# *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. 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 peptidases[2] 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 Pfleiderer and Celliers from pig kidney[5] 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 thyroid[23] 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/dermatomyosytis, 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 1989[6] 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 enzyme[53] indicating that

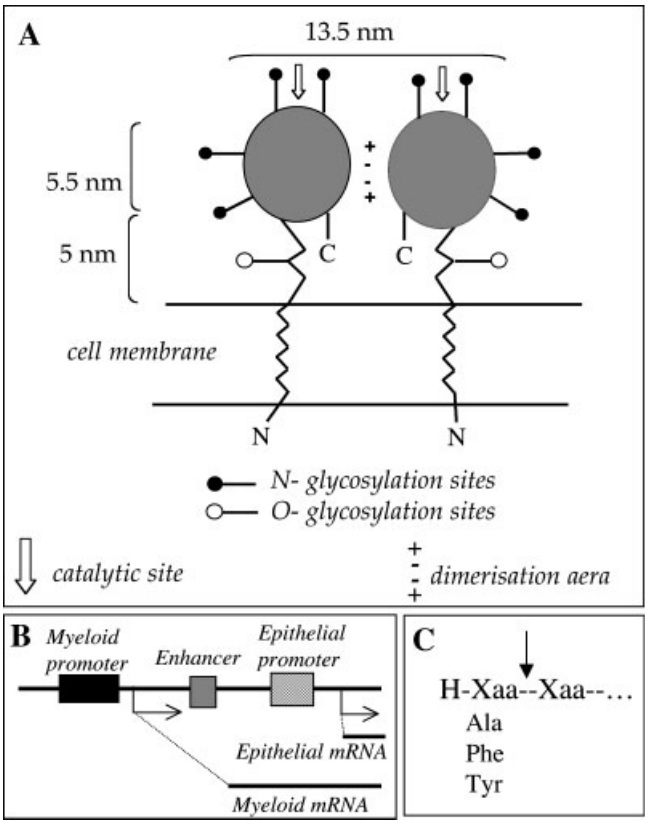



***Figure 1.*** Schematic diagrams showing protein (A) and promoter (B) structures of APN/CD13. The enzyme is a dimer of two non covalently associated monomers. The gene is controlled by two promoters, with an epithelial promoter and a myeloid promoter (in the hematopoietic system). (C) substrate specificity. 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-$NH_2$) 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 ($Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Ni^{2+}$) inhibit APN activity.[40] (for review) Moreover, molecules with a broad spectrum of action such as KCN, $NaN_3$,

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[$CH_2$]Tyr (**5**),[84] pseudoglutamyl aminophosphinic peptides such as Glu$\Psi$($PO_2CH_2$)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 (DPPIV/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, Ki, $IC_{50}$ 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*

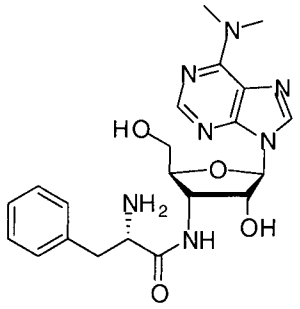
The most widely used among the naturally occuring APN/CD13 inhibitors are microorganism-produced and have been purified from microbial culture filtrates. A large part of them are generated by bacteria belonging to the order *Actinomycetales*, especially of the genera *Streptomyces*:

#### *1. Actinonin*

(2*R*)-$N^4$-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 *Actynomycetes.* 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-phenyl-butanoyl-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



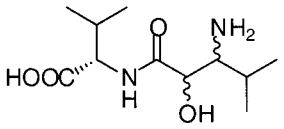
**Puromycin (1)**

$IC_{50}$ APM from rat blood plasma : 60 μM[67]

$IC_{50}$ APN : 50 μM, $IC_{50}$ AAP-S : 0.6 μM μM[75]

67% inhibition of APN from human seminal plasma at 100 μM[74]

$IC_{50}$ APN : 4.8 μM, $IC_{50}$ PSA : 0.6 μM[83]



**Lapstatin (2)**
(The absolute configuration is not completely elucidated)

$IC_{50}$ APM from hog kidney : 203 μM[76]

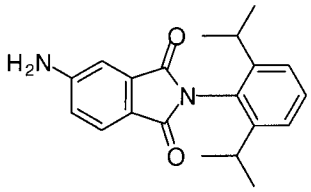
$IC_{50}$ APN from egg white : > 122 μM[76]

$IC_{50}$ LAP from porcine kidney : 41 μM[76]

$IC_{50}$ LAP from *Streptomyces rimosus*: 2.4 μM[76]

$IC_{50}$ LAP from *Aeromonas proteolytica*: 0.3 μM[76]

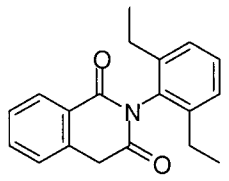
$IC_{50}$ API from *Streptomyces griseus*: 0.85 μM[76]



