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Review Article

NOVEL SMALL MOLECULE NONPEPTIDE AMINOPEPTIDASE N INHIBITORS WITH A CYCLIC IMIDE SKELETON

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A novel series of small molecule nonpeptide aminopeptidase N (APN) inhibitors with a N-phenylphthalimide or N-phenylhomophthalimide skeleton were prepared. Evaluation of their protease inhibitory activities revealed that (i) some N-phenylphthalimide analogs are potent APN inhibitors, but they are also inhibitors of another protease, dipeptidylpeptidase IV (DPP-IV), and (ii) some N-phenylhomophthalimide analogs, including 2-(2,6-diethylphenyl)-1,2,3,4-tetrahydroisoquinoline-1,3-dione (PIQ-22), are potent and specific inhibitors of APN without DPP-IV-inhibitory activity. The structure–activity relationship studies of N-phenylphthalimides and N-phenylhomophthalimides are reviewed. PIQ-22 showed potent tumor-cell invasion-inhibitory activity.

Keywords: Aminopeptidase N; Inhibitor; Phthalimide; Homophthalimide; Structure–activity relationship

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INTRODUCTION

Aminopeptidases are enzymes which catalyze the cleavage of amino acids from the N-terminus of protein or peptide substrates. They are widely distributed and have been found in many tissues or cells, on cell surfaces, and in soluble or secreted forms in plants and animals. Aminopeptidases play important roles in the turnover of proteins/peptides leading to uptake or re-use of amino acids in various tissues. They also modulate the regulation of various physiological events by peptide hormones.

Classification of aminopeptidases has often depended on the field of interest of the investigator. Criteria such as the number of residues removed from the substrate, the rate of cleavage of peptide analogs, metal ion content, location and inhibitor susceptibility have all been used for classification, resulting in a series of nonexclusive names.[1–5] Substrate-specificity is widely used for the classification of the enzymes. However, the specificities of many of these enzymes are broad, and it has become clear that the rates of cleavage of physiological substrates do not parallel the rates of cleavage of peptide analogs.[6–8] Also, several aminopeptidases that were originally thought to be distinct enzymes have been shown to be identical. For example, the *Escherichia coli xerB* gene product, *E. coli* aminopeptidase I, now called aminopeptidase A, and *Saccharomyces typhimurium* aminopeptidase appear to be the same,[9] as is the case for aminopeptidases N and M.

Aminopeptidase N (APN, also referred to as aminopeptidase M: APM, alanyl aminopeptidase, arylamidase, and acyl-peptide hydrolase; E.C. 3.4.11.2) is an aminopeptidase which has been the subject of a number of studies. APN is called neutral aminopeptidase, based on the pH at which its maximal activity is observed. The most favorable substrates of APN are peptides having an alanine residue (Ala) at the amino-terminal; the enzyme can also rapidly release Leu, Phe, Tyr, Arg, Met, Lys, Trp, Gly, Gln, Ser and His. However, when the amino-terminal is Pro or pyroglutamyl, the hydrolysis catalyzed by APN is limited.[10]

APN is mainly located in the small intestine and kidney brush borders, but is also found in brain, lung, liver and primary cultures of fibroblasts.[11-16] In the brain, APN, though the content is relatively low in the tissue and is predominantly located on blood vessels, is thought to be involved in the degradation of neuropeptides, particularly the endogenous opioid peptides, enkephalins,[17,18] in association with neutral endopeptidase,[19] another zinc metallopeptidase.[20] Another candidate of the aminopeptidase that primarily is relevant for degrading enkephalins is puromycin-sensitive aminopeptidase

(PSA), which is present in brain in much higher amounts than APN and is approximately 100-fold more sentitive to puromycin than APN.[21–23]

APN is a zinc-containing proteolytic ectoenzyme. APN and another zinc-dependent aminopeptidases have the typical zinc-binding active site with the consensus amino acid sequence of His(1)–Glu(2)–Xaa(3)–Xaa(4)–His(5)–(18 residues)–Glu(24), which is similar to that of metalloproteinases such as thermolysin. Metallopeptidases with this type of active site have been named gluzincins. In the aminopeptidases belonging to the gluzincin-class, His(1) (the first His of the consensus amino acid sequence of the zinc-binding active site shown above), His(5) and Glu(24) are the Zn^{2+}-coordinating residues, while Glu(2) is involved in catalysis.

Among mammals, most of the gluzincin aminopeptidases so far known are type II integral membrane proteins, i.e., they are N-terminally anchored in microsomes or cell membranes. APN from human,[24] rat,[25,26] mouse,[27] rabbit[28] and swine[29] have been cloned and sequenced, and their respective amino acid sequences show high degrees of similarity.

