Cancer Science
The official journal of the Japanese Cancer Association

Review Article

Aminopeptidase N (CD13) as a target for cancer chemotherapy

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(Received November 5, 2010/Accepted December 3, 2010/Accepted manuscript online December 9, 2010/Article first published online January 30, 2011)

The enzyme aminopeptidase N (APN, also known as CD13) is a Zn^{2+} dependent membrane-bound ectopeptidase that degrades preferentially proteins and peptides with a *N*-terminal neutral amino acid. Aminopeptidase N has been associated with the growth of different human cancers and suggested as a suitable target for anti-cancerous therapy. Different approaches have been used to develop new drugs directed to this target, including enzyme inhibitors as well as APN-targeted carrier constructs. This review discusses the prevalence and possible function of APN in malignant diseases, mainly solid tumors, as well as its ''drugability'' evaluated in preclinical *in vivo* models, and also provides a brief overview of current clinical trials focused on APN. (*Cancer Sci* 2011; 102: 501–508)

Increased expression of various hydrolytic enzymes like peptidases, esterases and proteases has been described in several types of human malignancies, especially those characterized by fast-growing and aggressive phenotypes.[1] The scientific literature provides numerous examples, diagnostic as well as therapeutic, of implications and possibilities related to these changes. With respect to peptidases, one of the most studied candidates is aminopeptidase N (APN; EC 3.4.11.2, also known as CD13, microsomal aminopeptidase, aminopeptidase M, alanine aminopeptidase, particle-bound aminopeptidase, p146, p161 or gp150), a Zn^{2+} dependent membrane-bound ectopeptidase that degrades preferentially proteins and peptides with a *N*-terminal neutral amino acid. Aminopeptidase N, although widely expressed in mammalian cells, has been associated with different aspects of normal (e.g. myeloid progenitor cells) as well as malignant development. The protein indeed has multiple functions, including enzymatic regulation of peptides as well as characteristics associated with malignant cells, like tumor cell invasion, differentiation, proliferation and apoptosis, motility and angiogenesis. Furthermore APN has been described as a viral receptor and may be involved in cholesterol turnover. Thus, APN has multiple functions and has consequently been designated to be a ''moonlighting ectoenzyme''.[2]

This review discusses the prevalence and possible functions of APN in malignant diseases, as well as its ''drugability'' evaluated in preclinical *in vivo* models, and also provides a brief overview of clinical trials focused on APN.

Structure and function of APN in malignancy

Aminopeptidases are widely distributed enzymes catalyzing the cleavage of amino acids from the amino terminus of protein or peptide substrates, and may localize as subcellular organelles in cytoplasm or as membrane components. Some are monomeric and others are assemblies of relatively high mass (50 kDa) subunits. Many, but not all, of these peptidases are zinc metalloenzymes (M1 family) and are inhibited by the transition-state analog bestatin.[3,4] Among this family, APN (EC 3.4.11.2, also known as CD13, microsomal aminopeptidase, aminopeptidase M, alanine aminopeptidase, particle-bound aminopeptidase, p146, p161 or gp150) has been extensively investigated. The full length APN 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.[4,5]

Aminopeptidase N is an ubiquitous enzyme present in several human organs, tissues and cell types. It is described as a multifunctional (''moonlighting'') protein with enzymatic as well as other functions, including antigen presentation and a receptor for some human viruses (e.g. coronaviruses).[6] Notably, the aminopeptidase inhibitor bestatin was actually designated ''immunomodulating agent'' in early clinical trials (see Treatment section below).

These functions facilitate the modulation of bioactive peptide responses, influence immune functions and major biological events, thereby providing treatment options for many kinds of diseases.[5] The various functions of APN, including the biological mechanisms, were recently reviewed and a number of outstanding questions were raised to be interpreted for a more rational design of APN-targeting agents.[2]

With respect to malignant cell growth, APN has been associated with a number of characteristics of the malignant phenotype (e.g. cell proliferation, secretion, invasion and angiogenesis).[2,7–9] These functions and the relation to different diagnoses are discussed below.

APN and the malignant phenotype

Angiogenesis. During studies designed to identify peptides that home specifically to solid tumors, Pasqualini and co-workers identified phages expressing the asparagine–glycine–arginine (NGR) motif binding strictly to the endothelium of angiogenic blood vessels.[10] A subsequent study revealed APN as the principal receptor for the NGR peptide motif and it was demonstrated that this receptor is expressed exclusively on the endothelial cells of angiogenic but not normal vasculature.[11] Further studies definitely established APN as an important regulator of endothelial morphogenesis during angiogenesis: (i) treatment of animals with APN inhibitors significantly impaired retinal neovascularization, chorioallantoic membrane angiogenesis and xenograft tumor growth; (ii) APN levels in primary cells and cell lines are upregulated in response to hypoxia, angiogenic growth factors and signals regulating capillary tube formation during angiogenesis; (iii) transcription of reporter plasmids containing CD13/APN proximal promoter sequences