**3**

$IC_{50}$ APN : 5.4 μg/mL, $IC_{50}$ DPP-IV : 14.1 μg/mL, WI-38 : 17.4 μg/mL[77,80]

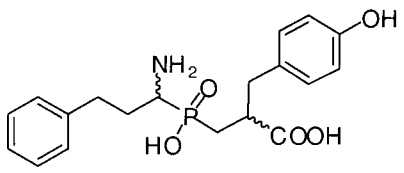
$IC_{50}$ APN : 16.8 μM, $IC_{50}$ DPP-IV : 251.2 μM[79]



**PIQ-22 (4)**

$IC_{50}$ APN : 0.12 μg/mL, $IC_{50}$ DPP-IV : 14.1 μg/mL[77,80]

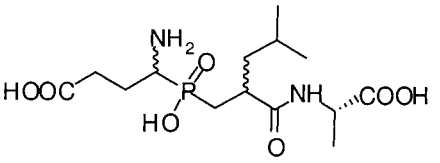
Inactive towards LAP, DPP-IV, Trypsin and Chymotrypsin[80,82]



**hPheP[$CH_2$]Tyr (5)**
(mixture of four diastereoisomers)

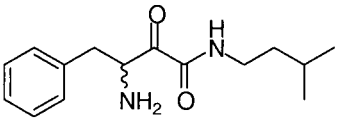
$K_i$ APN : 36 nM, $K_i$ LAP : 67nM[84]

***Figure 2.*** Miscellaneous inhibitors of APN/CD13.



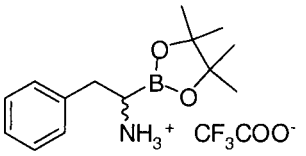
**GluΨ(PO$_2$CH$_2$)Leu-Ala (6)**
(mixture of four diastereoisomers)

$K_i$ APN : 31 μM, $K_i$ APA : 0.8nM, $K_i$ NEP : 62 nM, $K_i$ $K_i$ ACE : 53 μM[85]



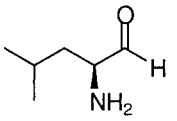
**7** (racemate)

$K_i$ APN : 2.5 μM, $K_i$ LAP$_c$ : 1 μM, $K_i$ APB : 1.5 μM[86]



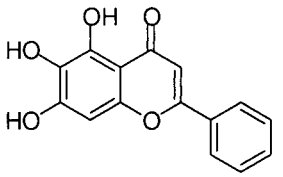
**8** (racemate)

$IC_{50}$ APN : 20 nM, $IC_{50}$ HEDA : 50 nM[87]



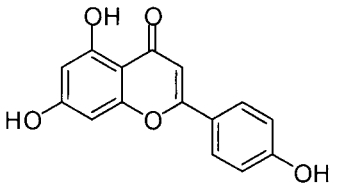
**9**

$K_i$ APN : 760 nM, $K_i$ LAP$_c$ : 60 nM[88]



**Baicalein (10)**

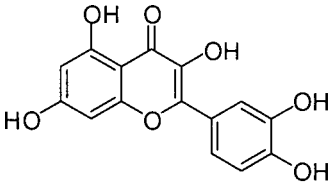
% inhibition (at maximal concentration of 300 μM)
APN : 57 %, ACE : 10 %, NEP : 36 %[90]



**Apigenin (11)**

% inhibition (at 100 μM) : APN : 26 %, inactive on LAP[89]
% inhibition (at maximal concentration of 300 μM) : APN : 42 %, ACE : 18 %, NEP : 31 %[90]

***Figure 2.*** (*Continued*)



**Myricetin (12)**

% inhibition (at 100 μM) : APN : 25 %, inactive on LAP[89]

% 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 $LTA_4$ 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 performed[131] 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]

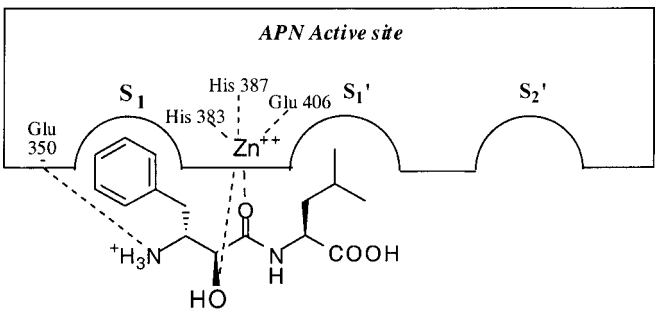



***Figure 3.*** Binding of Bestatin to the active site of APN.

*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 APN[138] 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 occuring APN inhibitors are of vegetal origin:

*8. Benzo[c]phenantridines*

Benzo[c]phenantridines 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 *Macleaya 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 inhibitors[207] through their ability to inhibit endothelial cell proliferation.[208]

### *10. Betulinic Acid*

(3β-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 revues.[211–213] Several hemisynthesis of **28** starting from betulin via betulonic acid[214–217] or from various naturally occuring betulinic acid derivatives such as glycosides,[218–221] sulfates,[222] or dihydroxycinnamic esters[223] have been reported.

