

Aminopeptidase-N/CD13 (EC 3.4.11.2) Inhibitors: Chemistry, Biological Evaluations, and Therapeutic Prospects

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Abstract: Aminopeptidase N (APN)/CD13 (EC 3.4.11.2) is a transmembrane protease present in a wide variety of human tissues and cell types (endothelial, epithelial, fibroblast, leukocyte). APN/CD13 expression is dysregulated in inflammatory diseases and in cancers (solid and hematologic tumors). APN/CD13 serves as a receptor for coronaviruses. Natural and synthetic inhibitors of APN activity have been characterized. These inhibitors have revealed that APN is able to modulate bioactive peptide responses (pain management, vasopressin release) and to influence immune functions and major biological events (cell proliferation, secretion, invasion, angiogenesis). Therefore, inhibition of APN/CD13 may lead to the development of anti-cancer and anti-inflammatory drugs. This review provides an update on the biological and pharmacological profiles of known natural and synthetic APN inhibitors. Current status on their potential use as therapeutic agents is discussed with regard to toxicity and specificity. © 2005 Wiley Periodicals, Inc. *Med Res Rev*, 26, No. 1, 88–130, 2006

Key words: aminopeptidase; ectoenzyme; natural inhibitor; synthetic inhibitor; bestatin; cancer; inflammation

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1. INTRODUCTION

Aminopeptidase N (EC 3.4.11.2, APN) is a metallo-dependent integral membrane protease.¹ The enzyme belongs to the M1 family of the MA clan of peptidases² also called gluzincins.³ Aminopeptidase N consists of 967 amino acids with a short N-terminal cytoplasmic domain, a single transmembrane part, and a large cellular ectodomain containing the active site.⁴ This enzyme was first isolated in 1963 by Pfeleiderer and Celliers from pig kidney⁵ and is known under several different names (alanine aminopeptidase; microsomal aminopeptidase; microsomal leucine aminopeptidase aminopeptidase M; amino oligopeptidase; GP 150). In the last few years, certain surface molecules identified as cluster differentiation (CD) antigens were found to be identical to some membrane proteins. Thus, CD13 is identical to APN.^{6,7} Soluble APN is detectable in plasma/serum and urine^{8–11} but the mechanism of release of membrane APN remains unknown.

Membrane-bound APN/CD13 is widely distributed outside the hematopoietic system (epithelial-, endothelial-, fibroblast-cell types) with main sources being brush border membranes of kidney proximal tubule cells and enterocytes, and in the hematopoietic compartment is not confined to a particular lineage.^{1,12,13} APN/CD13 is predominantly expressed on stem cells and on cells of the granulocytic and monocytic lineages at distinct stages of differentiation and is therefore considered as a marker of differentiation.^{14,15}

Dysregulated expression of membrane and/or soluble forms of APN/CD13 is observed in many diseases. Compiled observations indicate enhanced APN levels in tumor cells such as melanoma,^{16,17} renal,¹⁸ pancreas,¹⁹ colon,²⁰ prostate,²¹ gastric,²² and thyroid²³ cancers. Tumor-infiltrating T cells in renal and lung cancers are CD13-positive.^{24,25} APN activity is elevated in plasma and effusions of cancer patients.¹¹ APN activity on neutrophils from patients affected by a rare adrenal gland tumor, adrenal pheochromocytoma, is significantly increased as compared with healthy controls.²⁶ CD13 is overexpressed in acute and chronic myeloid leukemias^{1,12,27–29} and in anaplastic large cell lymphomas.^{30,31} Overexpression of APN/CD13 in T lymphocytes or neutrophils occurs in several inflammatory diseases (chronic pain, various forms of joint effusions, rheumatoid arthritis, multiple sclerosis, systemic sclerosis, systemic lupus erythematosus, polymyositis/dermatomyositis, pulmonary sarcoidosis).^{32–39}

APN/CD13 may be therefore considered as a useful clinical marker. Whether this protease critically contributes to the pathological behavior remains however unknown. In this review, we briefly summarise knowledge on the structure and the mechanisms of cleavage of APN/CD13 to integrate current knowledge in natural and synthetic APN inhibitors. The reader is referred to excellent reviews for the characteristics of APN/CD13 and substrate specificity.^{25,40–45} Various aspects on the roles of APN/CD13 are reviewed here in the context of the *in vitro* and *in vivo* use of certain APN inhibitors.

2. AMINOPEPTIDASE N/CD13

APN is anchored to the plasma membrane, *via* an uncleaved signal sequence, by the C-terminus (type II) facing extracellularly.¹ Membrane APN/CD13 is found as a dimer of two non covalently associated subunits with a relative molecular mass of 160 kDa (Fig. 1A).^{40,41,43} The human CD13 gene, cloned in 1989⁶ and subsequently mapped to chromosome 15 q25-26,⁴⁶ possesses two promoters (Fig. 1B).^{46–51}

The cDNA sequence reveals the presence of the amino acid sequence His-Glu-Xaa-Xaa-His which is a Zn⁺⁺ binding motif found in one class of metallo-peptidases.³ Site-directed mutagenesis indicates that extracellular cysteines in the molecule confer correct structure and consequently enzymatic activity and surface expression of APN.⁵² Mutation of glutamic acid 355 in an aminopeptidase conserved region (the GAMEN motif) leads to an inactive enzyme⁵³ indicating that

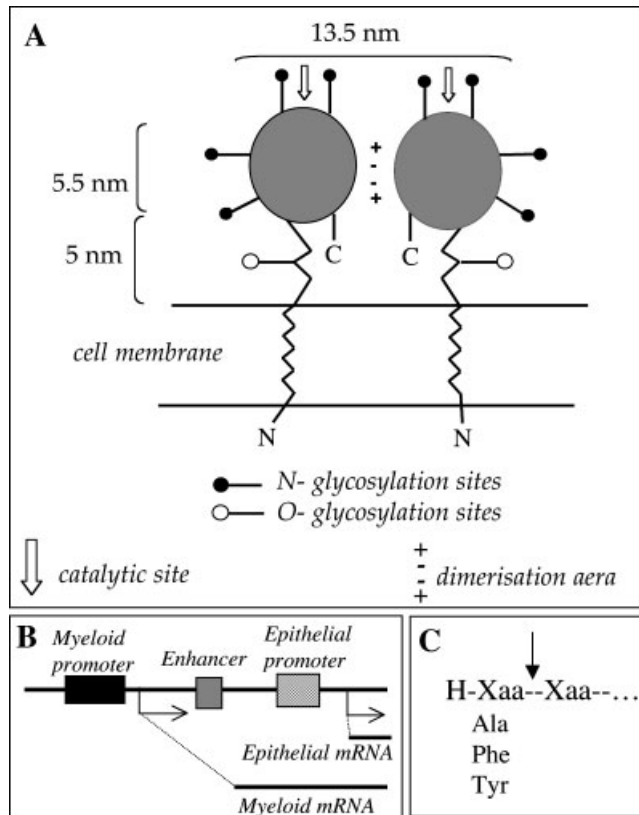


Figure 1. Schematic diagrams showing protein (A) and promoter (B) structures of APN/CD13. The enzyme is a dimer of two non covalently associated monomers. The gene is controlled by two promoters, with an epithelial promoter and a myeloid promoter (in the hematopoietic system). (C) substrate specificity. The arrow indicates the bond cleaved.

this glutamic acid belongs to the anionic binding site in APN and interacts with the N-terminal α -amino group of the substrate. APN/CD13 cleaves preferentially neutral amino acids (with the exception of proline) (Fig. 1C) from the unsubstituted N-terminus of oligopeptides.^{1,12} Biologically active peptide substrates cleaved by APN/CD13 are neuropeptides (Met- and Leu-enkephalins, neurokinin A, Met-lys-bradykinin, and endorphins such as spinorphin),^{41,54-59} vasoactive peptides (kallidin, somatostatin, and angiotensins)⁶⁰⁻⁶⁷ and chemotactic peptides (monocyte chemotactic protein/MCP-1 and N-formyl methionine leucine phenylalanine/f-MLP).^{40,68}

Apart from its hydrolytic ability, APN serves as a receptor for coronaviruses.⁶⁹⁻⁷² In humans, the 229E corona virus uses APN to enter alveolar cells and establish an upper respiratory tract infection.⁷²

3. APN/CD13 INHIBITORS

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) are natural peptides capable of inhibiting APN in micromolar concentrations.⁷³ Similarly, elevated concentrations of leucine, proline, L-alanine, L-arginine, L-glutamine, L-methionine, as well as divalent cations (Co²⁺, Zn²⁺, Mn²⁺, Ca²⁺, Ni²⁺) inhibit APN activity.⁴⁰ (for review) Moreover, molecules with a broad spectrum of action such as KCN, NaN₃,

ammonium oxalate, *N*-ethyl-maleimide, and 8-hydroxyquinoline inhibit APN/CD13.⁴⁰ (for review) APN activity is also inhibited by puromycin (**1**),^{74,75} lapstatin (**2**),⁷⁶ some *N*-phenylphthalimide derivatives such as compound **3**,^{77–80} several *N*-phenylhomophthalimide derivatives like PIQ-22 (**4**)^{77,78} which has later been described as a rather puromycin-sensitive aminopeptidase (PSA) inhibitor by the same group,^{80–83} phosphinate dipeptide analogues illustrated by hPheP[CH₂]Tyr (**5**),⁸⁴ pseudoglutamyl aminophosphinic peptides such as GluΨ(PO₂CH₂)Leu-Ala (**6**),⁸⁵ several variously substituted 3-amino-2-oxobutyramide exemplified by compound **7**,⁸⁶ α-aminoboronic derivatives such as the benzyl derivative **8**,⁸⁷ or α-aminobenzaldehydes illustrated by (*S*) 2-amino-5-methylpentanal (**9**).⁸⁸ An eclectic set of compounds has been described and used for the biochemical characterization or/and inhibition of other proteases—e.g.: urokinase-type plasminogen activator, dipeptidylpeptidase IV (DPP/CD26), or other different aminopeptidases including human enkephalin degrading aminopeptidase (HEDA), cytosolic leucine aminopeptidase (LAPc), glutamyl aminopeptidase (APA), and arginyl aminopeptidase (AP-B). In this context, it is also worth mentioning two systematic studies devoted to hydroxylated naturally occurring flavonoids such as baicalein (**10**), apigenin (**11**), or myricetin (**12**) and related compounds which, aside their activity on neutral endopeptidase (NEP/CD10) or angiotensin-converting enzyme (ACE/CD143), exhibited a significant *in vitro* inhibitory effect toward APN.^{89,90} Formulas, K_i, IC₅₀ or inhibition percentages of enzymes for compounds **1–12** are depicted in Figure 2. Two recent publications describing either the irreversible inhibition of both APN/CD13 and DPP IV/CD26 enzymatic activities by aqueous extracts of a *Cistus incanus* L.⁹¹ or ACE, NEP, and APN inhibition by extracts of *Epilobium angustifolium*⁹² deserve also quotation.