Recently, APN has been shown to be the major receptor for the enteropathogenic coronavirus TGEV[30] and for human coronavirus 229E,[31] and has also been shown to be involved in invasion and metastasis of a variety of tumor cells.[32,33]

Human APN is identical to the myeloid differentiation antigen CD13 (cluster of differentiation antigen 13), which is found in human leukemia cell line HL-60 and in many other types of cells.[34] APN is expressed in malignant melanoma cells, but is absent from normal melanocytes. A high level of plasma membrane-associated aminopeptidase activity was observed in rat mammary adenocarcinoma cells, which spontaneously metastasize from the mammary fat-pad to lymph nodes and lungs. APN/CD13 is also expressed in macrophages and fibroblasts that exhibit high mobility in solid tissues. Thus, the enzyme may also play a role in the degradation and invasion of the extracellular matrix (ECM) by these normal cells.[33,35,36]

These findings indicated that inhibitors of APN could be useful to block tumor cell invasion, metastasis and the progression of various kinds of proteolytic damage. In fact, APN inhibitors have been reported to inhibit significantly the invasion of murine and human metastatic tumor cells into reconstituted basement membranes.[37,38] It has also been reported that APN inhibitors inhibit the type IV collagenolytic activity elicited by metastatic tumor cells.[39,40] Anti-aminopeptidase N/CD13 monoclonal antibody, which blocks APN activity, has effects similar to those of APN inhibitors on several human tumor cell lines.[33] These findings suggest that neutral APN plays a crucial role in matrix degradation and invasion by tumor cells, and

that APN inhibitors may be useful for preventing the spread of malignant cells.

A number of reviews covering aminopeptidases and their inhibitors have appeared in the literature. The purpose of this article is to summarize current knowledge about APN inhibitors as well as to describe the structure–activity relationships of our newly developed APN inhibitors with a nonpeptide cyclic imide skeleton.

NATURAL APN INHIBITORS

Some of the most intriguing APN inhibitors found to date are natural products such as bestatin, amastatin and actinonin (Figure 1). Bestatin (also known as ubenimex) strongly inhibits APN, APB, Ala-AP, Leu-AP, and tripeptidyl and tetrapeptidyl aminopeptidases. Amastatin strongly inhibits APN, APA, Leu-AP, Tyr-AP and tripeptidyl and tetrapeptidyl aminopeptidases. Actinonin inhibits only APN and Leu-AP. Puromycin has been known to be a competitive inhibitor of an extrinsic membrane-associated aminopeptidase referred to as aminopeptidase MII. Though puromycin also elicits inhibiting activity on APN, the activity is relatively low and the antibiotics is a relative specific inhibitor of aminopeptidase MII and of puromycin-sensitive aminopeptidase (PSA).[21–23]

Bestatin

Bestatin (3-amino-2-hydroxy-4-phenylbutanoyl-L-leucin), a potent aminopeptidase inhibitor, was isolated from culture filtrates of *Streptomyces olivorsticuli* in 1976.[41] It has attracted considerable attention since its discovery due to its numerous biological activities, i.e., most notably as an

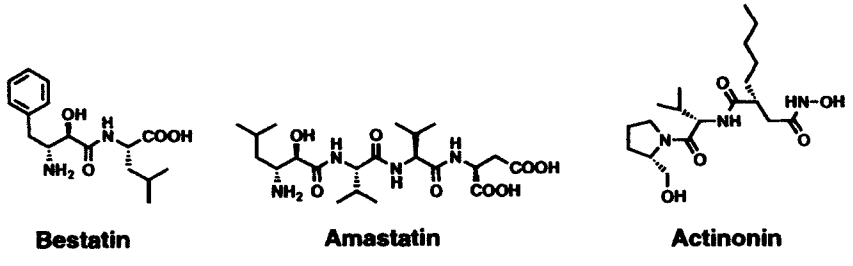


FIGURE 1 Some aminopeptidase N inhibitors.

immune response modifier[42] and a potential analgesic,[43,44] and due to the presence of a novel amino acid residue, 3(*R*)-amino-2(*S*)-hydroxy-4-phenylbutanoic acid (AHPBA) in its structure.

The inhibition by bestatin of P388 leukemia cell metastasis in mice was first demonstrated in 1981.[45] The administration of high doses of bestatin was reported to result in a significant inhibition of experimental and spontaneous metastasis of melanoma in mice.[37] It was also found that bestatin inhibits the invasion and degradation of type IV collagen by tumor cells *in vitro*.[39,40] It is now widely employed clinically as an antitumor agent with host-mediated actions.