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

### *11. Psammaplin 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 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 $Ca^{2+}$ 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]

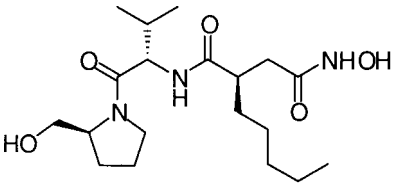
Chemical structures of APN inhibitors **13**–**30**, and enzyme inhibition values are depicted in Figure 4.

### *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)**

$IC_{50}$ APN : 0.4 μg/mL, $IC_{50}$ LAP : 1 μg/mL, Inactive towards APA, APB, Methionine aminopeptidase and formylmethionine aminopeptidase[94]

$IC_{50}$ APN : 1.99 μM, $IC_{50}$ APA : >100 μM[355]

$IC_{50}$ APN : 0.4 μg/mL, $IC_{50}$ APA : >100 μg/mL, $IC_{50}$ APB : >100 μg/mL[137]

$IC_{50}$ APN from *Bombyx mori* : 148 μM[368]

$IC_{50}$ APN : 0.32 μg/mL[77]

$IC_{50}$ Enkephalinaminopeptidase : 0.39 μM, $IC_{50}$ dipeptidylaminopeptidase : 1.1μM, $IC_{50}$ Enkephalinase A : 5.6μM[336]

91% inhibition of APN, 16.5% inhibition of APA and 13% inhibition of MDP at 100 μM, inactive towards APW[369]

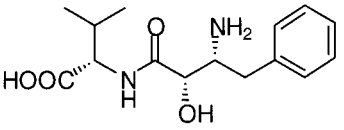
100% inhibition of APN from human seminal plasma at 100 μM[74]

$IC_{50}$ Bacterial PDF 0.8-90 nM depending on the metal cation form of the enzyme[370]

$IC_{50}$ Human PDF : 43 nM[358]

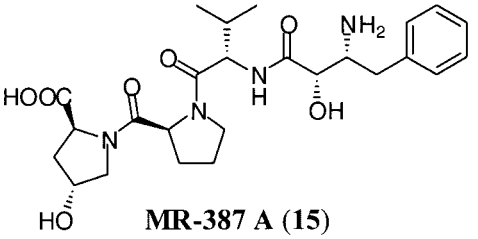
$K_i$ Meprin α : 20 nM, $K_i$ Meprin β : 2 μM[360]

***Figure 4.*** Natural inhibitors of APN/CD13.



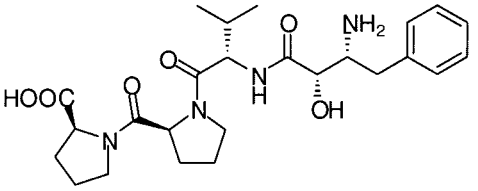
**AHPA-Val (SL-387) (14)**

$IC_{50}$ APN from porcine kidney microsome : 1.2 μg/mL, $IC_{50}$ APN from human fibrosarcoma HT1080 : 5.6 μg/mL, $IC_{50}$ APN from human myelogenous leukemia K562 : 7.8 μg/mL[104]



**MR-387 A (15)**

$IC_{50}$ APN from porcine kidney microsome : 198 nM, $IC_{50}$ APN from human fibrosarcoma HT1080 : 218 nM, $IC_{50}$ APN from human myelogenous leukemia K562 : 17 μM, APB from human myelogenous leukemia K562 : 651 nM[105]



**MR-387 B (16)**

$IC_{50}$ APN from porcine kidney microsome : 164 nM, $IC_{50}$ APN from human fibrosarcoma HT1080 : 201 nM, $IC_{50}$ APN from human myelogenous leukemia K562 : 4.6 μM, APB from human myelogenous leukemia K562 : 260 nM[105]

***Figure 4.*** (*Continued*)

*2. Alkyl D-Cysteinates*

Alkyl D-cysteinates 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*

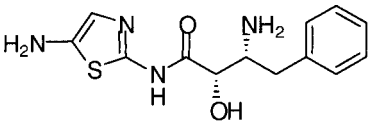
3-amino-2-tetralone derivatives such as the 2-amino-1,4-dihydro-2*H*-phenanthren-3-one hydrochloride (**33**) have been reported to be efficient and selective competitive inhibitors of APN. These compounds do not affect AP-A or AP-B and poorly inhibit LAPc.[245]

*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]

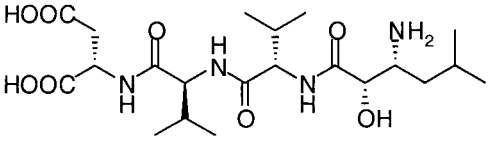
*5. Flavone-8-Acetic Acid Derivatives*

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 μM[371]