Although the borderline is not easy to position, leaving out the above-mentioned studies dealing with non-specific compounds targeting other enzymes and, incidentally, revealing an inhibitory activity on APN, we have chosen to focus the present review on the data tightly dedicated to natural and synthetic inhibitors of APN/CD13 itself.

A. Naturally Occurring APN/CD13 Inhibitors

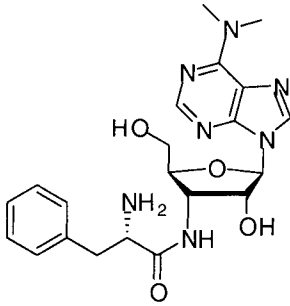
The most widely used among the naturally occurring APN/CD13 inhibitors are microorganism-produced and have been purified from microbial culture filtrates. A large part of them are generated by bacteria belonging to the order *Actinomycetales*, especially of the genera *Streptomyces*:

1. Actinonin

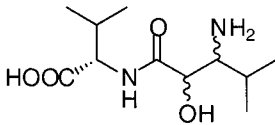
(2*R*)-*N*¹-hydroxy-*N*¹-[(1*S*)-1-[[2*S*]-2-(hydroxymethyl)-1-pyrrolidinyl]carbonyl]-2-methylpropyl]-2-pentylbutanediamide (**13**) was first isolated by R. Green and R. Bhagwan Singh from a Malayan strain of *Actinomycetes*. This compound was then listed as *Streptomyces* Cutter C/2 (N.C.I.B. 8845).⁹³ About 20 years later, actinonin was also obtained from another strain referenced MG848-hF6 and its inhibition against APN was found to be competitive with the substrate.⁹⁴ The structural study and the chemical synthesis of **13** and some analogues have aroused numerous works^{95–102} completed by a structure–activity relationship investigation dealing with anti bacterial properties observed in this actinonin series.¹⁰³

2. AHPA-Val

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valine (**14**) and two closely related derivatives: AHPA-Val-Pro-Hyp (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-(*trans*-4-hydroxy-L-proline) (MR387A) (**15**) and AHPA-Val-Pro-Pro (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline) (MR387B) (**16**) were obtained from the culture broth of *Streptomyces neyagawaensis* SL-387.^{104–106} The preparation of several novel synthetic AHPA derivatives (exemplified by **17**) bearing, for most of them, heterocyclic moieties and exhibiting

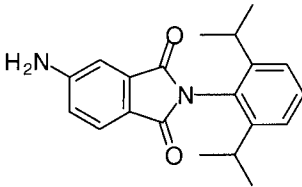


Puromycin (1)

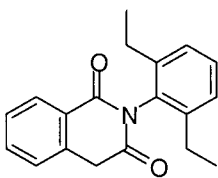


Lapstatin (2)

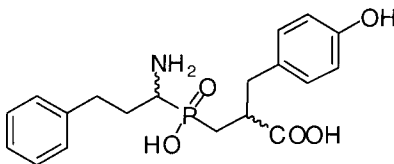
(The absolute configuration is not completely elucidated)



3



PIQ-22 (4)



hPheP[CH₂]Tyr (5)

(mixture of four diastereoisomers)

IC₅₀ APM from rat blood plasma : 60 μM⁶⁷

IC₅₀ APN : 50 μM, IC₅₀ AAP-S : 0.6 μM μM⁷⁵

67% inhibition of APN from human seminal plasma at 100 μM⁷⁴

IC₅₀ APN : 4.8 μM, IC₅₀ PSA : 0.6 μM⁸³

IC₅₀ APM from hog kidney : 203 μM⁷⁶

IC₅₀ APN from egg white : > 122 μM⁷⁶

IC₅₀ LAP from porcine kidney : 41 μM⁷⁶

IC₅₀ LAP from *Streptomyces rimosus*: 2.4 μM⁷⁶

IC₅₀ LAP from *Aeromonas proteolytica*: 0.3 μM⁷⁶

IC₅₀ API from *Streptomyces griseus*: 0.85 μM⁷⁶

IC₅₀ APN : 5.4 μg/mL, IC₅₀ DPP-IV : 14.1 μg/mL, WI-38 : 17.4 μg/mL^{77,80}

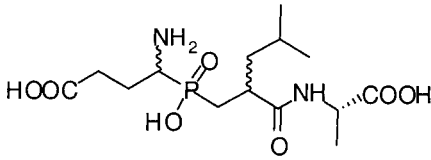
IC₅₀ APN : 16.8 μM, IC₅₀ DPP-IV : 251.2 μM⁷⁹

IC₅₀ APN : 0.12 μg/mL, IC₅₀ DPP-IV : 14.1 μg/mL^{77,80}

Inactive towards LAP, DPP-IV, Trypsin and Chymotrypsin^{80,82}

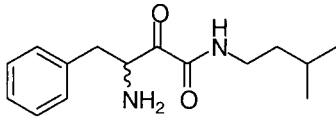
K_i APN : 36 nM, K_i LAP : 67nM⁸⁴

Figure 2. Miscellaneous inhibitors of APN/CD13.



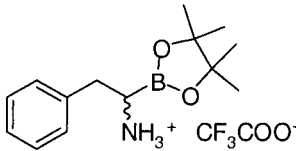
GluΨ(PO₂CH₂)Leu-Ala (6)
(mixture of four diastereoisomers)

K_i APN : 31 μ M, K_i APA : 0.8nM, K_i NEP : 62 nM, K_i ACE : 53 μ M⁸⁵



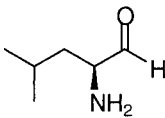
7 (racemate)

K_i APN : 2.5 μ M, K_i LAP_c : 1 μ M, K_i APB : 1.5 μ M⁸⁶



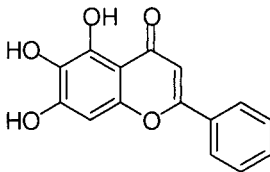
8 (racemate)

IC₅₀ APN : 20 nM, IC₅₀ HEDA : 50 nM⁸⁷



9

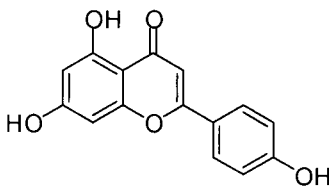
K_i APN : 760 nM, K_i LAP_c : 60 nM⁸⁸



Baicalein (10)

% inhibition (at maximal concentration of 300 μ M)

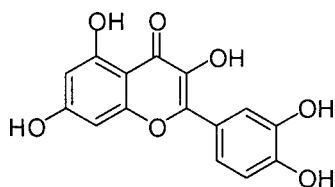
APN : 57 %, ACE : 10 %, NEP : 36 %⁹⁰



Apigenin (11)

% inhibition (at 100 μ M) : APN : 26 %, inactive on LAP⁸⁹

% inhibition (at maximal concentration of 300 μ M) : APN : 42 %, ACE : 18 %, NEP : 31 %⁹⁰

**Myricetin (12)**

% inhibition (at 100 μM) : APN : 25 %, inactive on LAP⁸⁹

% inhibition (at maximal concentration of 300 μM) : APN :

48 %, ACE : 26 %, NEP : 68 %⁹⁰

Figure 2. (Continued)

interesting *in vivo* antitumor potencies (30–40% inhibitory rate on S180 sarcoma) has been recently reported.¹⁰⁷

3. Amastatin

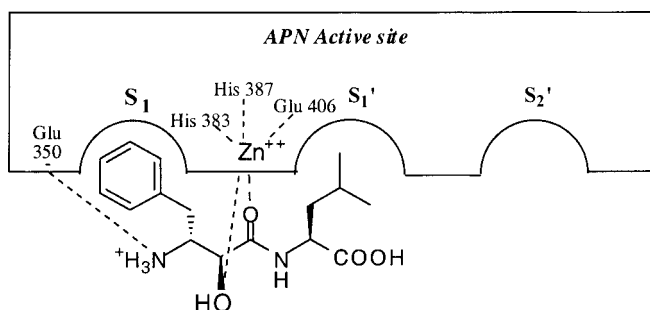
(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valine-L-valine-L-aspartic acid (**18**) has been reported to be a slow-binding competitive inhibitor of APN.¹⁰⁸ It was first isolated from the culture filtrate of *Streptomyces* sp. ME98-M3.¹⁰⁹ and its structure has been unambiguously determined.¹¹⁰ Several enantioselective syntheses of this tetrapeptide have been reported,^{110,111} and some of its analogues have also been prepared in the context of a SAR study.¹¹²

4. Bestatin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine (Ubenimex[®]) (**19**) is an inhibitor of various leucine and arginine aminopeptidases,¹¹³ and an efficient inhibitor of LTA₄ hydrolase.^{114–117} However, in spite of its marked toxicity and of its relative lack of selectivity toward exopeptidases, it is one of the most used compound for its APN/CD13 inhibitory effects.¹¹⁸ Bestatin has been described as a slow-binding competitive inhibitor of APN,¹⁰⁸ and a schematic representation of **19** within the active site of APN^{53,84} is depicted in Figure 3. Bestatin was first isolated from a culture filtrate of *Streptomyces olivoreticuli* (MD976-C7)¹¹⁹ and its chemical structure has been subsequently ascertained.¹²⁰ Several stereoselective total syntheses of **19** have been reported,^{121–130} the preparation of its stereoisomers has been performed¹³¹ and some ubenimex derivatives or analogues such as the *para*-hydroxybestatin (**20**),¹³² the 2-thiolbestatin (**21**),^{133,134} the bestatin thioamide (**22**),^{133,135} or the reduced bestatin **23**¹³⁶ have also been prepared.