Inhibitory Mechanism of Bestatin

In order to design novel APN inhibitors rationally, it would be helpful to elucidate the molecular structure of the active site of APN. Techniques of chemical modifications by selective reagents[46] and site-directed mutagenesis[47] have been used for this purpose. Also, structural modification of bestatin and the structure–activity relationships of the analogs have been studied extensively.

It has been suggested that bestatin (3-amino-2-hydroxy-4-phenylbutanoyl-L-leucin) might be a transition state-analogue inhibitor of aminopeptidases, in which the tetrahedral sp^3 geometry and alcohol at the C-2 carbon (2-hydroxy group) of the inhibitor mimic the features of the tetrahedral intermediate formed during substrate hydrolysis in the active site of the enzyme. Two models of bestatin binding to the active site of aminopeptidase were proposed (Figure 2). In one of them, an essential zinc ion in the active site is chelated by the 2(*S*)-hydroxyl group and the 3-amino group of bestatin, when the inhibitor is bound to aminopeptidases (Figure 2A).[46] The other, postulates an alternative mechanism, in which the

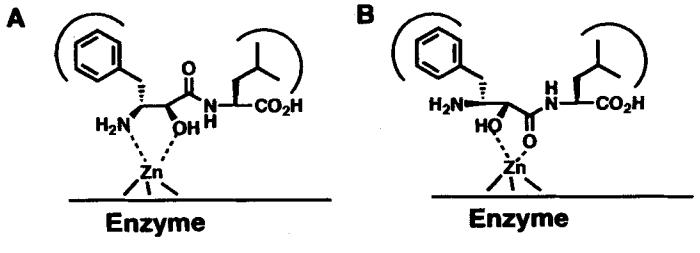


FIGURE 2 (A) Hypothetical model of bestatin binding to the active site of aminopeptidases as proposed by Nishizawa *et al.* (B) Hypothetical model of bestatin binding to the active site of aminopeptidase as proposed by Nishino and Powers.[48,49,51]

C-2 hydroxyl group and the amide carbonyl group of bestatin bind to zinc ion in the active site (Figure 2B).[49] Both mechanisms suggest an interaction between the 2(*S*)-hydroxyl group of the AHPBA moiety in bestatin and the zinc ion which is presumed to be present in the active site of aminopeptidases.

Thereafter, the two groups prepared sulfur-containing analogs of bestatin. The most important of these analogs has the 2(*S*)-hydroxyl group replaced with a sulfhydryl group.[50,51] Both research groups found that this substitution reduces the inhibitory potency of the compound toward leucine aminopeptidase, and concluded that the 2(*S*)-hydroxyl group does not interact with a metal ion.

The three-dimensional structures of some complexes formed between bestatin or amastatin and the cytosolic leucine aminopeptidase have been analyzed by X-ray crystallography.[52–57] The X-ray crystallographic results confirmed a structure similar to that postulated in the former model (Figure 2A), in which the 3-amino and the 2-hydroxyl groups of the AHPBA moiety of bestatin are in the coordination sphere of the zinc atom. Amastatin binds in the active site of leucine aminopeptidase in a manner similar to that of bestatin. Leucine aminopeptidase is considered to be a prototypical aminopeptidase, because it displays the properties common to this group of enzymes, i.e., a requirement for metal ion for activity. Though there has been no direct structural analysis of APN, the X-ray crystallographic results on leucine aminopeptidase could provide a basis for the design of novel APN inhibitors.

SYNTHETIC INHIBITORS

Potent APN inhibitors known at present are all peptides or peptide mimics in structure. In general, peptides have drawbacks for clinical application, i.e., low bioavailability, proteolytic lability, rapid biliary excretion, short duration of action, etc. From a medicinal–chemical point of view, non-peptide derivatives are preferable.

Studies on the development of inhibitors for the metalloproteases have mostly been focused on peptide mimics which are assumed to act as transition state analogs by binding to the catalytic zinc ion via amine and aldehyde (or hydrate) functions.[58] Without exception, the effective aminopeptidase inhibitors reported to date have been designed to incorporate a ligand to bind to zinc in the active site.

During our studies on tumor necrosis factor-alpha (TNF-α) production regulators,[59–63] we noticed that some TNF-α production regulators exhibit APN-inhibitory activities. Because TNF-α is a deleterious factor in tumorigenesis, APN inhibitors possessing TNF-α production-inhibitory activity might be superior clinical tools for cancer chemotherapy.