$IC_{50}$ APN : 3.16 μM, $IC_{50}$ APA : 2 μM,

$IC_{50}$ APW : 1.58 μM[355]

$IC_{50}$ APN : 150 nM[372]

Ki APN : 50 nM, Ki $LAP_c$ : 30 nM, Ki APA :0.15 μM [245]

$IC_{50}$ APN : 0.58 μg/mL, $IC_{50}$ APA : 0.54 μg/mL, $IC_{50}$ APB : >100 μg/mL[137]

$IC_{50}$ APN from *Bombyx mori* : 7.6 μM[368]

100% inhibition of APN, 100% inhibition of APA, 100% inhibition of APW and 18% inhibition of MDP at 100 μM[369]

100% inhibition of APN from human seminal plasma at 100 μM[74]

Ki APN : 52 nM, Ki $LAP_c$ : 30 nM, Ki *Aeromonas* AP : 0.26 nM[357]

Ki* APN : 20 nM, Ki* LAP : 0.2 μM[108]

$IC_{50}$ APM from rabbit kidney cortex : 0.4 μM[373]

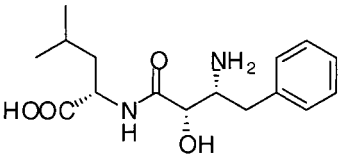
$IC_{50}$ APM from rat blood plasma : 0.2 μM, $IC_{50}$ APA from rat blood plasma : 8 μM [67]

$IC_{50}$ APA : 0.54 μg/mL, $IC_{50}$ APL : 0.5 μg/mL, $IC_{50}$ APB : >250 μg/mL [109]

$IC_{50}$ APA : 1.1 μM, $IC_{50}$ LAP : 1.1 μM[112,374]

$IC_{50}$ APW : 2 μM[375]

***Figure 4.*** (*Continued*)




**Bestatin (19)**

Ki APN : 3.03 μM, Ki $LAP_c$ : 9 nM[362]

Ki* APN : 4.1 μM[108]

$IC_{50}$ APN from pig kidney : 16 μM[371]

Ki APN : 1.45 μM, Ki $LAP_c$ : 0.4 nM,

Ki *Aeromonas* AP : 18 nM[357]

Ki APN : 4.1 μM, Ki $LAP_c$ : 20 nM,

Ki APB: 14 nM[136]

$IC_{50}$ APM from rabbit kidney cortex : 6 μM[373]

$IC_{50}$ APN : 89.1 μM, $IC_{50}$ APW : 7.9 μM[355]

$IC_{50}$ APN : 43 μM[372]

Ki APN : 3.5 μM, Ki $LAP_c$ : 0.6 nM,

Ki APB : 6 μM[245]

Ki APN : 3.5 μM, Ki $LAP_c$ : 0.6 nM, Ki AP *Aeromonas* P. : 20 nM, Ki APB: 6 μM[246]

$IC_{50}$ APN : 6.2 μg/mL, $IC_{50}$ APA : >100 μg/mL, $IC_{50}$ APB : 0.05 μg/mL[137]

$IC_{50}$ APN from *Bombyx mori* : 3.25 mM[368]

96% inhibition of APN from human seminal plasma at 100 μM[74]

52% inhibition of APN, 13.4% inhibition of APA, 89% inhibition of APW and 29% inhibition of MDP at 100 μM[369]

$IC_{50}$ APN from rat blood plasma : 30 μM[67]

$IC_{50}$ APN : 16.9 μM[210]

$IC_{50}$ APN : 2.5 μM[190]

$IC_{50}$ APN : 3.9 μM, $IC_{50}$ A-LAP : 11.2 μM[249]

$IC_{50}$ APB : 0.05 μg/mL, $IC_{50}$ LAP : 0.01 μg/mL[119]

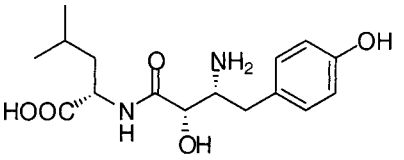
Ki APB : 60 nM, Ki LAP : 20 nM[353,356]

$IC_{50}$ Enkephalin aminopeptidase : 1.1 μM[336]

$IC_{50}$ APB : 0.05 μg/mL, $IC_{50}$ LAP : 0.003 μg/mL[132]

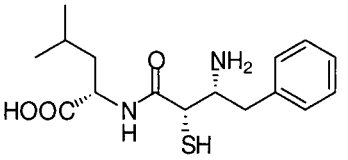
$IC_{50}$ APW : 6 μM[375]

***Figure 4.*** (*Continued*)



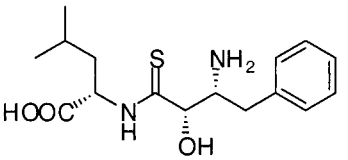
**para-hydroxybestatin (20)**

$IC_{50}$ APB : 0.007 μg/mL, $IC_{50}$ LAP : 0.02 μg/mL[132]



