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Review

The moonlighting enzyme CD13: old and new functions to target

Paola Mina-Osorio

The Feinstein Institute for Medical Research, Autoimmune Disease Center, 350 Community Drive, Manhasset, NY 11030, USA

Aminopeptidase N (CD13) is a widely expressed ectoenzyme with functions that do not always depend on its enzymatic activity: an aspect that has been overlooked. Numerous CD13-targeting tools have been developed in the last few years. Several of them are already undergoing clinical trials, and there are promising reports on the effectiveness of others in animal models of disease. However, their efficacy might be obscured by their effects on unrecognized functions of CD13, resulting in unexpected complications. The purpose of this review is (i) to discuss the various functions ascribed to CD13 and the possible mechanisms behind them and (ii) to consider some of the questions that need to be answered to achieve a better understanding of the biological relevance of these functions, a more precise interpretation of the results obtained after their manipulation and a more rational design of CD13-targeting agents.

CD13, a moonlighting ectoenzyme

Proteins recognized to have multiple functions have been termed 'moonlighting proteins' [1]. Ectoenzymes are perfect examples because the number of functions in which they are implicated is rapidly growing [2]. Here, a variety of functions of one member of this family, aminopeptidase N (APN, also known as CD13; EC.3.4.11.2), will be discussed, demonstrating that this protein can be considered a moonlighting ectoenzyme. Importantly, many of the functions attributed to CD13 have been observed after crosslinking with monoclonal antibodies (mAbs) [3,4], overexpressing or silencing the protein in various cell lines [5] or blocking its enzymatic activity with chemical inhibitors [6], but a CD13-deficient animal has only recently been described [7]. Therefore, although the *in vivo* relevance of many of these functions has not yet been determined, the interest in CD13 as a therapeutic target has been progressively increasing [8,9]. Surprisingly, one aspect that is rarely considered is the fact that CD13 is a multifunctional protein and thus its ligation or the inhibition of its enzymatic activity might result in complex and systemic effects. This is a common limitation of enzyme inhibition therapy, but it was not anticipated for CD13-targeting drugs because of a lack of knowledge of some functions of CD13. This problem might be exacerbated by the also unrecognized fact that enzymatic activity-dependent and -independent functions appear to act in concert. For these reasons, it is important to review the data on all putative and established functions of CD13 and to discuss the possible molecular mechanisms involved so that further investigation aimed towards the clarification of some of these aspects is stimulated.

Mechanisms of action

As shown in Figure 1, CD13 performs its known functions by one or more of the following mechanisms:

Enzymatic cleavage of peptides

CD13 is also named aminopeptidase N because of its preference for neutral amino acids. It removes N-terminal amino acids from unsubstituted oligopeptides, amide or arylamide, with the exception of peptides with Pro in the penultimate position [10]. The order of favored substrates is: Ala>Phe>Tyr>Leu>Arg>Thr>Trp>Lys>Ser>Asp>His>Val. CD13 belongs to the M_1 family of zinc metallopeptidases called glunzincins, which are characterized by the presence of the consensus sequence HEXXH and a glutamic acid (GXMEN motif) as a third zinc-binding domain [10].

Glossary

Angiogenesis: the process of developing new blood vessels. It is important in the normal development of the embryo, but it is also fundamental for tumor growth.
Chemokines: cytokines that activate and mobilize leukocytes. Most chemokines belong to one of four major subfamilies (i.e., the C, CC, CXC and CX3C subfamilies), and they are grouped according to the arrangement of the cysteine residues. Their receptors are designated by the chemokine subfamily name followed by the letter 'R' (e.g. CXCR1).
Ectoenzyme: an enzyme situated on the outer surface of a cell's membrane so that its active site is available to the exterior environment of the cell.
Endocytosis: a process of internalization of extracellular material (liquids or small solid particles) into various intracellular compartments by invagination of the cell membrane.
Fcγ receptors: receptors for immunoglobulins of the IgG subtype. They are expressed in most leukocytes and are responsible for the effector functions of antibodies. They are important modulators of the immune response.
Glycoforms: versions (isoforms) of a protein that differ on the type of glycan attached to them (i.e. two proteins would be of the same glycoform if they carried the same glycan). These differences in glycan composition occur during post-translational or co-translational modifications. Therefore, the amino acid sequence does not vary between glycoforms of the same protein.
Human coronavirus 229E (HCoV-229E): a species in the genus *Coronavirus* that is one of the major viral pathogens causing upper respiratory tract illness in humans. It belongs to the coronavirus phylogenetic group 1.
Inflammatory bowel disease (IBD): a group of chronic intestinal diseases characterized by inflammation of the large or small intestine. The most common types are ulcerative colitis and Crohn's disease.
Isoforms: different versions of a protein corresponding to differences in the amino acid sequence that originate from different gene loci, multiple alleles, different subunit interactions, different splice forms or different post-translational modifications.
Phagocytosis: the process by which cells ingest large particles, such as apoptotic cells or bacteria. The particle is internalized into a vacuole known as the phagosome.

Corresponding author: Mina-Osorio, P. (pminaoso@nshs.edu).