APN-inhibitory activity has been evaluated in assay systems using intact cells by measuring 7-amino-4-methylcoumarin (AMC) liberated from alanine-4-methylcoumaryl-7-amide (Ala-AMC) or by measuring *p*-nitroaniline liberated from alanine *p*-nitroanilide. In our studies aiming at the structural development of novel APN inhibitors, we adopted the former method using human acute lymphoblastic leukemia cells, MOLT-4, that possess APN on their surface. To confirm the specificity of the inhibition, the compounds were also assayed for inhibition of dipeptidyl peptidase IV (DPP-IV) (EC 3.4.14.5/CD26), a membrane-associated ectoenzyme (serine protease) in certain subsets of leukocytes, particularly $CD4^+$ T cells. The DPP-IV enzymatic activity of intact cells was determined by using MOLT-4 cells with glycyl-L-proline 4-methylcoumaryl-7-amide (Gly-Pro-AMC) as a substrate. Their growth-inhibitory (cytotoxic) effects were also measured by using human embryonic lung fibroblast WI-38 cells.

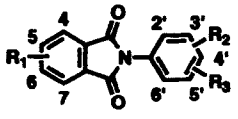
Though the assay systems using MOLT-4 cells that we adopted are established methods to measure APN and DPP-IV activity, the protease-specificity of the substrates (Ala-AMC and Gly-Pro-AMC, respectively) has not been verified to be exclusive. Therefore, our assay systems are, more precisely, methods to measure APN- and DPP-IV-like/type proteolytic activities. In the adopted assay systems, bestatin (**1**) and actinonin (**2**), which are known to inhibit APN but not DPP-IV, and Pro-boroPro (PBP: **3**), which is known to inhibit DPP-IV specifically, showed clearly distinct patterns of protease inhibition, i.e., bestatin (**1**) and actinonin (**2**) showed potent APN-inhibitory activity without DPP-IV-inhibitory activity, while PBP (**3**) showed DPP-IV-inhibitory activity without APN-inhibitory activity (Tables I–V).[64,65]

Structure–Activity Relationships of a Series of N-Phenylphthalimides[64,65]

In the course of our studies, we have synthesized a number of potent TNF-α production regulators with a 2′,6′-dialkylphenylphthalimide structure.[59–63] Some compounds among them show effects (induction of morphological changes) similar to those elicited by protease inhibitors, including bestatin and actinonin, on cultured cells. Therefore, we assayed our compounds for APN-inhibitory activity.

Unsubstituted dialkylphenylphthalimides (PP-11, PP-22 and PP-33: **4**–**6**) did not show any APN-inhibitory activity (IC_{50} values of $>100\,\mu g/ml$) (Table I). Introduction of an electron-withdrawing nitro group at the fused benzene ring (**7**–**12**) did not affect the activity. However, introduction of an electron-donating group, such as an amino (**14**–**18**) or a hydroxyl group (**19**–**24**), caused the appearance of moderate or potent APN-inhibitory activity. The effect of amino and hydroxyl substitution was independent of the site (position 4 or 5 in the structure presented in Table I) at which the

TABLE I Protease-inhibitory activity of phenylphthalimide derivatives



Compound	R_1	R_2	R_3	*APN*[a] IC_{50} (μg/ml)	*DPP*-IV[b] IC_{50} (μg/ml)	*WI*-38[c] IC_{50} (μg/ml)
4: PP-11	H	2′-Me	6′-Me	>100	>100	>100
5: PP-22	H	2′-Et	6′-Et	>100	>100	>100
6: PP-33	H	2′-iPr	6′-iPr	>100	>100	>100
7	4-NO_2	2′-Me	6′-Me	>100	>100	>100
8	4-NO_2	2′-Et	6′-Et	>100	>100	>100
9	4-NO_2	2′-iPr	6′-iPr	>100	>100	93.1
10	5-NO_2	2′-Me	6′-Me	>100	>100	>100
11	5-NO_2	2′-Et	6′-Et	>100	>100	>100
12	5-NO_2	2′-iPr	6′-iPr	>100	>100	42.7
13	4-NH_2	2′-Me	6′-Me	29.0	16.0	>100
14	4-NH_2	2′-Et	6′-Et	2.5	5.0	>100
15	4-NH_2	2′-iPr	6′-iPr	2.6	3.5	8.1
16	5-NH_2	2′-Me	6′-Me	15.0	23.4	>100
17	5-NH_2	2′-Et	6′-Et	5.6	>100	27.0
18	5-NH_2	2′-iPr	6′-iPr	5.4	81.0	17.4
19	4-OH	2′-Me	6′-Me	70.7	>100	>100
20	4-OH	2′-Et	6′-Et	10.2	12.8	>100
21	4-OH	2′-iPr	6′-iPr	4.3	14.1	>100
22	5-OH	2′-Me	6′-Me	10.3	19.8	>100
23	5-OH	2′-Et	6′-Et	9.6	12.8	52.0
24	5-OH	2′-iPr	6′-iPr	15.0	21.3	12.7
25	4-NHAc	2′-iPr	6′-iPr	>100	>100	7.2
26	5-tBu	2′-iPr	6′-iPr	>100	>100	>100
27	4-F	2′-iPr	6′-iPr	>100	>100	>100
28	5-F	2′-iPr	6′-iPr	>100	>100	6.4
29	4,7-diF	2′-iPr	6′-iPr	>100	>100	>100
30	fused-Ph	2′-iPr	6′-iPr	>100	>100	>100
1: bestatin				0.81	>100	>100
2: actinonin				0.32	>100	>100
3: PBP				>100	18.7	>100