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doi: 10.1111/j.1349-7006.2010.01826.x

is significantly increased in response to the same angiogenic signals that regulate the expression of the endogenous gene both *in vitro* and in human tumor xenografts; and (iv) functional antagonists of CD13/APN interfere with tube formation but not proliferation of primary vascular endothelial cells, suggesting that CD13/APN controls endothelial cell morphogenesis.[12]

The results were later confirmed and also extended to ''normal'' proliferating endothelial cells by demonstration of selective expression of APN in vascular endothelial cells, including human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC), which was not detectable in a majority of normal cells and tumor cell lines, and inhibition of capillary tube formation of HUVEC on Matrigel by RNA interference (RNAi) of APN.[7]

Distinct Ras-effector pathways regulate the cytokine induction of APN in endothelial cells, and phosphorylation of Ets-2 by RAS/MAPK is a prerequisite for APN endothelial induction of Ets-2 and its targets, which play essential roles in endothelial cell function.[13,14] Experiments to identify the transcription factors responsible for this regulation demonstrated that exogenous expression of the proto-oncogene c-Maf potently activates transcription from a critical regulatory region of the CD13 proximal promoter, with an atypical Maf response element.[15]

The high expression of APN in tumor vessels may be used for non-invasive *in vivo* imaging and monitoring of tumor growth and angiogenesis. Use of a NGR peptide labeled with the cyanine dye Cy 5.5 has been successfully shown to image tumor growth in nude mice; APN-positive xenografts were clearly visualized by 2-D planar fluorescence reflectance imaging (FRI) and 3-D fluorescence mediated tomography (FMT) up to 24 h after injection. The FMT also allowed quantification of fluorochrome distribution in deeper sections of the tumor. The authors suggested this might be a promising strategy for a sensitive evaluation of tumor angiogenesis *in vivo*.[16]

Besides APN, two other aminopeptidases, type 2 methionine aminopeptidase and adipocyte-derived leucine aminopeptidase/puromycin insensitive leucyl-specific aminopeptidase, have been suggested to be involved in the process of angiogenesis.[17,18]

Proliferation, invasion and metastasis. The aminopeptidase inhibitor bestatin inhibits tumor–cell invasion, as well as aminopeptidase activities of murine and human metastatic tumor cells,[19] an effect that has been attributed to inhibition of APN. Indeed, monoclonal antibodies specific for APN inhibit the invasion of human metastatic tumor cells (SN12M renal cell carcinoma, HT1080 fibrosarcoma and A375M melanoma) into reconstituted basement membrane (Matrigel filters), in a concentration-dependent manner. The mechanism was suggested to be direct inhibition of APN activity and reduced degradation of type-IV collagen.[20]

In the search for cell surface proteins controlling cell motility and angiogenesis in human colon cancer specimens, Hashida and co-workers[8] established a murine monoclonal antibody, MH8-11, which inhibits cell motility and *in vitro* angiogenesis. This epitope was a 165 kDa protein and the sequencing analysis revealed that it was almost identical to APN.[8] Aminopeptidase N-silencing RNA suppressed the migration of HUVEC through a fibronectin-coated transwell membrane, and reduced the cellular adhesion to matrigel and various adhesion molecules including collagens and fibronectin.[7]

In the osteosarcoma cell line MNNG/HOS, APN antisense cDNA transfection significantly decreased the invasive potential *in vitro*, as judged by adhesion and migration through a reconsituted basal membrane, but appeared to have no effect on proliferation, motility or collagen I adhesion. Under *in vivo* conditions, a reduced potency to metastasize to the lung was shown in an experimental metastasis assay in nude mice.[21] In the undifferentiated thyroid carcinoma cell line 1736, APN mRNA expression increased after exposure to epidermal growth factor, basic fibroblast growth factor, interleukin-6 and tumor necrosis factor alpha. FTC-133 cells stably transfected with an expression vector for APN showed a higher migration rate. The authors concluded that APN-associated downregulation of specific genes in thyroid carcinoma cells is an important step of tumor progression to more malignant phenotypes, suggesting an important role for APN as a mediator in a multimolecular process regulating cell migration.[22]

However, in normal human embryonic kidney cells the expression of wt APN was associated with a significant decrease in proliferation, migration and also reduced anchorage-independent growth when compared with enzymatically inactive APN variants and controls. This appeared to be due to downregulated mRNA and protein expression of the chemokine receptor CXCR4 and inhibition of the stromal cell-derived factor (SDF)-1alpha/CXCL12-mediated migration.[23]

Association between APN/CD13 and cells of solid malignancies

Several studies[24–34] have demonstrated an association of measurable APN (in tissues or in blood) and tumors in patients, which will be discussed in the following sections. Indeed, soluble APN is elevated in plasma and effusions of cancer patients compared with healthy controls. There is also a strong correlation between plasma APN and tumor load, suggesting that plasma APN partly originates from cells in, or related to, the tumor, such as tumor endothelium.[24]

APN activity and expression levels. For a long time CD13 was considered to be a specific surface antigen of myeloid cells and related neoplasms. The first effort to categorize the expression pattern was made by Mechtersheimer[25] in 1990, who immunohistochemically examined non-neoplastic mesenchymal cells along with 33 benign and 83 malignant mesenchymal tumors (MET) using CD13 monoclonal antibodies. In most, but not all, tumors studied the pattern of expression of CD13 was found to mirror that in their tissue of origin. Differential expression of CD13 in only some MET was suggested to reflect a special functional state of these neoplasms.[25]

Dixon and coworkers[26] studied APN expression in different tissues and found expression in breast epithelium and 20% of 37 breast cancer samples, and suggested a correlation with doxorubicin resistance.

