22**: 447-452.
5. Chung, M. C., H. K. Chun, H. J. Lee, and Y. H. Kho. 1995. The novel synthetic substance [(2S,3R)-2-hydroxy-3-amino-4-phenylbutanoyl-L-valyl-L-prolyl-L-leucine] as an aminopeptidase M inhibitor. *J. Biochem. Mol. Biol.* **28**: 83-86.
6. Fugiwara, H., M. Maeda, K. Imai, M. Fukuoka, K. Yasuda, K. Horie, K. Takakura, S. Taii, and T. Mori. 1992. Defferential expression of aminopeptidase N on human ovarian granulosa and theca cells. *J. Clin. Endocrinol. Metab.* **74**: 91-95.
7. Gros, C. G., B. Giros, and J. -C. Schwartz. 1985. Identification of aminopeptidase M as an enkephalin-inactivating enzyme in rat cerebral membranes. *Biochemistry* **24**: 2179-2185.
8. Harbeson, S. L. and D. H. Rich. 1989. Inhibition of aminopeptidases by peptides containing ketomethylene and hydroxyethylene amide bond replacements. *J. Med. Chem.* **43**: 1378-1392.
9. Harries, M. A. and B. W. Mattews. 1981. Binding of hydroxamic acid inhibitors to crystalline thermolysin suggests a pentacoordinate zinc intermediate in catalysis. *Biochemistry* **20**: 6912-1920.
10. Helene, A., A. Beaumont, and B. P. Roques. 1991. Functional residues at the active site of aminopeptidase N. *Eur. J. Biochem.* **196**: 385-393.
11. Imai, K., M. Maeda, H. Fugiwara, N. Okamoto, M. Kariya, N. Emi, K. Takakura, H. Kauzaki, and T. Mori. 1992. Human endometrial stromal cells and decidual cells express cluster of differentiation (CD) 13 antigen/aminopeptidase N and CD10 antigen/neutral endopeptidase. *Biol. Reprod.* **46**: 328-334.
12. Ino, K., S. Goto, T. Okamoto, S. Nomura, A. Nawa, K. Isobe, S. Mizutani, and Y. Tomoda. 1994. Expression of aminopeptidase N on human choriocarcinoma cells and cell growth suppression by the inhibition of aminopeptidase N activity. *Jpn. J. Cancer Res.* **85**: 927-933.
13. Kenny, A. J., S. L. Stephenson, and A. J. Turner. 1987. p. 169. *In* A. J. Kenny, and A. J. Turner (ed.), *Mammalian Ectoenzymes*, Elsevier, New York.
14. Ko, H. R., H. K. Chun, M. C. Chung, and Y. H. Kho. 1995. Valistatin(3-amino-2- hydroxy-4-phenylbutanoyl-valyl-valine), a new aminopeptidase M inhibitor, produced by *Streptomyces* sp. SL-20209. *J. Microbiol. Biotechnol.* **5**: 36-40.
15. Lejczak, B., P. Kafarski, and J. Zygmunt. 1989. Inhibition of aminopeptidases by aminophosphonates. *Biochemistry* **28**: 3549-3555.
16. Look, A. T., R. A. Ashmun, L. H. Shapiro, and S. C. Peiper. 1989. Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. *J. Clin. Invest.* **83**: 1299-1307.
17. Menrad, A., D. Speicher, J. Wacker, and M. Herlyn. 1993. Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. *Cancer Res.* **53**: 1450-1455.
18. Nishizawa, R., T. Saino, T. Takita, H. Suda, T. Aoyagi, and H. Umezawa. 1977. Synthesis and structure-activity relationships of bestatin analogues, inhibitors of aminopeptidase B. *J. Med. Chem.* **20**: 510-515.
19. Ocain, T. D. and D. H. Rich. 1988. Synthesis of sulfer-containing analogues of bestatin. Inhibition of aminopeptidases by α-thiolbestatin analogues. *J. Med. Chem.* **31**: 2193-2199.
20. Ocain, T. D. and D. H. Rich. 1992. α-Keto amide inhibitors of aminopeptidases. *J. Med. Chem.* **35**: 451-456.
21. Rich, D. H., B. J. Moon, and S. Harbeson. 1984. Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. *J. Med. Chem.* **27**: 417-422.
22. Saiki, I., H. Fujii, J. Yoneda, F. Abe, M. Nakajima, T. Tsuruo, and I. Azuma. 1993. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. *Int. J. Cancer* **54**: 137-143.
23. Schalk, C., H. d'Orchymont, M. -F. Jauch, and C. Tarnus. 1994. 3-Amino-2-tetralone derivatives: Novel potent and selective inhibitors of aminopeptidase N(EC 3.4.11.2). *Arch. Biochem. Biophys.* **311**: 42-46.
24. Shenvi, A. B. 1986. α-Aminoboronic acid derivatives: Effective inhibitors of aminopeptidases. *Biochemistry* **25**: 1286-1291.
25. Stewart, J. M. and J. D. Young. 1969. *Solid phase peptide synthesis*, W. H. Freeman and co., San Francisco.
26. Tobe. H., H. Morishima, T. Aoyagi, H. Umezawa, K. Ishiki, K. Nakamura, T. Yoshioka, Y. Shimauchi, and T. Inui. 1982. Synthesis and structure-activity relationships of amastatin analogues, inhibitors of aminopeptidase A. *Agric. Biol. Chem.* **46**: 1865-1872.
27. Umezawa, H., T. Aoyagi, H. Suda, M. Hamada, and T. Takeuchi. 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiotics* **29**: 97-99.

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