5. Phebestin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylalanine (**24**) is a tripeptide produced by *Streptomyces* sp. MJ716-m3.¹³⁷ Some stereoselective syntheses of **24** have been recently reported.^{125,128,129}

**Figure 3.** Binding of Bestatin to the active site of APN.

6. Probestin

(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline) (**25**) is a tetrapeptide isolated from the culture of *Streptomyces azureus* (MH663-2F6)¹³⁸ and its structure has been unambiguously established.¹³⁹ Probestin has been described as a competitive inhibitor of APN¹³⁸ and, here also, some total syntheses have been lately described.^{125,128,129}

An overview of the formulas of compounds **14–25** reveals that, except the synthetic analogue **23** prepared in its racemic form, they all possess the absolute configuration (2*S*,3*R*) which appears crucial for activity.¹³⁶ A comparable chiral framework is also existent in the side chain of the pharmacologically important series constituted by taxoids and, in this context, it is worth pointing out that numerous and various synthetic approaches to building blocks liable to lead to enantiomerically pure (2*S*,3*R*)-3-amino-2-hydroxyalkanoic structures and/or their diastereomers have attracted considerable attention.^{140–185}

7. Leuhistin

(2*R*,3*S*)-3-amino-2-hydroxy-2-1*H*-(imidazol-4-ylmethyl)-5-methylhexanoic acid (**26**) has been isolated in 1991 by Takeuchi and co-workers from the culture broth of a bacteria belonging to the phylum *Firmicutes*: *Bacillus laterosporus* BM156-14F1.^{186,187} This compound inhibits APN in a competitive manner with the substrate.¹⁸⁶ The structure of **26** and its absolute configuration have been thereafter ascertained by the same group.¹⁸⁸

Several naturally occurring APN inhibitors are of vegetal origin:

8. Benzo[*c*]phenanthridines

Benzo[*c*]phenanthridines such as 1,2-Dimethoxy-12-methyl 1,3-dioxolo[4',5':4,5]benzo[1,2-*c*]phenanthridin-12-ium chloride or Chelerythrine (**27**) and some closely related alkaloids have recently been isolated from extracts of the Papaveraceae *Macleania cordata* (Wild.) R. Br. Some of these compounds showed an efficacy against APN similar to that of amastatin (**18**) or bestatin (**19**). A weaker inhibitory effect on DPP-IV has also been reported.¹⁸⁹

9. Curcumin

(*E,E*-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (**28**) is a yellow natural phenolic compound isolated from the rhizomes of asian perennial herbs extensively cultivated in tropical areas and belonging to the Zingiberaceae family. All these plants are of the genera *Curcuma*. The most exploited representative is *Curcuma longa* L., whose dried rhizome is the source of the spice turmeric which is widely employed in food and has a long tradition of use in folk medicine. In addition to its irreversible APN/CD13 inhibition potencies,¹⁹⁰ curcumin is now considered by oncologists as a potential cancer chemopreventive agent,^{191,192} and clinical trials in this context are carried out in several laboratories.¹⁹³ Furthermore, curcumin possesses anti-inflammatory activity and is a potent inhibitor of reactive oxygen-generating enzymes (e.g. lipooxygenase/cyclooxygenase-2, xanthine dehydrogenase/oxidase and inducible nitric oxide synthase).¹⁹⁴ Curcumin hinders also the initiation of carcinogenesis by inhibiting the cytochrome P-450 enzyme activity and increasing the levels of glutathione-S-transferase. Its anti-tumor effect in the promotion and progression stages has been attributed, in part, to the arrest of cancer cells in S, G2/M cycle phase, and induction of apoptosis.¹⁹⁵ It has also been proposed that curcumin may suppress tumor promotion by blocking signal transduction pathways in the target cells.¹⁹⁶ Curcumin is a potent inhibitor of protein kinase C, EGF-receptor tyrosine kinase and I-κB kinase. In addition, curcumin inhibits the activation of NF-κB and the expression of c-jun, c-fos, c-myc.^{194,197} Last, curcumin has been

proposed as a HIV-1 or HIV-2 protease inhibitor,¹⁹⁸ as a HIV-1 integrase inhibitor,¹⁹⁹ and proved to be radioprotectant.^{200,201} Several chemical synthesis of **28**, involving 2,4-pentanedione and vanillin, have been reported^{202–206} as well as the preparations of some of its analogues designed as angiogenesis inhibitors²⁰⁷ through their ability to inhibit endothelial cell proliferation.²⁰⁸

10. Betulinic Acid

(3 β -hydroxylup-20(29)-en-28-oic acid) (**29**) is a pentacyclic compound widely present in the plant kingdom. This oxidized derivative of betulin owes its trivial name to the fact that this class of lupane type triterpenes was first isolated from *Betula* ssp. (birch trees). Afterwards, betulinic acid has been obtained from various other vegetal species including *Ancistrocladus* ssp., *Arbutus* ssp., *Diospyros* ssp., *Paeonia* ssp., *Picramnia* ssp., *Syzygium* ssp., *Tetracera* ssp., *Tryphillum* spp., *Zizyphus* ssp. One of the main current sources of betulinic acid from natural origin is the bark of plane trees (e.g. *Platanus acerifolia*) by employing a patented procedure.²⁰⁹ In addition to its APN inhibitory activity in a dose-dependent manner,²¹⁰ and possibly as a partial consequence of this inhibitory potency, betulinic acid has been shown to modulate the immune response, to exhibit anti-inflammatory properties and to block HIV-1 entry into cells. It has also been reported to be a selective inhibitor of DNA polymerase β and to induce apoptosis in tumor cells. The wide range of biological properties linked to betulinic acid have recently been recapitulated and analyzed in three excellent reviews.^{211–213} Several hemisynthesis of **28** starting from betulin via betulonic acid^{214–217} or from various naturally occurring betulinic acid derivatives such as glycosides,^{218–221} sulfates,²²² or dihydroxycinnamic esters²²³ have been reported.

To our knowledge, only one naturally occurring APN inhibitor originates from animal kingdom:

11. Psammalin A

((*E,E*)-*N,N'*-Bis[(3-(3'-Bromo-4'-hydroxyphenyl)-2-oximidopropionyl)cystamine] (**30**) is a symmetrical disulfide compound bearing two hydroxyimino functional groups. This bis-bromotyrosine derivative was, almost simultaneously, first isolated in 1987 by three groups: from an unidentified marine sponge (probably of the Verongidae family) collected in Guam,²²⁴ from a *Psammaplysilla* sp.,²²⁵ and from *Thorectopsamma xana*.²²⁶ Its structure has been unambiguously and independently established by these different authors. Thereafter, psammalin A has also been extracted from other sponges: *Psammaplysilla purpurea*,^{225,227} *Dysidea* spp. (in this case, the authors have erroneously named «bisprasin»—the misspelled name of the psammalin A dimer—a compound which is obviously the psammalin A itself as judged by the reported formula)²²⁸ *Aplysinella rhax*,^{229–231} *Pseudoceratina purpurea*,²³² or from a two-sponge association: *Poecillastra wondoensis* and *Jaspis wondoensis*.^{233,234} A biosynthetic pathway has been proposed for the formation of **30** involving modified cysteine and bromotyrosine^{227,232} and, to our knowledge, only one laboratory preparation of psammalin A has been carried out starting from L-tyrosine through its *N,N'*-bis-(tetrahydropyran-2-yl)oxime derivative.²³⁵ It is also worth pointing out that a library comprising about two hundred psammalin A type derivatives has recently been prepared by Nicolaou and his co-workers by using solution phase combinatorial synthesis with the aim to evaluate their antibacterial activity.^{235,236}

In addition to its very recently reported ability to inhibit APN in a non-competitive manner thus inducing a suppression of *in vitro* angiogenesis,²³⁷ **30** has been shown to induce a variety of biological effects: (i) a significant *in vitro* antibacterial activity against *Staphylococcus aureus*²²⁶ and methicillin-resistant *Staphylococcus aureus*^{235,236,238} which is assumed to be due to its ability to inhibit DNA gyrase,²³⁸ (ii) a cytotoxicity against various human tumor cell lines,^{229,231–233} (iii) an increase in Ca²⁺ release from the heavy fraction of skeletal muscle sarcoplasmic reticulum,²²⁸ (iv) an inhibition of topoisomerase II,²³⁹ Leucine aminopeptidase and farnesyl protein transferase,²²⁹ mycothiol-*S*-conjugate amidase,²⁴⁰ chitinase,²³¹ histone deacetylase and DNA methyltransferase,²³²

and DNA replication by targeting polymerase α -primase.²⁴¹ Some antifungal and insecticidal activities have been further reported.²³¹

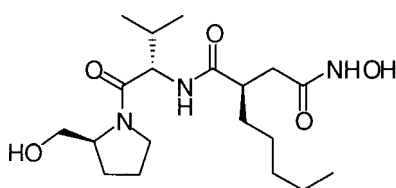
Chemical structures of APN inhibitors **13**–**30**, and enzyme inhibition values are depicted in Figure 4.