The aminopeptidase activity in breast cancer tissues was investigated by Martines and co-workers[27] using a fluorometric assay with naphtyl-amide substrates. Compared with the adjacent unaffected tissue, aminopeptidase activity, including that of soluble alanyl-aminopeptidase (i.e. sAPN), was significantly increased in tumors.[27] Another study also investigated the activity of APN in cancerous ($n = 16$) and non-cancerous ($n = 16$) breast tissue but found no difference between the activities in the tissues.[28]

Aminopeptidase N is also expressed in ovarian cancer cells. However, the largest study so far (73 patients; 43 primary laparotomies and 30 secondary cytoreductions) could not demonstrate any relationships between expression and clinical or other pathological variables. The expression, however, was more pronounced in samples obtained in the primary laparotomies compared with samples from the secondary cytoreductions ($P < 0.001$), and the authors suggested that a potential treatment of ovarian cancer with APN inhibitors should be performed before chemotherapy or in parallel to first-relapse chemotherapy.[29]

Another study quantified the expression of APN in the ovarian cancer tissue of 15 patients representing three different histological types (five patients each) by immunohistochemistry (IHC), and found an association with the histological subtype: APN expression in tumor cells was observed in 80–100% of patients

doi: 10.1111/j.1349-7006.2010.01826.x

with a serous or mucinous carcinoma, but in only one of the clear cell carcinoma patients. In all samples, APN-positive blood vessels were present.[30] In a stably APN-transfected ovarian cancer cell line, chemosensitivity and growth *in vitro* was not changed, but *in vivo* the growth rate of xenografts was reduced, the vascular architecture was different and resistance to cisplatin was observed.[30] Further cell line data suggest a correlation also with paclitaxel sensitivity, and a significant increase in paclitaxel-sensitivity of APN-expressing ovarian carcinoma cells was obtained by suppression of this enzyme (bestatin or the siRNA technique). Furthermore, in a peritoneal metastasis model using nude mice, combination treatment with paclitaxel and bestatin caused a synergistic increase in survival time.[31]

In human colon cancer specimens the APN/CD13 expression was associated with tumor status ($P = 0.025$). The disease-free and overall survival rate for patients with positive APN/CD13 expression tumors was significantly lower than that for patients with APN-negative tumors.[8] A study of oligonucleotide microarrays to monitor gene expression in left-sided sporadic colorectal carcinomas identified several chromosomal locations with clusters of either potential oncogenes or potential tumor suppressors. Some of these, such as APN, coincided with a high frequency of loss of heterozygosity.[32]

When the expression of APN was studied in thyroid carcinoma cell lines and in the tissues of patients with thyroid carcinomas, it was found that undifferentiated anaplastic thyroid carcinomas expressed more APN than differentiated thyroid carcinomas.[22]

While being ubiquitously expressed and exemplified in several tumor types as described above, renal carcinomas appear to make an exception in the case of APN. When studied in 16 samples of renal clear cell carcinomas, the activities and protein levels of aminopeptidases were significantly decreased compared with the adjacent normal tissues.[33] These results were later confirmed by Varona and co-workers,[34] who studied the activities and expression of different aminopeptidases in RCC. While several aminopeptidases were found to be increased, APN expression was lower in tumors compared with the adjacent tissue (1.3-fold).[34]

Evaluation of APN as a diagnostic and prognostic factor. In a biomarker study,[35] APN was measured in 40 patients with breast and thyroid cancer and in 40 patients with benign tumors or benign non-inflammatory diseases. Aminopeptidase N revealed sufficient sensitivity, good specificity, a sufficient predictive value of both positive and negative results and overall good accuracy. The authors suggested that an APN enzyme activity test from serum could be useful in the diagnosis of breast and thyroid cancer.[35]

Plasma levels of APN were found elevated in patients with non-small-cell lung cancer (NSCLC; 90 patients, 90 controls) and a significant correlation was found with tumor progression stage and serum concentrations ($r = 0.23$, $P = 0.029$). High serum APN ($n = 17$) was associated with advanced stage ($P = 0.004$) or poor performance status ($P = 0.001$). The overall survival rate for patients with high APN was significantly less than that of patients with low APN ($n = 73$, $P < 0.0001$) and, based on multivariate analysis, APN was considered as an independent prognostic factor in patients with NSCLC.[36]