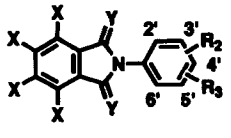
[a]APN-inhibitory activity was assayed by the L-Ala-MCA method.
[b]DPP-inhibitory activity was assayed by the glycyl-L-Pro-MCA method.
[c]Cytotoxicity was assayed by measuring the viability of human embryonic lung fibroblast WI-38 cells.

substituent was introduced. The results suggest that the electronic nature of the phthalimide moiety, rather than the shape, is crucial for potent APN-inhibitory activity.

On the other hand, the steric effect (shape) of the substituent around the imide nitrogen part of the molecule (positions 2′ and 6′ in the structure shown in Table I) seems to be important. In a series of active succinimide derivatives, a comparison of several sets of compounds with different 2′,6′-alkyl substituents (**13**–**15**, **16**–**18**, **19**–**21** and **22**–**24**) suggests that the APN-inhibitory activity tends to increase in the order of 2′,6′-dimethylphenyl $\ll$ 2′,6′-diethylphenyl $\cong$ 2′,6′-diisopropylphenyl (although **22**, **23** and **24** exhibited approximately the same APN-inhibitory activity). Although some of the N-phenylphthalimide analogs showed moderate/potent APN-inhibitory activities, their specificity was low, i.e., they also inhibited DPP-IV with IC_{50} values similar to those for the APN-inhibitory activity.

It is of great interest that conversion of the carbonyl groups (groups Y in the structure presented in Table II) of the succimide moiety to thiocarbonyl groups (mono- or dithiocarbonyl analogs: Table II, **32** and **34**, **35**) changed the enzyme-inhibitory specificity. In particular, PPS-33 (**32**) was shown to be a potent DPP-IV-specific inhibitor without APN-inhibitory activity. This might be consistent with an APN- or DPP-IV-inhibitory mechanism involving the imide carbonyl group, although it is not known how a thiocarbonyl would interact with the active site. The nature of the sulfur atom of a thioamide group, i.e., the steric bulkiness of the sulfur atom, which is larger than an oxygen atom, and the reduced hydrogen-bonding capability of the sulfur

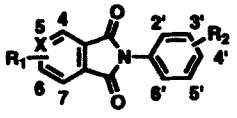
TABLE II Protease-inhibitory activity of thiocarbonyl-phenylphthalimide derivatives



Compound	*X*	*Y*		R_2	R_3	*APN*[a] IC_{50} (μg/ml)	*DPP*-IV[b] IC_{50} (μg/ml)	*WI*-38[c] IC_{50} (μg/ml)
31: PP-33	H	O	O	2′-iPr	6′-iPr	> 100	> 100	> 100
32: PPS-33	H	S	O	2′-iPr	6′-iPr	> 100	18.7	60.5
33: FPP-33	F	O	O	2′-iPr	6′-iPr	> 100	> 100	0.9
34: FPPS-33	F	S	O	2′-iPr	6′-iPr	11.4	12.0	> 100
35: FPPSS-33	F	S	S	2′-iPr	6′-iPr	40.5	15.1	1.4
1: bestatin						0.81	> 100	> 100
2: actinonin						0.32	> 100	> 100
3: PBP						> 100	18.7	> 100

[a–c]As for those of Table I.