B. Synthetic APN/CD13 Inhibitors

Several synthetic small molecules belonging to various chemical families have been reported to inhibit APN activity.

1. α -Aminomethylketones

α -Aminomethylketones such as (*S*)-3-Amino-4-methylpentan-2-one hydrochloride (valine methyl ketone hydrochloride) (**31**)²⁴² have been described as potent competitive inhibitors of APN.²⁴³



Actinonin (13)

IC₅₀ APN : 0.4 μ g/mL, IC₅₀ LAP : 1 μ g/mL,
Inactive towards APA, APB, Methionine
aminopeptidase and formylmethionine
aminopeptidase⁹⁴

IC₅₀ APN : 1.99 μ M, IC₅₀ APA : >100 μ M³⁵⁵

IC₅₀ APN : 0.4 μ g/mL, IC₅₀ APA : >100
 μ g/mL, IC₅₀ APB : >100 μ g/mL¹³⁷

IC₅₀ APN from *Bombyx mori* : 148 μ M³⁶⁸

IC₅₀ APN : 0.32 μ g/mL⁷⁷

IC₅₀ Enkephalinaminopeptidase : 0.39 μ M,

IC₅₀ dipeptidylaminopeptidase : 1.1 μ M,

IC₅₀ Enkephalinase A : 5.6 μ M³³⁶

91% inhibition of APN, 16.5% inhibition of
APA and 13% inhibition of MDP at 100
 μ M, inactive towards APW³⁶⁹

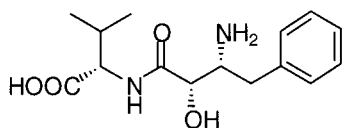
100% inhibition of APN from human
seminal plasma at 100 μ M⁷⁴

IC₅₀ Bacterial PDF 0.8-90 nM depending on
the metal cation form of the enzyme³⁷⁰

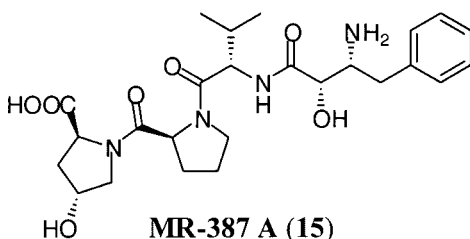
IC₅₀ Human PDF : 43 nM³⁵⁸

K_i Meprin α : 20 nM, K_i Meprin β : 2 μ M³⁶⁰

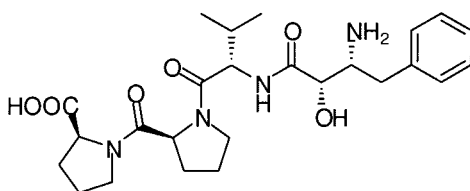
Figure 4. Natural inhibitors of APN/CD13.

**AHPA-Val (SL-387) (14)**

IC₅₀ APN from porcine kidney microsome :
1.2 μg/mL, IC₅₀ APN from human
fibrosarcoma HT1080 : 5.6 μg/mL, IC₅₀
APN from human myelogenous leukemia
K562 : 7.8 μg/mL¹⁰⁴

**MR-387 A (15)**

IC₅₀ APN from porcine kidney microsome :
198 nM, IC₅₀ APN from human
fibrosarcoma HT1080 : 218 nM, IC₅₀ APN
from human myelogenous leukemia K562 :
17 μM, APB from human myelogenous
leukemia K562 : 651 nM¹⁰⁵

**MR-387 B (16)**

IC₅₀ APN from porcine kidney microsome :
164 nM, IC₅₀ APN from human
fibrosarcoma HT1080 : 201 nM, IC₅₀ APN
from human myelogenous leukemia K562 :
4.6 μM, APB from human myelogenous
leukemia K562 : 260 nM¹⁰⁵

Figure 4. (Continued)

2. Alkyl D-Cysteines

Alkyl D-cysteines display also efficient competitive APN inhibitions. Among the five esters tested, an optimal inhibitory activity has been observed with the *n*-butyl derivative (**32**).²⁴⁴

3. 3-Amino-2-Tetralone Derivatives

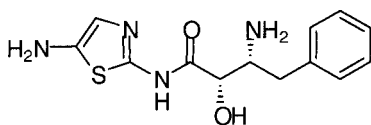
3-amino-2-tetralone derivatives such as the 2-amino-1,4-dihydro-2*H*-phenanthren-3-one hydrochloride (**33**) have been reported to be efficient and selective competitive inhibitors of APN. These compounds do not affect AP-A or AP-B and poorly inhibit LAPc.²⁴⁵

4. 3-Amino-2-Hydroxypropionaldehyde and 3-Amino-1-Hydroxypropan-2-One Derivatives

3-Amino-2-hydroxypropionaldehyde and 3-amino-1-hydroxypropan-2-one derivatives such as **34** and **35**, respectively. These competitive inhibitors of APN are very moderately active on LAPc or APB.²⁴⁶

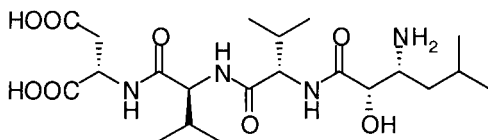
5. Flavone-8-Acetic Acid Derivatives

Flavone-8-acetic acid derivatives constitute a class of products whose the parent compound showed antiangiogenic properties.²⁴⁷ In this series, products bearing a nitro group in the 2-position such as the 2',3-dinitroflavone-8 acetic acid (**36**) proved the most potent APN inhibitors and act by reversibly binding to the catalytic site of the enzyme. These compounds present the advantage to exhibit no



(17)

Inhibits the growth of mouse S_{180} tumors by 38%¹⁰⁷

**Amastatin (18)**

IC_{50} APN from pig kidney : $0.5 \mu M$ ³⁷¹

IC_{50} APN : $3.16 \mu M$, IC_{50} APA : $2 \mu M$,

IC_{50} APW : $1.58 \mu M$ ³⁵⁵

IC_{50} APN : $150 nM$ ³⁷²

K_i APN : $50 nM$, K_i LAP_c : $30 nM$, K_i

APA : $0.15 \mu M$ ²⁴⁵

IC_{50} APN : $0.58 \mu g/mL$, IC_{50} APA :

$0.54 \mu g/mL$, IC_{50} APB : $>100 \mu g/mL$ ¹³⁷

IC_{50} APN from *Bombyx mori* : $7.6 \mu M$ ³⁶⁸

100% inhibition of APN, 100% inhibition of APA, 100% inhibition of APW and 18% inhibition of MDP at $100 \mu M$ ³⁶⁹

100% inhibition of APN from human seminal plasma at $100 \mu M$ ⁷⁴

K_i APN : $52 nM$, K_i LAP_c : $30 nM$, K_i

Aeromonas AP : $0.26 nM$ ³⁵⁷

K_i^* APN : $20 nM$, K_i^* LAP : $0.2 \mu M$ ¹⁰⁸

IC_{50} APM from rabbit kidney cortex :

$0.4 \mu M$ ³⁷³

IC_{50} APM from rat blood plasma : $0.2 \mu M$,

IC_{50} APA from rat blood plasma : $8 \mu M$ ⁶⁷

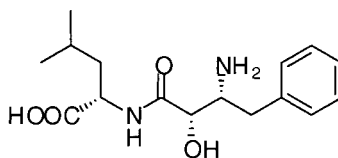
IC_{50} APA : $0.54 \mu g/mL$, IC_{50} APL : 0.5

$\mu g/mL$, IC_{50} APB : $>250 \mu g/mL$ ¹⁰⁹

IC_{50} APA : $1.1 \mu M$, IC_{50} LAP : $1.1 \mu M$ ^{112,374}

IC_{50} APW : $2 \mu M$ ³⁷⁵

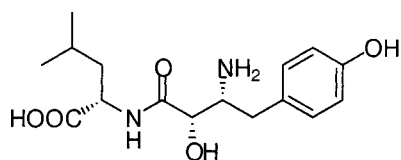
Figure 4. (Continued)



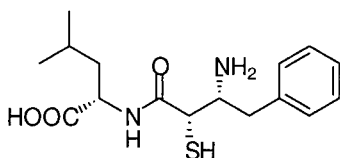
Bestatin (19)