An immunohistochemical study of 194 cases of NSCLC demonstrated a correlation between the expression of APN and angiogenesis ($r = 0.659$; $P < 0.0001$). Approximately one-third of the patients were APN-positive and the 5-year survival of these was significantly lower than in APN-negative tumors (48.3% *vs* 67.1%; $P = 0.0001$).[37] Another study investigated differential staining within the tumor; among 95 patients only 9% had APN-positive tumor cells, all of adenocarcinoma type. Stromal cell positivity was more prominent in squamous cell carcinoma than in adenocarcinoma ($P = 0.005$) and the microvessel density was significantly associated with APN-positive stromal cells ($P = 0.001$). However, the 5-year survival rates were not significantly different between groups, based on APN status in stromal cells.[38]

In a study on pancreatic cancer specimens ($n = 50$),[39] the APN expression, detected by reverse transcriptase-PCR and IHC, was positive in 50% and 48% of the tumors, respectively. Aminopeptidase N was also significantly associated with an increase of intratumor microvessel density. Although not directly associated with various prognostic factors, the median survival time of patients with APN expression was significantly shorter than that of patients without APN expression ($P = 0.009$), and multivariate analysis showed that the APN status was a significant independent factor ($P = 0.016$).[39]

In contrast, in an IHC analysis of 121 patients with gastric carcinoma, APN expression was negatively associated with lymph node metastasis, and the overall survival rate of patients with negative APN expression was significantly lower than that of patients with positive APN expression.[40]

The different expression correlation in patient materials is summarized in Table 1.

APN as a target for cancer therapy

Aminopeptidase N is clearly dysregulated in human malignancy, contributing to the neoplastic properties. Thus, interference with APN expression, function or signaling may lead to the development of novel anticancer drugs. The approach has been evalu-

Table 1. APN expression levels in patient tumor samples and clinical/pathological parameters

Diagnosis	Major findings	References
Breast cancer	Increased AP activity *vs* surrounding normal tissue	27
	Increased APN activity in patient serum	35
Colon cancer	Significant correlation IHC *vs* poor status, decreased DFS, poor survival	8
Gastric cancer	Significant negative correlation *vs* lymph node metastasis and survival	40
NSCLC	Circulating APN considered as an independent prognostic factor for poor survival	36
	Significant correlation of IHC with increased angiogenesis and poor survival	37
Ovarian cancer	Higher expression in early disease, no correlation with clinical parameters	29
	Expression in serous and mucinous but rarely in clear cell histology	30
Pancreatic cancer	Significant correlation of IHC *vs* high IMD and poor survival	39
Renal cancer	AP activities and IHC lower in tumor than surrounding tissue	33
	APN lower in RCC	34
Thyroid cancer	Higher expression in undifferentiated anaplastic tumors	22
	Increased activity in patient serum	35

AP, aminopeptidase; APN, aminopeptidase N; DFS, disease-free survival; IHC, immunohistochemistry; IMD, intratumor microvessel desnsity; NSCLC, non-small-cell lung cancer.

Table 2. APN-targeted therapy under clinical and late pre-clinical development against solid tumors

Prototype	Strategy	Status	References
Ubenimex	Direct inhibition	Clinical	As in text
Tosedostat	Direct inhibition	Phase I–II	53
TVT-DOX	NGR-coated liposomes	Preclinical	http://www.ambrilia.com
NGR-TNF	NGR-TNF fusion protein	Phase I–II, single and combination	64 62,63
tTF-NGR	NGR-tTF fusion protein	Phase I	67 68
Pt(IV)-NGR	NGR-platinum conjugate	Preclinical	66
CNF1/CNF2	NGR-linked prodrug of 5-FdUrd	Preclinical	81
siRNA	Gene silencing	Experimental	21
Endo-NGR	NGR-endostatin fusion protein	Preclinical	69
DOX-CNGRC	Doxorubicin-NGR conjugate	Preclinical	24,65
J1	APN-mediated activation of prodrug	Phase I–II	http://www.oncopeptides.se
NGR-PM-DTX	NGR-coated PEG-b-PLA polymeric micelles with docetaxel	Preclinical	74

DOX, doxorubicin; J1, melphalanyl-p-fluorophenylalanyl ethyl ester; NGR, asparagin–glycine–arginine tumor-homing peptide; TNF, tumor necrosis factor alpha; tTF, truncated tissue factor; TVT, tumor vessel target; 5-FdUrd, 5-fluoro-2′-deoxyuridine.

ated for some time and several Investigational New Drugs are currently under clinical investigation. The target may be used in several, principally different, ways to exert antitumoral effects, which are discussed next and summarized in Table 2.

Direct APN inhibition. In relation to its 3-D structure and function, the medicinal chemistry of different APN inhibitors, including the structural and electronic requirements of the enzyme active site and the binding pocket, have been reviewed recently.[9] The different aminopeptidases, including APN, generally have broad and overlapping substrate specificity, and inhibitors are not expected to be specific.[2,9,41] For example, bestatin inhibits at least 12 different aminopeptidases, most with a Ki below 1 μM.[42] Some of these aminopeptidase inhibitors with APN-inhibiting properties have entered clinical trials in the anticancer area and are discussed below.