**α-Thiolbestatin (21)**

Ki APN : 4.4 μM, Ki LAP : 0.55 μM, Ki APB : 4.6 nM[133]



**Bestatin thioamide (22)**

Ki APN : 40.3 μM, Ki LAP : 0.33 μM, Ki APB : 2.4 μM[133]

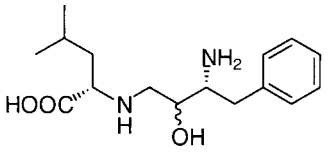
***Figure 4.*** (*Continued*)

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

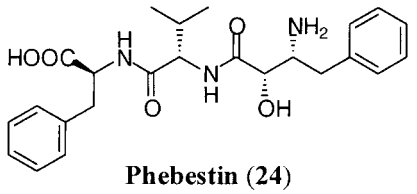
*N*-Hydroxy-2-(naphthalene-2-ylsulfanyl)acetamide (**37**) has recently been identified as a potent APN inhibitor. It acts in a dose-dependent manner and is inactive on metalloenzymes MMP-2, MMP-9, MMP-14, or A-LAP.[249]

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 $S_1$ 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 ($^{125}$I)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]



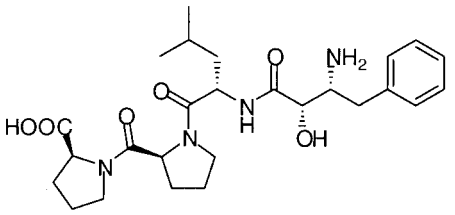
**23** (racemate)

Ki APN : 2.1 mM, Ki $LAP_c$ : > 1 mM, Ki APB: 14 nM[136]



**Phebestin (24)**

$IC_{50}$ APN : 0.18 μg/mL, $IC_{50}$ APA : 9 μg/mL, $IC_{50}$ APB : 9 μg/mL[137]



**Probestin (25)**

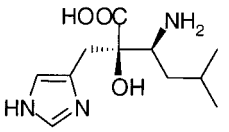
Ki APN : 19 nM[138]

$IC_{50}$ APN : 50 nM, $IC_{50}$ APA : 19.9 μM, $IC_{50}$ APW : 5 μM[355]

$IC_{50}$ APN : < 10 nM[372]

$IC_{50}$ APN : 0.03 μg/mL, $IC_{50}$ APA : >100 μg/mL, $IC_{50}$ APB : 37 μg/mL[137]

$IC_{50}$ APN from *Bombyx mori* : 74 μM[368]



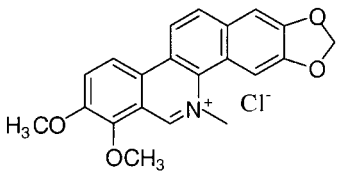
**Leuhistin (26)**

Ki APN : 0.23 μM[186]

$IC_{50}$ APN : 0.2 μg/mL, $IC_{50}$ APA : 10 μg/mL, $IC_{50}$ APB : 13 μg/mL[137]

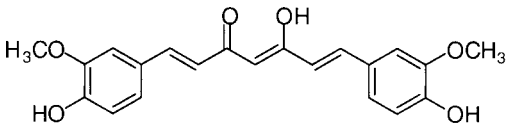
100% inhibition of APN from human seminal plasma at 100 μM[74]

$IC_{50}$ APN from *Bombyx mori* : 0.89 mM: 089 mM[368]



**Chelerythrine (27)**

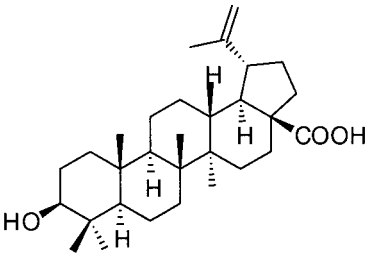
82% inhibition of APN and 38% inhibition of DPP IV at 50 μM[189]



**Curcumin (28)**

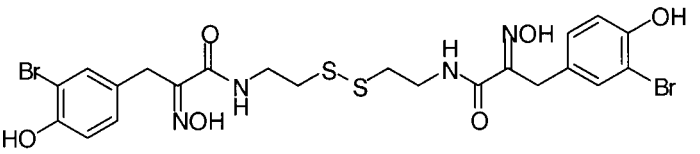
Ki APN : 11.2 μM[190]

***Figure 4.*** (*Continued*)




$IC_{50}$ APN : 7.3 μM[210]

**Betulinic acid (29)**




$IC_{50}$ APN : 18 μM[237]

**Psammaplin A (30)**

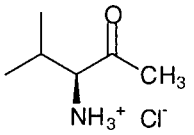
***Figure 4.*** (*Continued*)