Ki APN : 3.03 μ M, Ki LAP_c : 9 nM³⁶²
 Ki^{*} APN : 4.1 μ M¹⁰⁸
 IC₅₀ APN from pig kidney : 16 μ M³⁷¹
 Ki APN : 1.45 μ M, Ki LAP_c : 0.4 nM,
 Ki *Aeromonas* AP : 18 nM³⁵⁷
 Ki APN : 4.1 μ M, Ki LAP_c : 20 nM,
 Ki APB: 14 nM¹³⁶
 IC₅₀ APM from rabbit kidney cortex :
 6 μ M³⁷³
 IC₅₀ APN : 89.1 μ M, IC₅₀ APW : 7.9 μ M³⁵⁵
 IC₅₀ APN : 43 μ M³⁷²
 Ki APN : 3.5 μ M, Ki LAP_c : 0.6 nM,
 Ki APB : 6 μ M²⁴⁵
 Ki APN : 3.5 μ M, Ki LAP_c : 0.6 nM, Ki AP
Aeromonas P. : 20 nM, Ki APB: 6 μ M²⁴⁶
 IC₅₀ APN : 6.2 μ g/mL, IC₅₀ APA :
 >100 μ g/mL, IC₅₀ APB : 0.05 μ g/mL¹³⁷
 IC₅₀ APN from *Bombyx mori* : 3.25 mM³⁶⁸
 96% inhibition of APN from human seminal
 plasma at 100 μ M⁷⁴
 52% inhibition of APN, 13.4% inhibition of
 APA, 89% inhibition of APW and 29%
 inhibition of MDP at 100 μ M³⁶⁹
 IC₅₀ APN from rat blood plasma : 30 μ M⁶⁷
 IC₅₀ APN : 16.9 μ M²¹⁰
 IC₅₀ APN : 2.5 μ M¹⁹⁰
 IC₅₀ APN : 3.9 μ M, IC₅₀ A-LAP :
 11.2 μ M²⁴⁹
 IC₅₀ APB : 0.05 μ g/mL, IC₅₀ LAP :
 0.01 μ g/mL¹¹⁹
 Ki APB : 60 nM, Ki LAP : 20 nM^{353,356}
 IC₅₀ Enkephalin aminopeptidase : 1.1 μ M³³⁶
 IC₅₀ APB : 0.05 μ g/mL, IC₅₀ LAP :
 0.003 μ g/mL¹³²
 IC₅₀ APW : 6 μ M³⁷⁵

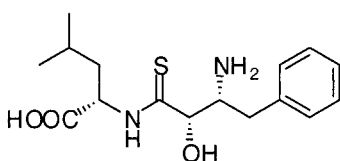
Figure 4. (Continued)

***para*-hydroxybestatin (20)**

IC₅₀ APB : 0.007 μg/mL, IC₅₀ LAP :
0.02 μg/mL¹³²

**α-Thiolbestatin (21)**

Ki APN : 4.4 μM, Ki LAP : 0.55 μM,
Ki APB : 4.6 nM¹³³

**Bestatin thioamide (22)**

Ki APN : 40.3 μM, Ki LAP : 0.33 μM,
Ki APB : 2.4 μM¹³³

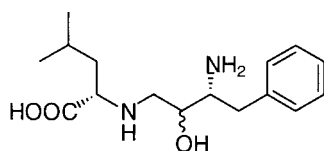
Figure 4. (Continued)

toxicity towards cultured human cells, to induce no apoptosis, and to be inactive on other proteases such as MMP-9, ACE, NEP, γ-glutamyl transpeptidase, cathepsin G, or DPPIV.²⁴⁸

6. *N*-Hydroxy-2-(naphthalene-2-ylsulfanyl)Acetamide

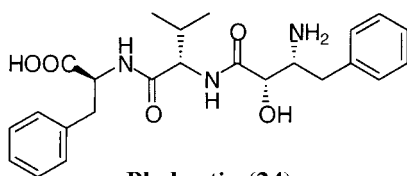
N-Hydroxy-2-(naphthalene-2-ylsulfanyl)acetamide (**37**) has recently been identified as a potent APN inhibitor. It acts in a dose-dependent manner and is inactive on metalloenzymes MMP-2, MMP-9, MMP-14, or A-LAP.²⁴⁹

The design of synthetic APN inhibitors has often been relied to structure–activity studies based on active site models derived from structural data obtained on the zinc-dependent protease thermolysin crystallized with a variety of inhibitors.²⁵⁰ Molecules capable of interacting with at least the S₁ subsite of APN and which have a strong zinc-chelating group^{251,252} were designed. According to these criteria, some α-aminophosphinic acids and derivatives such as **38** or **39**²⁵³ have been prepared and proved to be very potent APN inhibitors. According to the patterns of these models, synthesis of analogs such as the iodo derivative **40** (*RB 129*) have next been performed to give rise to the radiolabelled (¹²⁵I)*RB 129*²⁵⁴ which represents a useful probe to investigate the physiological role of APN.^{13,255,256} In the same context, several β-aminothiols exemplified by **41**²⁵⁷ or **42**²⁵¹ have been conceived and synthesized. The research in this field has then been extended to more elaborated series by Roques and co-workers, and novel sulfur-containing molecules capable of inhibiting APN such as **43**, **44**,²⁵⁸ **45**, **46**²⁵⁹ or **47**²⁵³ were prepared. From these works on β-aminothiols, two products emerged: *PC 18* (*S*)(2-amino-4-methylthiobutanethiol) (**48**)²⁵³ and *EC 27* (*S*)(2-aminopentan-1,5-dithiol) (**49**).²⁵⁹ These products have essentially aroused deeper studies because they are able to induce vasopressin release by acting on the half-life of angiotensin III.^{61,66,260,261}



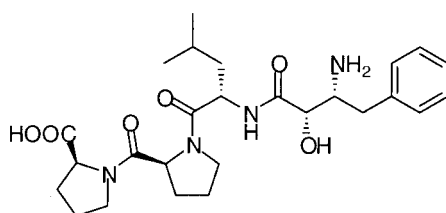
23 (racemate)

Ki APN : 2.1 mM, Ki LAP₀ : > 1 mM,
Ki APB: 14 nM¹³⁶



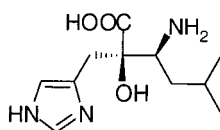
Phebestin (24)

IC₅₀ APN : 0.18 µg/mL, IC₅₀ APA :
9 µg/mL, IC₅₀ APB : 9 µg/mL¹³⁷



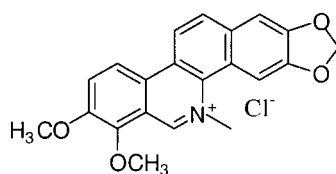
Probestin (25)

Ki APN : 19 nM¹³⁸
IC₅₀ APN : 50 nM, IC₅₀ APA : 19.9 µM,
IC₅₀ APW : 5 µM³⁵⁵
IC₅₀ APN : < 10 nM³⁷²
IC₅₀ APN : 0.03 µg/mL, IC₅₀ APA : >100
µg/mL, IC₅₀ APB : 37 µg/mL¹³⁷
IC₅₀ APN from *Bombyx mori* : 74 µM³⁶⁸



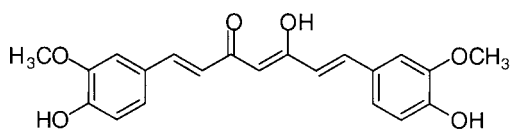
Leuhistin (26)

Ki APN : 0.23 µM¹⁸⁶
IC₅₀ APN : 0.2 µg/mL, IC₅₀ APA :
10 µg/mL, IC₅₀ APB : 13 µg/mL¹³⁷
100% inhibition of APN from human
seminal plasma at 100 µM⁷⁴
IC₅₀ APN from *Bombyx mori* : 0.89
mM : 089 mM³⁶⁸



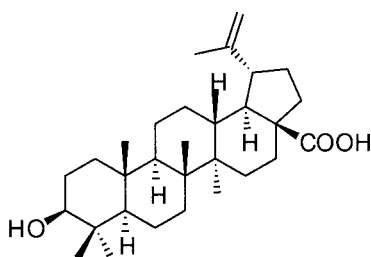
Chelerythrine (27)

82% inhibition of APN and 38% inhibition
of DPP IV at 50 µM¹⁸⁹



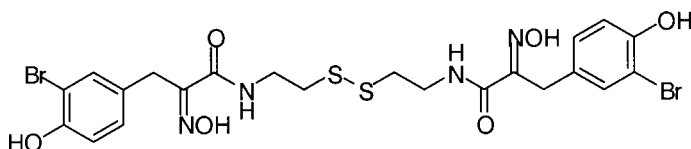
Curcumin (28)

Ki APN : 11.2 µM¹⁹⁰



IC₅₀ APN : 7.3 μM²¹⁰

Betulinic acid (29)



IC₅₀ APN : 18 μM²³⁷

Psammaphin A (30)

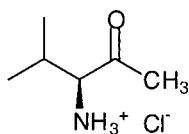
Figure 4. (Continued)

Formulas of the synthetic APN inhibitors **31–49**, and enzyme inhibition values are outlined in Figure 5.

C. Synthetic Dual APN/CD13 and E-24.11/CD10 (NEP) Inhibitors

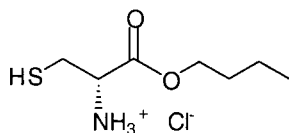
The similarities between the active sites of APN and the membrane-bound protease neutral endopeptidase 24.11 (EC3.4.24.11, CD10, NEP) led to the idea that mixed inhibitors could be developed by selecting frameworks bearing a strong zinc-chelating group and a residue able to interact with at least one subsite (S₁, S₁', and S₂') of each peptidase.^{65,251,262–265} The first dual E24.11/APN inhibitors developed were hydroxamate-containing molecules such as *Kelatorphan* (**50**) or *RB 38A* (**51**)^{262,266,267} whose several analogs have been synthesized and found to be also potent inhibitors of leukotriene A₄ hydrolase.²⁶⁸ However, the important water solubility of these compounds is an impediment for crossing the blood–brain barrier and, consequently, for obtaining a good bioavailability. Another strategy, involving more lipophilic derivatives, led to the synthesis of *RB 101* (*N*-((*R,S*)-2-benzyl-3((*S*)-2-amino-4-methylthio)butylthio)-1-oxopropyl)-*L*-phenylalanine benzyl ester (**52**) and *RB 120* (*N*-((*S*)-2-benzyl-3((*S*)-2-amino-4-methylthio)butylthio)-1-oxopropyl)-*L*-alanine benzyl ester (**53**), two dual inhibitors in which a disulfide bridge links the APN inhibitor PC 18 with analogs (the phenylalanine analog (ST 43) in the case of **52**, or the alanine analogue in the case of **53**) of the benzyl ester of Thiorphan, a specific NEP inhibitor²⁶⁹ (Fig. 6).^{251,263,270} Such mixed inhibitors present the advantage to possess the above-mentioned disulfide bond which is relatively stable in plasma, in contrast to its rapid cleavage in brain, thus allowing the delivery of the NEP and APN inhibitors in their active form toward their respective target.²⁶³ The development of such mixed inhibitors has constituted an important advance in the research of new antihypertensives and novel antinociceptive drugs devoid of opioid side effects.^{264,271} (for reviews) More recently, a new generation of phosphinic acid derivatives have been prepared as NEP/APN dual inhibitors, and compounds such as **54** have been successfully tested in this context.^{252,272}

Chemical structures of APN inhibitors **50–54**, and enzyme inhibition values are outlined in Figure 7.