TABLE III Protease-inhibitory activity of methylthiophenylphthalimide derivatives



Compound	*X*	R_1	R_2	*APN*[a] IC_{50} (μg/ml)	*DPP*-IV[b] IC_{50} (μg/ml)	*WI*-38[c] IC_{50} (μg/ml)
36	CH	H	2′-SMe	47.8	> 100	> 100
37	CH	H	3′-SMe	> 100	> 100	> 100
38	CH	H	4′-SMe	> 100	> 100	> 100
39	CH	4-NO_2	2′-SMe	> 100	> 100	> 100
40	CH	4-NH_2	2′-SMe	10.7	26.0	> 100
41	CH	4-OH	2′-SMe	38.2	64.4	> 100
42	CH	5-Me	2′-SMe	28.1	> 100	> 100
43	N	H	2′-SMe	> 100	> 100	> 100
1: bestatin				0.81	> 100	> 100
2: actinonin				0.32	> 100	> 100
3: PBP				> 100	18.7	> 100

[a–c] As for those of Table I.

atom compared to the oxygen atom of an amide group, might contribute to the decrease in the APN-binding affinity of the thiocarbonyl analogs. The geometrical and conformational properties of the thioamide unit are considered to closely resemble those of an amide unit, except for the bond length.

In a series of methylthiophenylphthalimide derivatives (Table III, **36**–**38**), only the 2′-methylthio derivative (**36**) showed moderate APN-inhibitory activity, while the corresponding 3′- or 4′-regioisomers were inactive, suggesting highly specific recognition of the compound by APN. Introduction of an electron-withdrawing nitro group (**39**) or a nitrogen atom at the fused benzene ring (**43**) reduced the activity (Table III). As in the case of a series of dialkylphenylphthalimide analogs (Table I), introduction of an electron-donating amino (**40**), a hydroxyl (**41**), or a methyl group (**42**) augmented the APN-inhibitory activity, though the extent of the augmentation was not high.

Structure–Activity Relationships of a Series of N-Phenylhomophthalimides[64,65]

Structural development based on the phenylphthalimide skeleton did not result in the separation of APN- and DPP-IV-inhibitory activities (Tables I–III). Furthermore, some phenylphthalimide analogs were shown

to be moderately cytotoxic (Tables I and II). Therefore, we focused on structural development of the succinimide skeleton itself.

When the cyclic imide part was changed from a five-membered ring system (phthalimides) to a six-membered one (homophthalimides), an extraordinary enhancement of the APN-inhibitory activity was obtained (Table IV). For example, while 2′,6′-dialkylphenylphthalimides (PP-11, PP-22 and PP-33: **4**–**6**, Table I) were inactive, the corresponding homophthalimide analogs (PIQ-11: **58**, PIQ-22: **64** and PIQ-33: **67**, Table IV) showed extremely potent APN-inhibitory activity with IC_{50} values of 0.1–10 μg/ml.

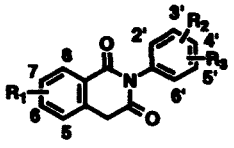
Nonsubstituted N-phenyhomophthalimide (**44**) was almost inactive. The steric effect of substituents introduced around the imide nitrogen of the homophthalimides (positions 2′ and 6′ in the structure shown in Table IV) is extremely important for potent activity, as in the case of the phthalimide analogs (Table I). In a series of N-(2′,6′-disubstituted phenyl)homophthalimides (PIQ-11: **58**, PIQ-22: **64** and PIQ-33: **67**), the 2′,6′-diethylphenyl derivative (PIQ-22: **64**) showed the most potent APN-inhibitory activity with an IC_{50} value of 0.12 μg/ml, being more potent than bestatin ($IC_{50} = 0.81$ μg/ml) or actinonin ($IC_{50} = 0.32$ μg/ml). The effect of the two ethyl groups introduced at the 2′- and 6′-positions seems to be specific, because the corresponding regioisomers (2′,5′-isomer: **65** and 2′,4′-isomer: **66**, IC_{50} values 4.3 and 19.5 μg/ml, respectively), as well as the 2′,6′-dimethyl (PIQ-11: **58**, $IC_{50} = 8.7$ μg/ml) and 2′,6′-diisopropyl (PIQ-33: **67**, $IC_{50} = 3.5$ μg/ml) analogs all have much weaker APN-inhibitory activity than PIQ-22 (**64**, $IC_{50} = 0.12$ μg/ml). A similar tendency was also observed for monoalkyl-substituted N-phenylhomophthalimides, i.e., the potency of APN-inhibitory activity decreased in the order of 2′-ethyl (**46**, $IC_{50} = 17.8$ μg/ml) $\gg$ 2′-methyl (**45**, $IC_{50} = 41.5$ μg/ml) > 2′-isopropyl (**49**, $IC_{50} = 54.3$ μg/ml) analogs. The corresponding 4′-alkylated regioisomers (**48** and **50**, IC_{50}-values > 100 μg/ml) did not show APN-inhibitory activity.

In a series of monosubstituted N-phenylhomophthalimides, the introduction of a 2′-methylthio group (**54**) resulted in very potent inhibition with an IC_{50} value of 0.9 μg/ml.