Bestatin (ubenimex). Several clinical trials in hematological malignancies have been conducted with this agent that originally was described as an immunomodulant. Therapeutic efficacy, survival benefit, has been demonstrated in acute myeloid leukemia (AML) and lymphomas.[43] Regarding efficacy in solid tumors, clinical studies have been conducted in carcinoma of the lung, bladder, stomach, head and neck, esophagus and skin (malignant melanoma).

Several trials in NSCLC have indicated activity of bestatin in patients with squamous carcinoma.[44–46] A randomized study of bestatin in 153 patients with NSCLC (72 with squamous cell carcinoma, 66 with adenocarcinoma and 15 with other types of cancer) demonstrated significantly prolonged survival (compared with the control group without treatment) in patients with squamous cell carcinoma. No significant difference between the two groups was seen in adenocarcinoma.[44] Combination therapy with bestatin (30 mg daily, every day) was tested in a randomized trial with 238 patients with inoperable primary lung cancer. There was no statistically significant difference in the response rate or survival between groups; however, in squamous cell cancer, a response was observed in 34.5% of the bestatin group and 17.9% of the control group. The analysis, including Cox's proportional hazard model, revealed that survival tended to be longer in the bestatin group (median survival, 40 weeks) than in the control group (median survival, 24 weeks; $P = 0.051$).[45] A more recent prospective randomized, double-blind, placebo-controlled trial with adjuvant bestatin (30 mg daily orally for 2 years) was conducted in patients with completely resected stage I squamous-cell lung carcinoma. Four hundred and two patients were included (202 bestatin and 198 placebo), and the median follow up was 76 months. The 5-year overall survival was 81% in the bestatin group and 74% in the placebo group. The 5-year cancer-free survival was 71% in the bestatin group and 62% in the placebo group. Overall survival ($P = 0.033$, log-rank test) and cancer-free survival ($P = 0.017$, log-rank test) were statistically significantly different by Kaplan–Meier analysis. Few adverse events were observed in either group.[46]

Ninety-six patients with resectable gastric cancer were randomized to adjuvant treatment with intravenous mitomycin C (MMC) plus oral administration of tegafur with or without oral bestatin (60 mg daily). The survival benefit of bestatin addition observed was not significant, but it was for a subgroup of stage III + IV patients with positive histological serosal invasion ($P < 0.05$, log-rank test). Moreover, in patients with positive histological serosal invasion, the recurrence of peritoneal dissemination was significantly suppressed in the MMC + FT + bestatin group.[47]

In patients with non-metastatic transitional cell carcinoma of the bladder, scheduled for full-dose local irradiation therapy (64 Gy), adjuvant oral bestatin treatment (30 mg daily for at least 1 year) did not have any positive effects on the outcome (194 evaluable patients).[48]Adjuvant combination therapy with UFT (5-fluoro-1-[2-tetrahydrofuryl]-2,4 [1H,3H]-pyrimidinedione) ± bestatin was evaluated in 45 patients with bladder cancer (oral administration initiated 4 weeks postoperatively and continued for 1 year). After a median follow-up period of 628 days, the relapse-free rate was 43.5% for the group given UFT alone and 81.8% for the group given a combination of UFT plus bestatin. In patients with a solitary and papillary tumor, the relapse-free rate was significantly better in the UFT plus bestatin group compared with the group of UFT alone.[49]

Furthermore, it was recently demonstrated that bestatin enhanced the effectiveness of radiotherapy (i.e. acting as a radiosensitizer) in human cervical cancer *in vitro* and *in vivo* models in nude mice.[50]

In summary, it may be concluded that bestatin has been repeatedly studied for the treatment of various solid malignancies, and several studies indicate a possible benefit of the addition of this treatment. However, most studies are small or with few recorded events, and in several cases the positive results are restricted to sub-group analyses.

CHR-2797 (tosedostat). CHR-2797 is a novel metalloenzyme inhibitor that is converted into a pharmacologically active acid product (CHR-79888) inside cells. CHR-79888 is a potent inhibitor of a number of intracellular aminopeptidases, and exerts antiproliferative effects against a range of tumor cell lines

doi: 10.1111/j.1349-7006.2010.01826.x

in vitro and *in vivo*.[51] A phase I–II study with tosedostat included 51 patients with AML, showing an overall response rate of 31.4% (including six patients with complete responses), prompting for further development in this diagnosis. The OPAL study is ongoing and will further evaluate the efficacy and safety of tosedostat in AML.[52] CHR-2797 is orally bioavailable and currently undergoing phase I–II clinical investigation in the treatment of solid tumors (in combination with paclitaxel) and myeloid leukemia. Forty patients were included in a reported phase I trial with accelerated titration design in patients with advanced solid tumors. CHR-2797 was administered once daily. The most commonly observed toxicities were fatigue, diarrhea, peripheral edema, nausea, dizziness and constipation. One patient had a partial response (renal cell carcinoma) and four patients had stable disease for >6 months.[53]