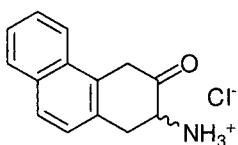
31

Ki APN : 0.55 μM ²⁴³



32

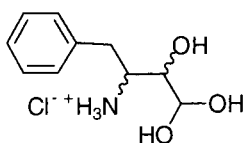
Ki APN : 0.18 μM ²⁴⁴



33

Ki APN : 0.5 μM , Ki LAP_c : 120 μM , Ki APA : > 1 mM,
Ki APB : > 1 mM²⁴⁵

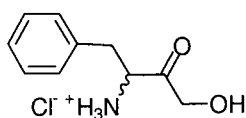
(absolute stereochemistry not specified)



34

Ki APN : 3 μM , Ki LAP_c : 0.1 mM, Ki AP *Aeromonas p.* :
30 μM ²⁴⁶

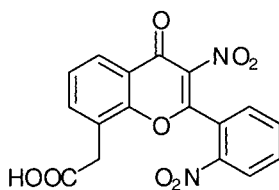
(absolute stereochemistry not specified)



35

Ki APN : 1 μM ²⁴⁶

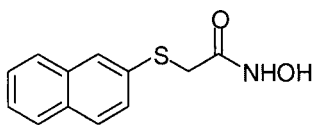
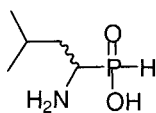
(absolute stereochemistry not specified)



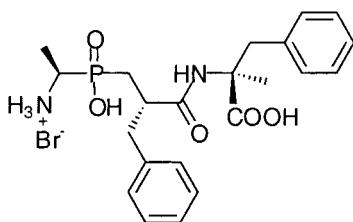
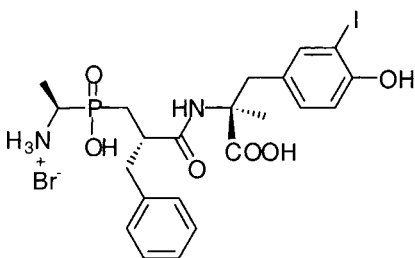
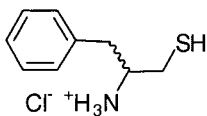
36

IC₅₀ APN : 25 μM ²⁴⁸

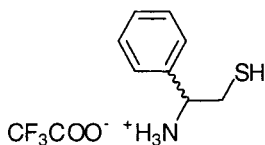
Figure 5. Synthetic inhibitors of APN/CD13.

**37**IC₅₀ APN : 3.4 μM²⁴⁹**38**

(mixture of R + S isomers)

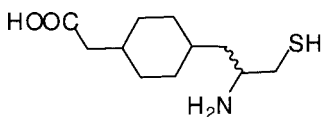
K_i APN : 1.2 μM²⁵³**39**K_i APN : 0.6 nM, K_i APA : 0.13 μM, K_i APB : >10 μM²⁵³**RB 129 (40)**K_i APN : 0.95 nM²⁵⁴**Phetiol (41)**

(absolute stereochemistry not specified)

K_i APN : 5 nM, K_i PSA : 10 nM²⁵⁷**42**

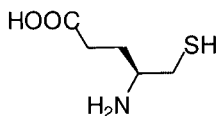
(absolute stereochemistry not specified)

IC₅₀ APN : 25 nM²⁵¹



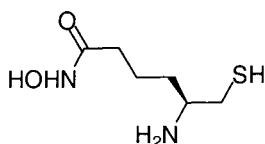
43
(mixture of R + S isomers)

Ki APN : 35 nM, Ki APA : 2.8 μM ²⁵⁸



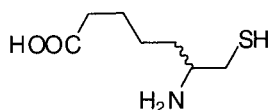
44

Ki APN : 0.12 μM , Ki APA : 0.14 μM ²⁵⁸



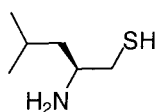
45

Ki APN : 37 nM, Ki APA : 2.0 μM ²⁵⁹



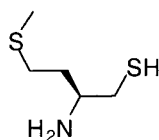
46
(mixture of R + S isomers)

Ki APN : 0.28 μM , Ki APA : 1.6 μM ²⁵⁹



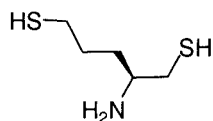
47

Ki APN : 22 nM²⁵³



PC 18 (48)

Ki APN : 8 nM, Ki APA : 17.2 μM , Ki APB : 1 μM ²⁶⁰
Ki APN : 11 nM³³⁹



EC 27 (49)

Ki APN : 32 nM, Ki APA : 2.4 μM ^{259,261}

Figure 5. (Continued)

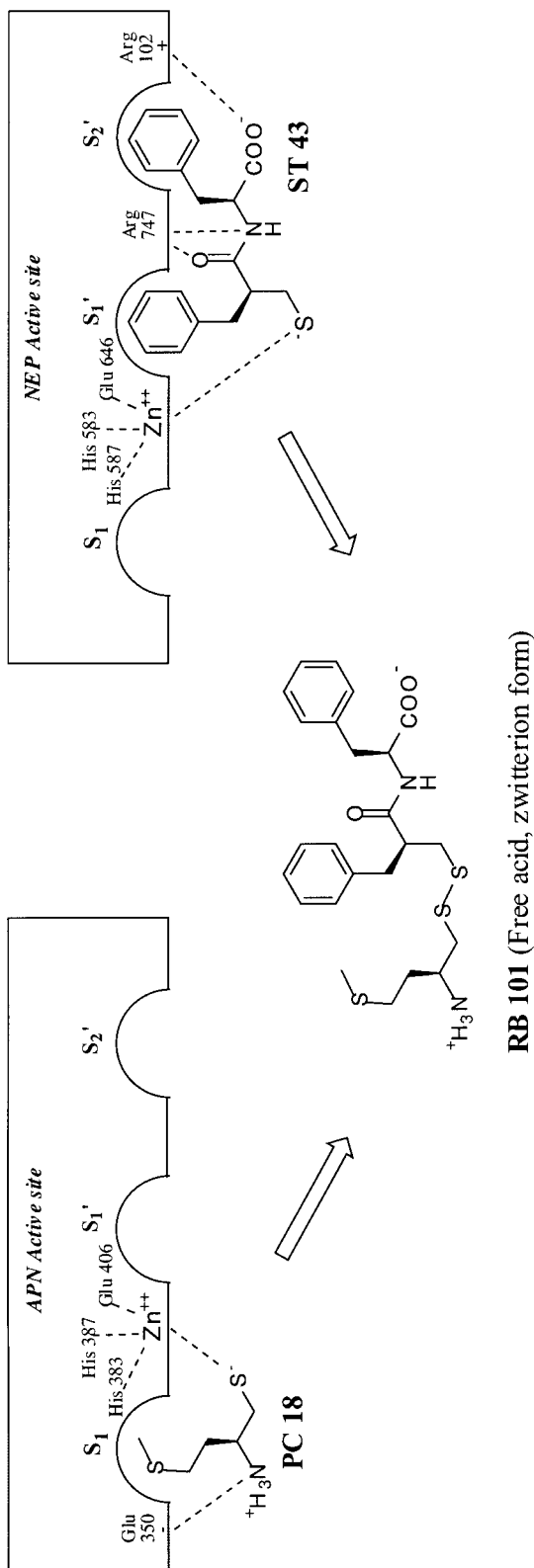
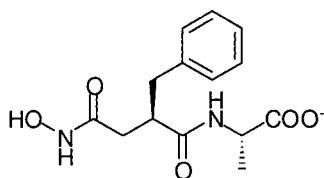


Figure 6. Conceptualization of APN/NEP dual inhibitors (example of **RB 101**).