Although some dialkyl- (**58**–**71**) and dimethoxyphenylhomophthalimides (**72**–**75**) are developed (Table IV), the structure–activity relationships of the regioisomers are unclear, except that 2′,6′-disubstitution resulted in potent APN-inhibitory activity. In dimethylphenylhomophthalimides (**58**–**63**), the 2′,6′-disubstituted one (**58**) is a potent APN inhibitor, but its regioisomer, the 3′,5′-disubstituted one (**63**), possesses even more potent activity than **58**.

Introduction of both an electron-withdrawing nitro group (**80**) and an electron-donating amino group (**81**) at the fused benzene ring of PIQ-22 (**64**)

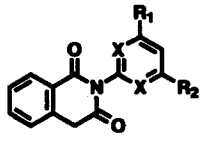
TABLE IV Protease-inhibitory activity of phenylhomophthalimide derivatives



Compound	R_1	R_2	R_3	*APN*[a] IC_{50} (μg/ml)	*DPP*-IV[b] IC_{50} (μg/ml)	*WI*-38[c] IC_{50} (μg/ml)
44	H	H	H	93.7	> 100	> 100
45	H	2′-Me	H	41.5	> 100	> 100
46	H	2′-Et	H	17.8	> 100	> 100
47	H	3′-Et	H	11.7	> 100	> 100
48	H	4′-Et	H	> 100	> 100	> 100
49	H	2′-iPr	H	54.3	> 100	> 100
50	H	4′-iPr	H	> 100	> 100	> 100
51	H	2′-OMe	H	6.2	> 100	> 100
52	H	3′-OMe	H	9.5	> 100	> 100
53	H	4′-OMe	H	7.8	11.6	> 100
54	H	2′-SMe	H	0.9	> 100	> 100
55	H	3′-SMe	H	8.0	> 100	> 100
56	H	4′-SMe	H	6.2	6.0	> 100
57	H	3′-CH_2OH	H	29.0	> 100	> 100
58: PIQ-11	H	2′-Me	6′-Me	8.7	23.7	> 100
59	H	2′-Me	5′-Me	11.6	> 100	> 100
60	H	2′-Me	4′-Me	16.6	> 100	> 100
61	H	2′-Me	3′-Me	30.0	> 100	> 100
62	H	3′-Me	4′-Me	22.8	> 100	> 100
63	H	3′-Me	5′-Me	1.5	> 100	> 100
64: PIQ-22	H	2′-Et	6′-Et	0.12	> 100	> 100
65	H	2′-Et	5′-Et	4.3	> 100	> 100
66	H	2′-Et	4′-Et	19.5	> 100	> 100
67: PIQ-33	H	2′-iPr	6′-iPr	3.5	> 100	> 100
68	H	2′-Me	6′-Et	0.43	> 100	> 100
69	H	2′-Me	6′-iPr	0.78	> 100	> 100
70	H	2′-tBu	5′-tBu	> 100	> 100	> 100
71	H	3′-tBu	5′-tBu	7.3	> 100	> 100
72	H	2′-OMe	5′-OMe	3.2	> 100	> 100
73	H	2′-OMe	4′-OMe	4.9	> 100	> 100
74	H	3′-OMe	4′-OMe	> 100	> 100	> 100
75	H	3′-OMe	5′-OMe	5.3	> 100	> 100
76	H	3′,4′,5′-tri-OMe		> 100	> 100	> 100
77	H	2′-Cl	6′-Cl	44.1	> 100	> 100
78	H	2′-F	6′-F	> 100	> 100	> 100
79	H	2′,3′-fused-Ph		1.7	> 100	> 100
80	7-NO_2	2′-Et	6′-Et	28.0	> 100	> 100
81	7-NH_2	2′-Et	6′-Et	8.2	> 100	22.0
1: bestatin				0.81	> 100	> 100
2: actinonin				0.32	> 100	> 100
3: PBP				> 100	18.7	> 100

[a–c] As for those of Table I.

TABLE V Protease-inhibitory activity of heterocyclic homophthalimide derivatives



Compound	*X*		R_1	R_2	*APN*[a] IC_{50} (μg/ml)	*DPP*-IV[b] IC_{50} (μg/ml)	*WI*-38[c] IC_{50} (μg/ml)
75	CH	CH	OMe	OMe	5.3	> 100	> 100
82	N	N	OMe	OMe	> 100	> 100	> 100
63	CH	CH	Me	Me	1.5	> 100	> 100
83	CH	N	Me	Me	3.6	> 100	> 100
84	N	N	Me	Me	> 100	> 100	> 100
85	N	N	Me	OMe	11.3	42.2	> 100
1: bestatin					0.81	> 100	> 100
2: actinonin					0.32	> 100	> 100
3: PBP					> 100	18.7	> 100

[a–c]As for those of Table I.

decreased the activity (Table IV). The activity increased in the order of nitro derivative (**80**) < amino derivative (**81**), and this order is the same as that in the case of phenylphthalimide analogs (Table I).