Other APN inhibitors. Several other compounds inhibiting APN are under preclinical development, including novel synthetic compounds or structure analogs, for example, the bestatin dimethylaminoethyl ester, LYP.[54] Others, like curcumin, a phenolic natural product, has, in addition to other effects, been described as an irreversible inhibitor of APN. Curcumin is currently being investigated for its effects on cancer patients in several clinical trials.[55,56]

Silencing APN. When the osteosarcoma cell line MNNG/HOS was stably transfected with an expression vector capable of expressing the antisense transcript of APN, the clones presented reduced potency to metastasize to the lung, as was shown in an experimental metastasis assay in nude mice.[21]

Use of APN for target-directed delivery. *Homing of NGR.* As described above, the NGR peptide (asparagine–glycine–arginine, which could be flanked by two cysteine moieties in a circular peptide [CNGRC]) was discovered in the search for tumor homing peptides, and was subsequently demonstrated as an APN ligand in proliferating endothelium.[10,11] As discussed in detail below, the NGR (or CNGRC) motif has been used in different ways to deliver different cytotoxins to an APN-rich environment, such as solid tumors. Given that APN is not only expressed in the angiogenic endothelium but also in other cell types, the mechanism(s) for the tumor-homing properties of NGR drug conjugates may also be associated with specific APN-dependant side-effects. However, studies on the expression patterns of APN in normal and neoplastic human tissues suggest that different APN forms are expressed in myeloid cells, epithelia and tumor-associated blood vessels.[57] Both direct binding assays with NGR-tumor necrosis factor (TNF) and competitive inhibition experiments with anti-CD13 antibodies showed that an APN isoform expressed in tumor blood vessels could function as a vascular receptor for the NGR motif. In contrast, APN expressed in normal kidney and in myeloid cells failed to bind to NGR-TNF. These findings may explain the selectivity and tumor-homing properties of NGR drug conjugates and may have important implications in the development of vascular-targeted therapies based on the NGR/CD13 system.[57]

NGR-TNF alpha conjugates. The clinical use of TNF alpha as an anticancer drug is limited to local treatments because of its dose-limiting systemic toxicity. In order to increase the therapeutic index by tumor targeting, TNF fusion proteins with the tumor-homing peptide NGR have been constructed. Murine TNF fused with the CNGRC peptide (NGR-TNF) is 12–15 times more efficient than murine TNF alone in decreasing the tumor burden in lymphoma and melanoma animal models, whereas its toxicity is similar. Similarly, human NGR-TNF induced stronger antitumor effects than human TNF, even with doses 30 times lower.[58] The fusion protein has been subject to numerous preclinical studies and clearly demonstrates activity in various animal models, including synergistic activity with some standard chemotherapeutic drugs.[59–61] Pretreatment with NGR-TNF enhanced the response to cisplatin, doxorubicin, gemcitabine, melphalan and paclitaxel in murine models, without apparent increased toxicity in the animals. The synergistic effect was transient, with maximal synergism being observed with a 2-h delay between NGR-TNF and drug administration. NGR-TNF did not increase the *in vitro* cytotoxicity of the chemotherapeutic drugs against tumor cells, suggesting that the *in vivo* synergism depends on NGR-TNF effects on host cells rather than on tumor cells.[60]

In the first clinical trial,[62,63] the pharmacokinetics, plasma biomarkers and dynamic contrast-enhanced magnetic resonance imaging were evaluated at baseline and after each cycle in 16 patients enrolled at four doubling-dose levels (0.2–0.4–0.8–1.6 $\mu g/m^2$) of NGR-hTNF, given intravenously q3w. The most frequent treatment-related toxicity was grade 1–2 chills (69%) occurring during the first infusions. Seventy-five percent of patients assessed with DCE-MRI showed a decrease over time of K(trans), which was more pronounced at 0.8 $\mu g/m^2$. Seven patients (44%) had stable disease for a median time of 5.9 months, including a colon cancer patient who experienced an 18-month progression-free time.[62,63]

Another phase 1b study[64] with 15 patients showed the combination of NGR-hTNF plus doxorubicin (Dox) to be administered safely and indications of activity (one partial response and 10 stable disease; median duration, 5.6 months) also in patients pre-treated with anthracyclines. No dose-limiting toxicity occurred and the combination was considered well tolerated; only 11% of the adverse events were related to NGR-hTNF. There was no apparent pharmacokinetic interaction and the shedding of soluble TNF-receptors did not increase to 0.8 $\mu g/m^2$. The dose level of 0.8 $\mu g/m^2$ NGR-hTNF plus Dox 75 mg/m^2 was selected for phase II development.[64]

NGR-doxorubicin conjugates. Van Hensbergen and co-workers[65] designed a Dox derivative, in which the cyclic CNGRC peptide was coupled via a hydrolysable spacer to the C-14 anthracycline-side chain. The antitumor activity was tested *in vitro* and in nude mice bearing human ovarian cancer xenografts. While cytotoxic effects were demonstrated in all systems, the authors concluded that the antiproliferative and anti-angiogenic effects of Dox-CNGRC were most likely caused by the cytostatic effects of the intracellularly released parent drug Dox, independent of CD13 expression/activity.[65]