Kelatorphan (50)

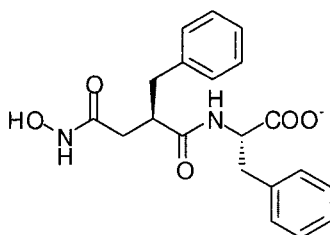
Ki APN : 0.38 μ M, Ki NEP : 1.8 nM²⁶⁴

Ki APN : 7 μ M, Ki NEP : 1.7 nM³³⁹

Ki APN : 0.38 μ M, Ki DAP : 0.9 nM, Ki NEP : 2.0 nM³⁷⁶

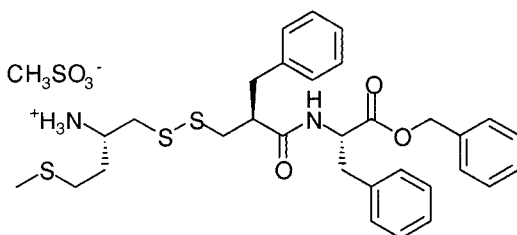
IC₅₀ NEP : 46 nM, IC₅₀ LTA₄ hydrolase : 5 nM, IC₅₀

Aminopeptidase : 7 nM, IC₅₀ ACE : >10 μ M²⁶⁸



RB 38A (51)

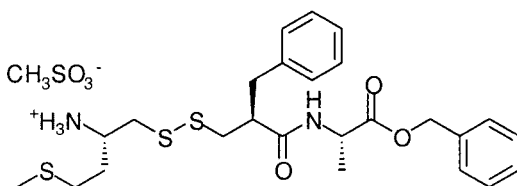
Ki APN : 0.12 μ M, Ki NEP : 0.9 nM²⁶⁴



RB 101 (52)

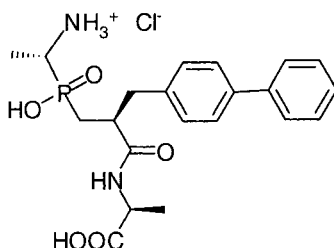
IC₅₀ APN : 16 μ M, IC₅₀ NEP : >100 μ M²⁶⁷

After 15 min of preincubation with whole rat brain membrane, IC₅₀ APN : 11 nM, IC₅₀ NEP : 4 nM²⁶⁷



RB 120 (53)

To our knowledge, no quantitative specific value dealing with APN or NEP inhibition has been published yet.



54

Ki APN : 2.9 nM, Ki NEP : 1.2 nM²⁷²

Ki APN : 2.9 nM, Ki NEP : 1.2 nM, Ki ACE : 0.12 μ M²⁵²

Ki APN : 2.9 nM, Ki NEP : 1.2 nM³³⁹

Ki APN : 2.9 nM, Ki APA : >1 μ M, Ki NEP : 1.2 nM,

Ki ACE : 0.12 μ M³⁷⁶

Figure 7. Dual inhibitors of APN/CD13.

4. APN/CD13 INHIBITORS IN MODULATION OF CELL FUNCTIONS

The effects of some of these above described inhibitors on cell behavior have been assayed in *in vitro* approaches. Table I provides a summary of most relevant studies in the human system.

A. APN Inhibitors as Modulators of Cell Growth and Maturation

Actinonin, bestatin, probestin, and psammaplin A (at 1–100 μ M concentrations) were shown to reduce the growth of human T/B lymphocytes, dendritic and cord blood CD34⁺ cells^{273–276} and human myeloid and lymphoid cell lines,^{273,274,276–282} as well as the proliferation of

Table I. Effects of APN/CD13 Inhibitors in *in vitro*, in Animal and Clinical Approaches

INHIBITOR	IN VITRO	IN ANIMALS	IN CLINICAL TRIALS
Actinonin	growth ↓ apoptosis ↑ migration ↑ invasion ↓	tumor growth ↓ neovascularization ↓ pain management ↓	
Amastatin	chemotaxis ↑ angiogenesis ↓	blood pressure ↓	
Bestatin (ubemimex)	growth ↓ differentiation ↑ apoptosis ↑ migration ↑↓ invasion ↓ angiogenesis ↑↓	tumor growth ↓ fetal growth ↓ monocyte activation ↑ placental apoptosis ↑ neutrophil migration ↓ neovascularization ↓ inflammation ↓	Remission in : AML lymphoma (<i>monocyte & lymphocyte activation</i>) lung carcinoma
Betulinic acid	apoptosis ↑ angiogenesis ↓	tumor growth ↓	
Curcumin	angiogenesis ↓		
Leuhistin	invasion ↓	neutrophil migration ↓ inflammation ↓	
Probestin	growth ↓		
Psammaplin A	growth ↓ angiogenesis ↓		
PC 18 & EC 27 RB 101& RB 120		blood pressure ↓ pain management ↓	

AML, acute myeloid leukemia.

↓ Decrease ; ↑ Increase.

keratinocytes^{283,284} and various tumor and endothelial cell lines.^{237,285–287} A question central to APN inhibition studies is how cell growth can be turned off by APN inhibitors. APN inhibitors may alter the processing of (unknown) growth factors directly involved in the regulation of growth. In addition, several studies indicate that inhibitors like actinonin and probestin may transmit intracellular-transduction signals by interfering with the MAP kinase signaling pathway.^{25,279,288,289} A second cell signaling pathway involving the Wnt-5a proto-oncogene appears also affected by inhibition of APN by actinonin.²⁹⁰

It has to be pointed out that actinonin (at a 10 μM concentration) inhibited the growth of both CD13-positive myeloid and CD13-negative lymphoma cell lines²⁸⁷ suggesting that the effects induced by actinonin are not likely to be mediated by CD13. Moreover, amastatin at a concentration which inhibits APN activity was found without any effect on the growth of human myeloid cell lines^{274,291}.

Bestatin-mediated cell growth arrest is associated with an induction of cell maturation of clonogenic GM-CFU (granulocyte-macrophage colony forming unit) cells from human immature derived-bone marrow cells.^{292,293} Similarly, treatment of human myeloid U937 and NB4 cell lines with bestatin induced phenotypic changes characteristic of macrophage (U937) or neutrophil (NB4) maturation.^{280,293,294}

B. Effects of APN Inhibitors on Cell Secretion

Cell growth arrest induced by APN inhibitors correlates with alternated secretion of proinflammatory and immunosuppressive cytokines involved in pathophysiological processes. Bestatin (2.9 μM) increased the levels of IL-8 secreted by endothelial cells,²⁹⁵ and of IL-1 release from mouse peritoneal macrophages and IL-2 release from concanavalin-stimulated T cells.²⁹⁶ Probestin induces the synthesis and release of TGF- β 1.^{41,297}

C. Effects of APN Inhibitors on Apoptosis

Recent observations point to the involvement of APN in the process of apoptosis (programmed cell death). Bestatin and actinonin (starting 30 μM) induce apoptosis in a large variety of cell lines, i.e. myeloid (P39/TSU, HL-60, U937, NB4) and lymphoid (Jurkat, BJAB, NALM6, BOE) cells, and carcinoma (fibrosarcoma, cervical, and lung carcinoma).^{274,282,287,291,298,299} Betulinic acid induces apoptosis in the HT29 colon cancer cell line (26 μM)⁸⁴ and in acute leukemia cells (50 μM).³⁰⁰

D. Effects of APN Inhibitors on Cell Motility

In a general way, cell motility (migration and invasion) may be influenced by the processing of chemokines and/or degradation of the extracellular matrix (ECM). The two small proteins with chemotactic activity, MCP-1 and f-MLP, are *in vitro* hydrolyzed by APN/CD13. With regard to MCP-1, there is no current data reporting the potential action of APN inhibitors on the MCP-1-mediated migration. Actinonin and amastatin were able to enhance the chemotactic response of human neutrophils toward f-MLP.³⁰¹ One explanation of the effects of actinonin or amastatin would be that both inhibitors prevent the inactivation of f-MLP by APN, to further enhance the f-MLP-mediated chemotactic response. It has however to underline that both inhibitors weakly inhibited APN enzymatic activity over the range from 10^{-8} to 10^{-4} M, concentrations that are effective on neutrophil migration.³⁰¹

APN inhibition by actinonin or bestatin significantly enhanced the *in vitro* migration of eosinophils across HUVEC monolayers.³⁰² Moreover, actinonin, bestatin as well as leuhistin (50–150 μM) significantly blocked the invasion of various human metastatic tumor cells into reconstituted basement membranes^{303,304} or into Matrigel.^{21,305–307} These latter data suggested that

APN could be indirectly involved in type IV collagen degradation by activating type IV procollagenase/proMMP-9.^{17,303,304} Recent studies demonstrated that soluble APN/CD13 induces *in vitro* chemotactic migration of T lymphocytes, and that bestatin at high concentration (580 μM) abolishes this process, suggesting that the enzymatic activity of APN was responsible for the chemotactic activity.^{34,36,304,308}

5. APN INHIBITORS AND ANGIOGENESIS

The demonstration of the participation of APN in angiogenesis has come from recent studies in which blocking APN activity by APN inhibitors resulted in the perturbation of “angiogenic” assays (Table I).

A. *In Vitro* Assays

APN/CD13 is expressed on the human umbilical vein endothelial cells (HUVECs) of angiogenic, but not normal, vasculature.³⁰⁹ Bestatin, betulinic acid, amastatin, curcumin, and psammaplin A (10–250 μM) abrogate the ability of the HUVECs cultured on matrigel to organize a capillary network^{20,190,237,310–312} without altering their proliferation rates.³¹⁰ In contrast, one study underlines the proangiogenic effect of bestatin (8–250 μM) which instead causes matrix degradation and stimulates the invasion of microvascular endothelial cells into a fibrin matrix.³¹³

B. *In Vivo* Assays

In the chorioallantoic membrane (CAM) assay, the angiogenic response is determined by measuring the number of avian extraembryonic capillary vessels that grow within a matrix polymer (containing an angiogenic molecule such as fibroblast growth factor-2/FGF-2) placed on the yolk sac membrane of a 4 day embryo in culture.³¹⁴ The chick vasculature expresses a phenotype APN/CD13, and subsequent treatment with bestatin or actinonin (200 μg) inhibited FGF-2-induced angiogenesis.³⁰⁹ In the mouse retinal neovascularization model, bestatin (200 $\mu\text{g}/\text{mouse}$) leads to the blockade of hypoxia-induced retinal neovascularization in mice.³⁰⁹ The intraperitoneal administration of bestatin (50–100 mg/kg/day) after the orthotopic implantation of B16-BL6 melanoma cells into mice reduces the number of vessels oriented toward the established primary tumor mass on the dorsal side of mice.³¹¹

6. EFFECTS OF APN/CD13 INHIBITORS IN ANIMAL MODELS

Compiled data documenting the involvement of APN/CD13 in pathophysiological events (cancer, inflammation, infection, pain suppression) have come from studies which blocked APN activity in rodent models (Table I).