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*

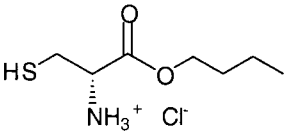
The similarities between the active sites of APN and the membrane-bound protease neutral endopeptidase 24.11 (EC3.4.24.11, CD10, NEP) led to the idea that mixed inhibitors could be developed by selecting frameworks bearing a strong zinc-chelating group and a residue able to interact with at least one subsite ($S_1$, $S_1'$, and $S_2'$) 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 $A_4$ 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)butyldithio)-1-oxopropyl)-L-phenylalanine benzyl ester (**52**) and *RB 120* (*N*-((*S*)-2-benzyl-3((*S*)(2-amino-4-methylthio)butyldithio)-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 inhibitor[269] (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.



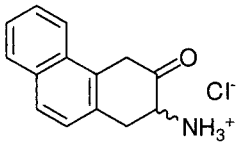
**31**

Ki APN : 0.55 μM[243]



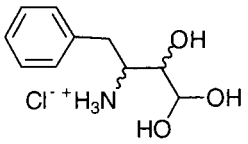
**32**

Ki APN : 0.18 μM[244]



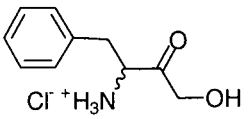
**33**
(absolute stereochemistry not specified)

Ki APN : 0.5 μM, Ki $LAP_c$ : 120 μM, Ki APA: > 1 mM, Ki APB: > 1 mM[245]



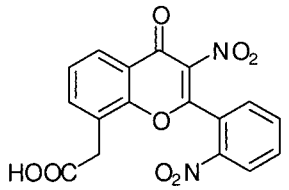
**34**
(absolute stereochemistry not specified)

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



**35**
(absolute stereochemistry not specified)

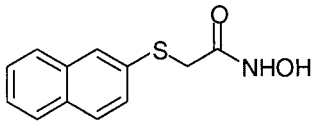
Ki APN : 1 μM[246]



**36**

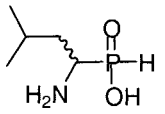
$IC_{50}$ APN : 25 μM[248]

***Figure 5.*** Synthetic inhibitors of APN/CD13.



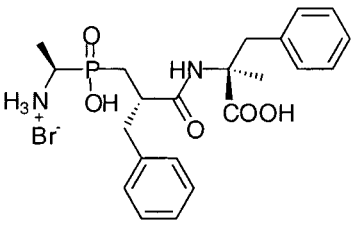
**37**

$IC_{50}$ APN : 3.4 μM[249]



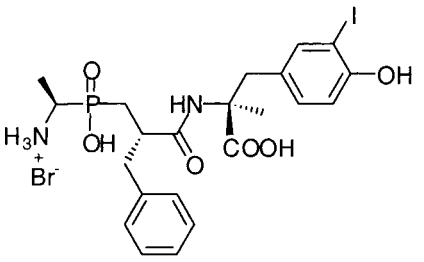
**38**
(mixture of R + S isomers)

Ki APN : 1.2 μM[253]



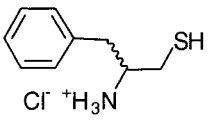
**39**

Ki APN : 0.6 nM, Ki APA : 0.13 μM, Ki APB : >10 μM[253]



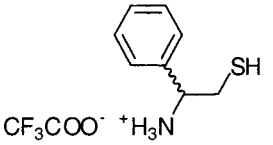
**RB 129 (40)**

Ki APN : 0.95 nM [254]



**Phetiol (41)**
(absolute stereochemistry not specified)

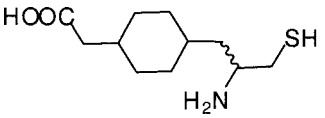
Ki APN : 5 nM, Ki PSA : 10 nM[257]



**42**
(absolute stereochemistry not specified)

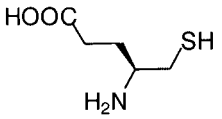
$IC_{50}$ APN : 25 nM[251]

***Figure 5.*** (*Continued*)



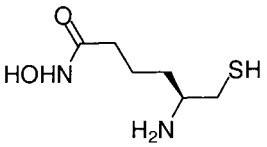

**43**
(mixture of R + S isomers)

Ki APN : 35 nM, Ki APA : 2.8 μM[258]



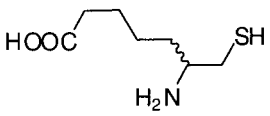

**44**

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



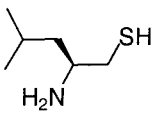

**45**

Ki APN : 37 nM, Ki APA : 2.0 μM[259]



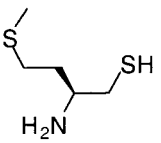

**46**
(mixture of R + S isomers)

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



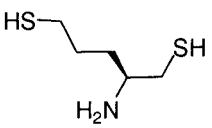

**47**

Ki APN : 22 nM[253]