NGR-cisplatin conjugates. Mukhopadhyay and co-workers[66] designed mono- and difunctionalized platinum(IV) complexes conjugated to the NGR peptide motif. Platinum(IV)-peptide complexes with nonspecific amino acids or peptide moieties were prepared as controls. Regarding the cytotoxic activity against cancer cell lines *in vitro*, the Platinum (IV)-NGR complexes were more active than the nonspecific Platinum-peptide controls.[66]

NGR-truncated tissue factor (tTF) conjugates. Fusion proteins consisting of the extracellular domain of tissue factor (truncated tissue factor, tTF) and the NGR motif were expressed in *Escherichia coli* BL21 (DE3). *In vivo* studies in different xenografted nude mice revealed that i.v. administration of tTF-NGR induced partial or complete thrombotic occlusion of tumor vessels, as shown by histological analysis, and significant tumor growth retardation without apparent side-effects.[67,68] In a human fibrosarcoma xenograft model, magnetic resonance imaging (MRI) revealed a significant reduction of tumor perfusion by administration of tTF-NGR. Clinical first-in-man application of low dosages of this targeted coagulation factor revealed good tolerability and decreased tumor perfusion as measured by MRI.[68]

NGR-endostatin fusion protein. Through genetic engineering, the NGR motif was introduced in human endostatin (NGR-endostatin) to be expressed in yeast. The addition of an NGR-sequence at the amino terminus resulted in strong binding and inhibition of endothelial cell APN, and NGR-endostatin showed increased binding to endothelial cells compared with the

native protein. Increased binding of endostatin also coincided with improved antiangiogenic properties of endostatin. The NGR modification improved tumor localization and, as a consequence, effectively inhibited ovarian carcinoma growth in athymic nude mice.[69]

NGR-coated liposomes and micelles. The Canadian biotech company Ambrilia Biopharma Inc. has developed NGR-coated liposomal carriers for target-directed delivery of cytotoxic agents or siRNA (http://www.ambrilia.com). Preclinical data has been published on Dox-containing liposomes (TVT-Dox), with several xenograft studies demonstrating superior effects (compared with liposomal doxorubicin without NGR) on cell proliferation, blood vessel density and microvessel area, and apoptosis.[59,70–73]

In a similar fashion, Wang and co-workers[74] have constructed NGR-modified docetaxel (DTX)-loaded PEG-b-PLA polymeric micelles (NGR-PM-DTX). It was demonstrated quantitatively by the spectrophotofluorometry and qualitatively by the confocal image analysis that NGR facilitates the uptake of micelles by CD13-overexpressed tumor cells (fibrosarcoma, HT1080) and endothelial cells (HUVEC), and this uptake could be inhibited by free NGR. In BALB/c mice bearing HT1080 tumor xenografts, stronger antitumor efficacy and less bodyweight changes were shown in the NGR-PM-DTX group.[74]

NGR-coated nanoparticles. NGR-modification of PEGylated LPD (liposome-polycation-DNA) nanoparticles has been designed for efficient delivery of small interfering RNA (siRNA) and/or doxorubicin into tumor cells *in vivo*. The LPD-PEG-NGR efficiently delivered siRNA to the cytoplasm and downregulated the target gene in HT-1080 cells but not CD13(-) HT-29 cells. Three daily injections (1.2 mg/kg) of c-myc siRNA formulated in the LPD-PEG-NGR effectively suppressed c-myc expression and triggered cellular apoptosis in the tumor, resulting in a partial tumor growth inhibition. When doxorubicin (DOX) and siRNA were co-formulated in LPD-PEG-NGR, an enhanced therapeutic effect was observed.[75]

NGR-gene therapy conjugates. Moffatt and colleagues[76] developed a polyethylenimine (PEI)-DNA vector formulation with CNGRC for tumor-specific delivery. *In vitro* assessment of targeting by the CNGRC/PEG/PEI/DNA vector carrying a beta-galactosidase (beta-Gal)-expressing plasmid showed as much as a fivefold increase in transduction, relative to the untargeted PEG/PEI/DNA-betagal vector, of CD13-positive lung cancer, fibrosarcoma, bladder cancer and human umbilical vein endothelial cells, which was specific for CD13-positive cells. *In vivo* delivery to both tumor cells and tumor endothelial cells was demonstrated. The method may thus be useful for developing tumor-targeted gene therapies for use in the clinical treatment of cancer.[76]

Pro-drug concept. Since APN has enzymatic capability it may be used for activation of prodrugs. However, the broad and often overlapping substrate specificity of aminopeptidases make true ''specific'' activation less likely.