Studies in rats indicate that administration of bestatin leads to the inhibition of fetal growth and the induction of placental apoptosis.^{315,316} The *in vivo* anti-cancer activities of bestatin and betulinic acid have been reported through their capacities to inhibit the growth of syngeneic tumor (leukemia/melanoma/ovarian/hepatoma/gastric carcinoma) cells implanted in mice^{16,213,309,310,317–325} and rats.^{319,326,327} Doses as low as 0.5 mg/kg for bestatin and 5 mg/kg for betulinic acid were used in these studies. Moreover, high doses (up to 500 mg/kg) did not lead to any cytotoxic effect in mice.

Bestatin, leuhistin, and betulinic acid have been investigated for anti-inflammatory properties. Betulinic acid possessed moderate ant-inflammatory abilities at relatively high concentrations

(100 mg/kg/mouse, i.v.).²¹³ In contrast, bestatin and leuhistin inhibit acute inflammation associated with the accumulation of polymorphonuclear neutrophils in a mouse model (2 mg/kg, i.v.).^{57,328} Moreover, oral administration of bestatin (5 mg/kg) in carcinoma-bearing mice induces generation of cytotoxic T cells and NK (natural killer) cells.³¹⁷

Angiotensins II and III are two peptide effectors of the brain rennin–angiotensin system that participate in the control of blood pressure, increase water consumption and vasopressin release. In hypertensive rats, infusion of amastatin (16 nmol/min i.v.) prevents degradation of angiotensins associated with blood pressure decrease.^{67,329} In the mouse brain, APN inhibition by PC18 or EC27 (10–300 µg injected intracerebroventricularly) increases the half life of angiotensin III, resulting in enhanced vasopressin release.^{61,66,260}

Several studies report that bestatin exerts anti-infectious properties by augmenting host resistance to bacterial, viral or fungal experimental infections in mice by inducing neutrophil and macrophage activation^{330,331} and enhancing antibody production.^{330–335}

Finally, in the central nervous system, enkephalins which modulate responses to painful stimuli, are inactivated by APN and the membrane-bound protease neutral endopeptidase 24.11 (EC3.4.24.11, CD10). This led to the idea that inhibition of these enzymes (alone or in combination) could achieve clinically efficient analgesia. Actinonin as well as the dual inhibitors RB101 and RB120 (9 mg/kg, i.v.; 80 mg/kg, i.p.) exhibited analgesic properties against chronic pain in rats and mice.^{261,263,267,336,341}

7. EFFECTS OF BESTATIN IN CLINICAL TRIALS

In first clinical trials, bestatin (30 mg/daily) has been used to treat patients with acute and chronic myeloid leukemias (AML, CML) and lymphomas.^{342–346} Therapeutic efficacy was demonstrated by a prolongation of survival in patients with AML^{345,346} and lymphomas,^{342,343,347} and in promoting graft versus leukemia effects in patients following allogeneic bone marrow transplant.³⁴⁸

In a phase Ib trial, activation of blood monocytes and increase in the CD4/CD8 lymphocyte ratio were observed in Hodgkin's and non-Hodgkin's lymphoma patients treated orally with high doses of bestatin (90–180 mg/daily/60 days) following autologous bone marrow transplantation.^{330,334,349}

In phase III trials in resected stage I squamous cell lung carcinoma, survival was statistically better for patients who were treated with bestatin (30 mg/daily/2 years) as a post-operative adjuvant therapy than those who received a placebo.^{350,351}

8. CONCLUDING COMMENTS AND PERSPECTIVES

APN/CD13, is useful in defining clinical subgroups of patients with various malignancies or inflammatory diseases. The use of natural and synthetic APN inhibitors has revealed that APN/CD13 participates to the control of major biological processes such as proliferation, secretion and apoptosis. Dysregulation of APN/CD13 in tumors is often linked to tumor invasion and angiogenesis. Studies on non-hematopoietic cells suggest that APN/CD13 may influence cell migration and invasion. APN/CD13 inhibitors have been shown to alter angiogenesis in *in vitro* and *in vivo* assays. Documented evidence underlines both the antiangiogenic and proangiogenic effects of bestatin.^{309,310,313} Figure 8 summarizes our current understanding of the involvement of APN inhibitors in the modulation of these events. The detailed molecular mechanisms underlying these effects are however yet unclear.

Importantly, the requirement for APN in these processes has been mostly confirmed with studies in which APN/CD13 expression was blocked by neutralizing CD13 antibodies^{20,285,303,309,310} or antisense CD13 oligonucleotides,^{20,41,352} or enhanced by the use of CD13 transfectants.¹⁷

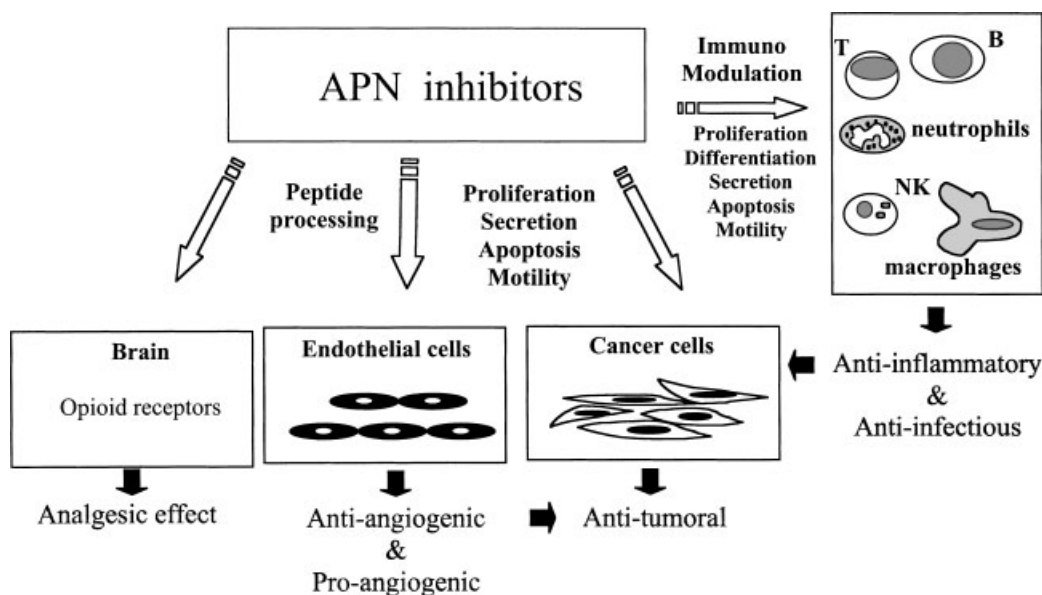


Figure 8. Biological effects of APN/CD13 inhibitors. The actions of APN inhibitors *in vitro* and *in vivo* (animal models) are diverse; they may directly target cancer cells, or act indirectly against targets by activation of immune cells (T, B, neutrophils, natural killer/NK cells, macrophages) or alteration of angiogenesis (endothelial cells). In the brain, APN inhibitors exhibit analgesic properties by increasing the levels of Met-enkephalin.

It has however to be pointed out that most of APN inhibitors lack tight specificity by inhibiting other membrane-bound metalloproteases or secreted matrix metalloproteinases (MMPs) (Table I). For example, bestatin interacts with leucyl-aminopeptidase (EC3.4.11.1, oxytocinase, Leu-AP), aminopeptidase B (EC 3.4.11.6, AP-B) and aminopeptidase W (EC 3.4.11.16, AP-W)^{136,353–357} thus suggesting that some of the observed chemotherapeutic actions of bestatin may be due to inhibition of other cell surface peptidases. Actinonin was recently shown to interact with human peptide deformylase,^{358,359} meprin α (EC 3.4.24.18, endopeptidase 24.18),³⁶⁰ and MMP-2.³⁶¹ Amastatin and probestin in the low micromolar range (1.5–20 μM) inhibit aminopeptidase A (EC 3.4.11.2, AP-A) and AP-W.^{109,355,362} Leuhistin inhibits AP-A and AP-B to the same degree than APN.¹⁸⁶ Curcumin and betulinic acid block MMP-9 expression and collagenase activity through inhibition of NF- κ B activation.^{363–367} In addition, the use of available APN inhibitors in some experimental situations has revealed complex effects on cell behavior. As mentioned in paragraph 4.A, CD13-positive and CD13-negative cell lines are equally sensitive to the growth-inhibitory effect of actinonin (50–260 μM)²⁸⁷ thus emphasizing that actinonin may induce unspecific cytotoxic side-effects. Moreover, betulinic acid inhibits tube formation of bovine aortic endothelial cells at a concentration which had no effect on the cell viability and *in vivo* APN activity of endothelial cells, thus indicating an APN-independent mode of action of betulinic acid.³¹²

Together, these observations emphasize the need for more specific and targeted APN inhibitors to (re)evaluate the actions of APN/CD13 in pathophysiological processes. Future consideration has to be given to the obtention of the three-dimensional structure of APN determined by NMR spectroscopy to help APN inhibitor design strategy. Further *in vitro* and *in vivo* studies with promising non cytotoxic APN inhibitors (such as psammaphin A, phosphonic derivatives, flavone-8-acetic acid derivatives) are also required before clinically prescribing an APN inhibitor as an anti-cancer or anti-inflammatory agent.

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