**PC 18 (48)**

Ki APN : 8 nM, Ki APA : 17.2 μM, Ki APB : 1 μM[260]
Ki APN : 11 nM[339]




**EC 27 (49)**

Ki APN : 32 nM, Ki APA : 2.4 μM[259,261]

***Figure 5.*** (*Continued*)

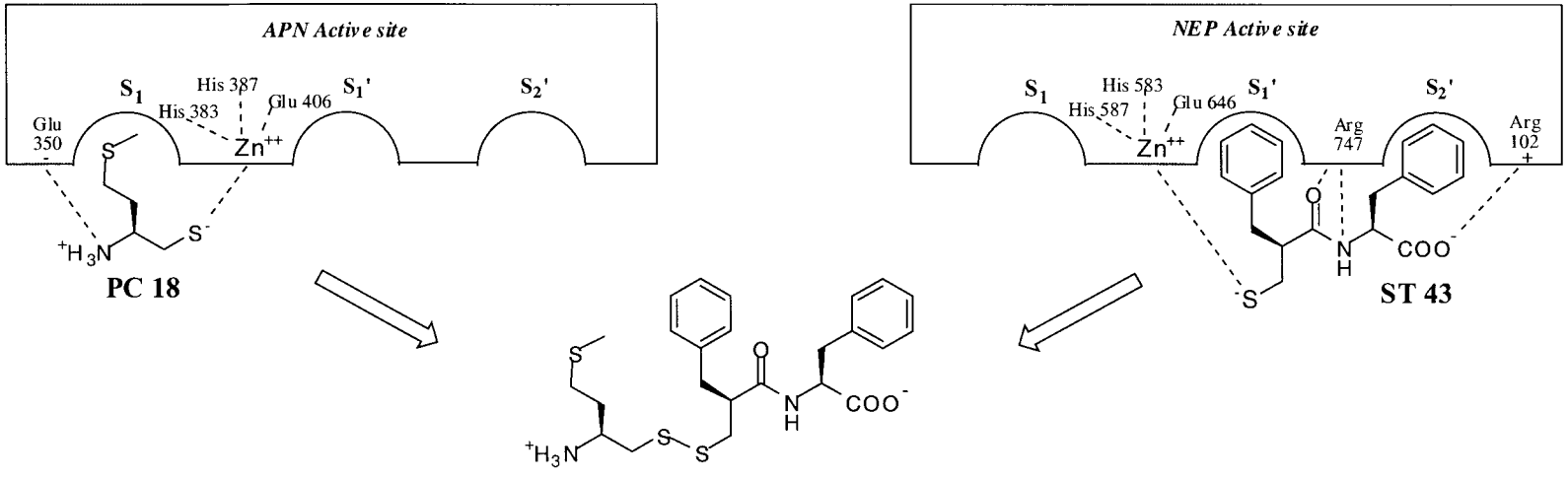
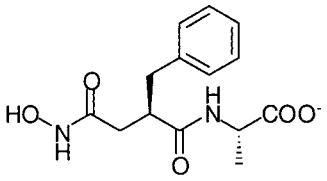



***Figure 6.*** Conceptualization of APN/NEP dual inhibitors (example of **RB 101**).



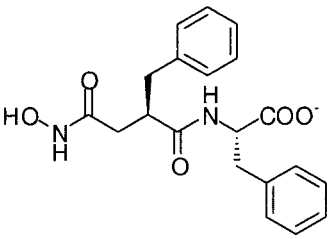
**Kelatorphan (50)**

Ki APN : 0.38 μM, Ki NEP : 1.8 nM[264]

Ki APN : 7 μM, Ki NEP : 1.7 nM[339]

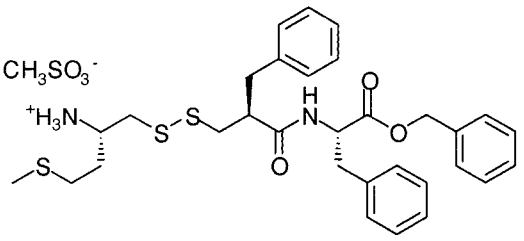
Ki APN : 0.38 μM, Ki DAP : 0.9 nM, Ki NEP : 2.0 nM[376]

$IC_{50}$ NEP : 46 nM, $IC_{50}$ $LTA_4$ hydrolase : 5 nM, $IC_{50}$ Aminopeptidase : 7 nM, $IC_{50}$ ACE : >10μM[268]



**RB 38A (51)**

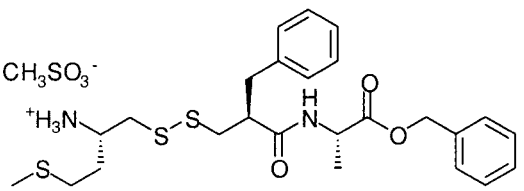
Ki APN : 0.12 μM, Ki NEP : 0.9 nM[264]