Melphalan prodrug J1. Recently, the prodrug J1 was demonstrated to be cleaved by APN to release the constitutive drug (melphalan) in its free form, in the tumor cell cytoplasm or in close vicinity related to the APN-rich environment.[77] J1 has shown activity in numerous *in vitro* and preclinical models,[78–80] superior of comparator melphalan indicating a targeting benefit. J1 is currently in clincal phase I–IIa trials.[77]

Fluoro-2′-deoxyuridine-prodrugs. Two tumor-targeting prodrugs denoted CNF1 and CNF2 were designed from 5-fluoro-2′-deoxyuridine (5-FdUrd) and the CNGRC motif connected by succinate and glutarate linkers, respectively. Both prodrugs released 5-FdUrd upon hydrolysis and were of lower cytotoxicity compared with 5-FdUrd, showing more selective cytotoxicity toward APN/CD13-positive cells (HT-1080) than APN/CD13-negative cells (HT-29, MDA-MB-231). The authors concluded that these prodrugs target APN to cause tumor–cell-selective cytotoxicity due to 5-FdUrd release, the rate of which could be controlled by the structure of the ester linker.[81]

Remarks and conclusions on the use of differential enzyme expression as a basis for cancer therapy

Increased expression of various hydrolytic enzymes like peptidases, esterases and proteases has been described in several types of human malignancies, especially those characterized by fast-growing and aggressive phenotypes.[1] These enzymes include, for example, the cathepsins, MMP and plasminogen activators. High expression may be related to metastatic potential as a result of degradation of the basement membranes and extracellular matrix, in addition to activation of enzymes, growth factors and other proteases as part of the metastatic cascade.[1] Consequently, this may provide a basis for selective chemotherapy, and there are numerous examples in the literature.[84] Targeting MMP in disease treatment is complicated by the fact that MMP are indispensable for normal development and physiology, and by their multifunctionality, possible functional redundancy or contradiction, and context-dependent expression and activity. This complexity was revealed by previous efforts to inhibit MMP activity in the treatment of cancer patients that yielded unsatisfactory results.[82] The pro-drug concept has also been thoroughly investigated but so far the results are mainly restricted to preclinical studies. For example, Masquelier and co-workers synthesized a series of amino acid and peptide derivatives of daunorubicin (DNR) to serve as prodrugs activated by lysosomal proteases inside or in the close vicinity of tumor cells.[83] Similarly, targeting cells with a high expression of plasminogen activators, Carl *et al.* constructed peptide conjugates with increased *in vitro* selectivity using three different cytotoxic agents: (i) the antimetabolite acivicin; (ii) the alkylator phenylenediamine mustard; [84] and (iii) the anthracyclin doxorubicin.[85] Specific proteolysis-targeted prodrugs may also be exemplified by the work of Denmeade *et al.*, in which heptapeptide conjugates of doxorubicin,[86] 5-fluorodeoxyuridine[87] and thapsigargin[88] were studied. These derivatives were targeted to activation by the serine prostate-specific antigen.

Transmembrane proteases are widely expressed and participate in extracellular proteolysis (degradation of extracellular matrix components, regulation of chemokine activity, release of membrane-anchored cytokines, cytokine receptors and adhesion molecules) and influence cell functions (growth, secretion of angiogenic molecules, motility).[89] Recent attention has been focused on ADAM-17, membrane type 1 (MT1)-MMP, the ectopeptidases aminopeptidase N, dipeptidyl peptidase IV and angiotensin-converting enzyme, which appear to have a critical role in angiogenesis.[89] This review describes the prevalence and possible functions of APN in malignancy (primarily focused on solid tumors), as well as attempts to target the enzyme pharmacologically.

It is concluded that APN has been associated with a number of characteristics of the malignant phenotype, for example, cell proliferation, secretion, invasion and angiogenesis. The dysregulation of APN can be monitored by IHC of tumor tissue or, at least in some cases, serum analysis, and for several diagnoses appear positively correlated with clinical measures.

Furthermore, the use of APN as a target for anticancer therapy was discussed. The literature presents different approaches including direct inhibition of the enzyme, use as a peptide homing receptor in the malignant environment (e.g. CNGRC-coupled derivatives), or as an enzymatic activator of prodrugs. A few compounds from all classes have reached or are currently being evaluated in clinical trials. For Bestatin, several trials

doi: 10.1111/j.1349-7006.2010.01826.x

present promising data, but most studies are small and positive effects restricted to sub-group analysis. For others, like Tosedostat, NGR-TNF or J1, additional clinical data are likely to be presented within the coming years. While APN is associated with several characteristics of the malignant phenotype and repeatedly being described as expressed or over-expressed in human tumors, it also attributed with several other ''moon-lighting'' functions that may complicate the development of the true target-specific anticancer drug. The future will tell what can be harvested from this target regarding prognostic and diagnostic measures, as well as anti-cancer therapy.

Disclosure Statement

Peter Nygren, Rolf Larsson and Joachim Gullbo are co-founders and minor shareholders of Oncopeptides AB, a small Swedish research and development company currently investigating the effects of J1 in clinical trials.

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doi: 10.1111/j.1349-7006.2010.01826.x