Heteroaromatic-substituted homophthalimide analogs (Table V: **82**–**85**) showed lower APN-inhibitory activity than the corresponding substituted phenylhomophthalimides (**63** and **75**).

The homophthalimide analogs investigated in our study showed no apparent cytotoxicity, except for the 7-amino derivative, **81** (Table IV).

The heteroatom-containing substituent at the position 4′ might be important for DPP-IV-inhibitory activity, because the 4′-methoxy analog (**53**) and 4′-methylthio analog (**56**) possess DPP-IV-inhibitory activity, while the corresponding 2′- (**51** and **54**, respectively) and 3′-regioisomers (**52** and **55**, respectively) are inactive (Table IV).

It should be noted that N-phenylhomophthalimides and N-phenylphthalimides with APN-inhibitory activity seem not to have any efficient zinc-chelating functionality in the molecule. Their structure–activity relationships, especially the effect of the 2′,6′-diethyl group of N-phenylhomophthalimides (PIQ-22: **64**), might mean that there is a specific steric requirement for tight binding of these types of compounds to APN.

Inhibition of Tumor-Cell Invasion

PIQ-22 (**64**) has a significant APN-inhibitory activity, which is more potent than those of bestatin and actinonin (Table IV). This high potency of

TABLE VI Inhibitory effect of PIQ-22 (**64**) and PIQ-11 (**58**) on tumor-cell invasion

Compound	*Percent inhibition of cell-invasion*[a]		
	1 μM	10 μM	100 μM
64: PIQ-22	27.7	68.5	98.9
58: PIQ-11	28.6	56.4	82.7
1: bestatin	nt[b]	−4	57.9
2: actinonin	nt	nt	42.1

[a]The number of cells that invaded in the absence of a test compound was taken as the control. The decrease of the invaded cell number in the presence of a test compound is shown as a percentage with respect to the control.
[b]nt: not tested.

PIQ-22 (**64**) and its low cytotoxicity led us to investigate the effect of PIQ-22 (**64**) and PIQ-11 (**58**) on tumor-cell invasion into reconstituted basement membrane (Matrigel). The invasion of mouse metastatic tumor cells (B16F10 mouse melanoma cells) into Matrigel-coated filters was inhibited by both PIQ-22 (**64**) and PIQ-11 (**58**), in a concentration-dependent manner (Table VI) (Miyata, K. *et al.*, in preparation).[65] Both of them were more effective than bestatin and actinonin. PIQ-22 (**64**) inhibited tumor-cell invasion almost completely at the concentration of 100 μM. Thus, PIQ-22 (**64**) appears to have potential as a novel tumor-cell invasion inhibitor. It is not clear why PIQ-11 (**58**), which has weaker APN-inhibitory activity than bestatin and actinonin, inhibits invasion almost as potently as PIQ-22 (**64**). There may be some target molecule(s) other than APN which mediates the tumor-cell invasion-inhibitory activity elicited by these homophthalimides.

CONCLUDING REMARKS

We have developed a novel, potent APN inhibitor, PIQ-22 (**64**), with a small molecule nonpeptide structure. PIQ-22 (**64**) was also shown to potently inhibit tumor-cell invasion. We are currently examining the mechanism of the neutral aminopeptidase-inhibitory activity. Novel neutral aminopeptidase inhibitors should be helpful in studies to investigate the roles of APN and other neutral aminopeptidases in tumor-cell invasion.

We have also developed many other APN inhibitors with phthalimide and homophthalimide skeletons as an extension of our work on TNF-α production regulators.[53-59] Some of the APN inhibitors described in this review article also possess TNF-α production-inhibitory activity.[53-59,66] Because TNF-α is a pathophysiological factor in many diseases, including tumors, AIDS, and rheumatoid arthritis, APN inhibitors concomitantly possessing

TNF-α production-regulatory activity might be lead compounds for novel biological response modifiers with potential clinical applicability.

A similar strategy, i.e., structural development based on phenylphthalimide and phenylhomophthalimide skeletons, has been successfully applied to derive novel, potent androgen antagonists.[59,67] A cyclic imide skeleton might be a promising protopharmacophore for various kinds of activity, modulating TNF-α production, androgen receptor- and other nuclear receptor-mediated actions, and protease activities.

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