**RB 101 (52)**

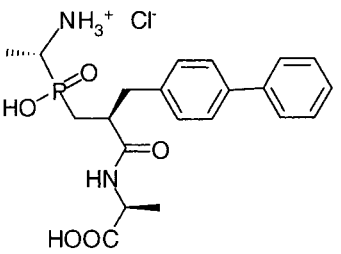
$IC_{50}$ APN : 16μM, $IC_{50}$ NEP : >100μM[267]

After 15 min of preincubation with whole rat brain membrane, $IC_{50}$ APN : 11 nM, $IC_{50}$ NEP : 4 nM[267]



**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 nM[272]

Ki APN : 2.9 nM, Ki NEP : 1.2 nM, Ki ACE : 0.12 μM[252]

Ki APN : 2.9 nM, Ki NEP : 1.2 nM[339]

Ki APN : 2.9 nM, Ki APA : >1μM Ki NEP : 1.2 nM, Ki ACE : 0.12 μM[376]

***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 μM 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 ↓<br>apoptosis ↑<br>migration ↑<br>invasion ↓ | tumor growth ↓<br>neovascularization ↓<br>pain management ↓ | |
| Amastatin | chemotaxis ↑<br>angiogenesis ↓ | blood pressure ↓ | |
| Bestatin<br>(ubenimex) | growth ↓<br>differentiation ↑<br>apoptosis ↑<br>migration ↑↓<br>invasion ↓<br>angiogenesis ↑↓ | tumor growth ↓<br>fetal growth ↓<br>monocyte activation ↑<br>placental apoptosis ↑<br>neutrophil migration ↓<br>neovascularization ↓<br>inflammation ↓ | Remission in :<br>AML<br>lymphoma *(monocyte & lymphocyte activation)*<br>lung carcinoma |
| Betulinic acid | apoptosis ↑<br>angiogenesis ↓ | tumor growth ↓ | |
| Curcumin | angiogenesis ↓ | | |
| Leuhistin | invasion ↓ | neutrophil migration ↓<br>inflammation ↓ | |
| Probestin | growth ↓ | | |
| Psammaplin A | growth ↓<br>angiogenesis ↓ | | |
| PC 18 & EC 27<br>RB 101& RB 120 | | blood pressure ↓<br>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 lines[287] 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-β1.[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 μg/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 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]

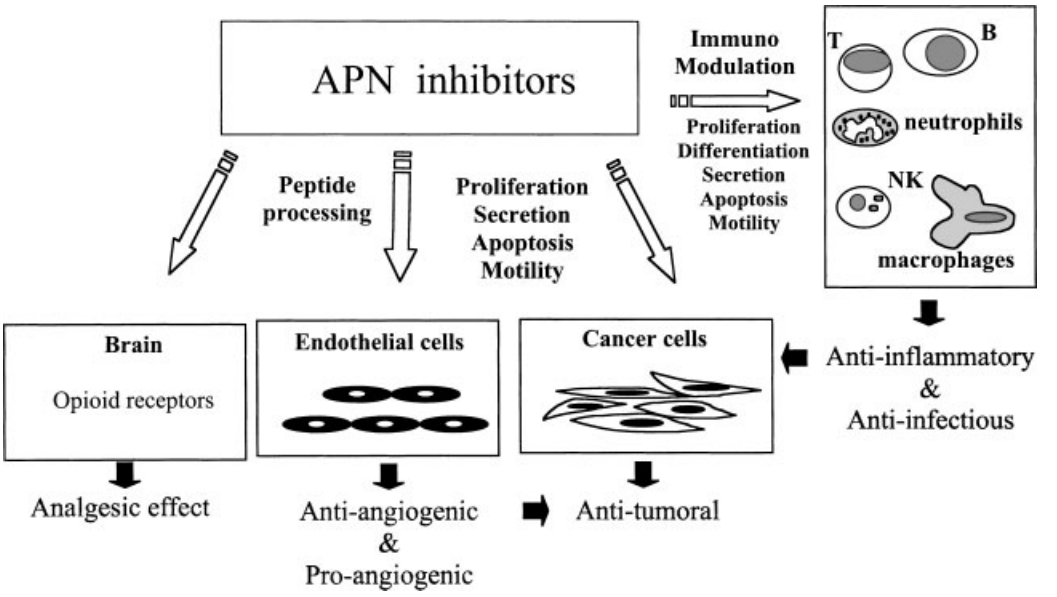



***Figure 8.*** Biological effects of APN/CD13 inhibitors. The actions of APN inhibitors in vitro and in vivo (animal models) are diverse; they may directly target cancer cells, or act indirectly against targets by activation of immune cells (T, B, neutrophils, natural killer/NK cells, macrophages) or 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-κB 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 cytototoxic APN inhibitors (such as psammaplin 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.

***ACKNOWLEDGMENTS***

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