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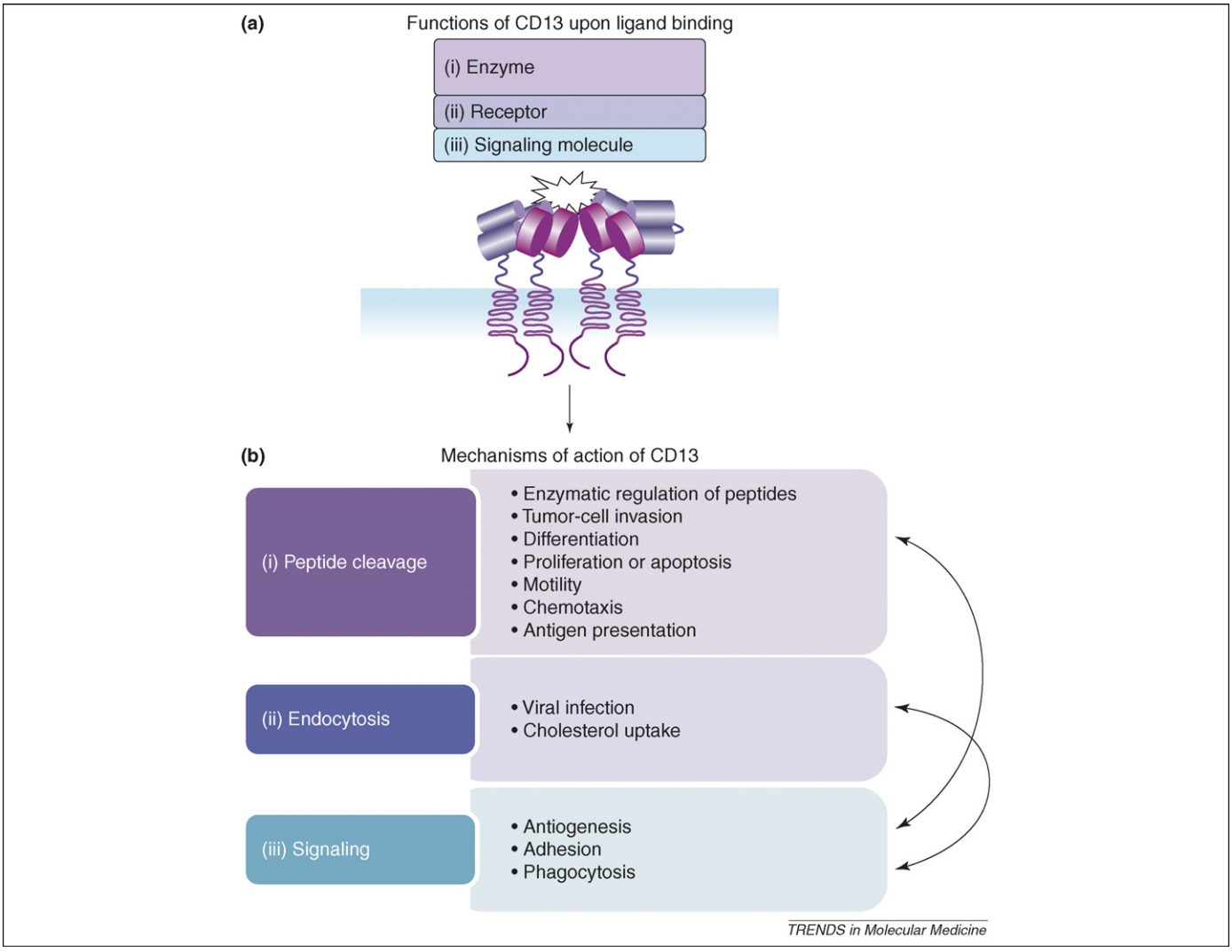

Figure 1. The functions and three mechanisms of action of human CD13. As shown in **(a)**, upon ligand binding, CD13 functions: (i) as an enzyme, (ii) as a receptor and/or (iii) as a signaling molecule. Each of these functions depends on at least one of the mechanisms of action listed in **(b)**, namely: (i) peptide cleavage, (ii) endocytosis and (iii) signal transduction. Each of these mechanisms result in the biological phenomena listed on the right side of part (b). Some complex phenomena, such as angiogenesis, invasion and chemotaxis, must occur as a result of the interplay between enzymatic activity and signaling functions. Similarly, upon virus or maybe even cholesterol and NGR-peptide binding (especially NGR-targeted liposomal drugs), the receptor functions could mediate signal transduction required for endocytosis. The functional interplay between mechanisms of action is represented by the arrows on the right of part (b).

Endocytosis

Some of the functions of CD13, such as the viral receptor function, either require or result in its internalization [11]. This phenomenon could explain some of the functions that are triggered by antibodies that do not block the enzymatic activity but that could reduce the membrane levels of CD13 [4] and, therefore, the aminopeptidase activity. Several molecules known to be associated with CD13 appear to regulate its trafficking as a mechanism to regulate its function [12,13]. The potential importance of endocytosis in the regulation of the functions of CD13 is underscored by the reported mutations, single site polymorphisms and differences in the splicing frequencies of the human CD13 gene in patients with hematological malignancies. Some of these mutations lead to altered trafficking of CD13 [14,15], which could have pathophysiological implications.

Signal transduction

Signal transduction has been proposed to account for some of the functions of CD13 that are independent of its enzymatic activity [4]. However, CD13 has been predicted to have a very short cytoplasmic domain that does not contain any known signaling motif (Figure 2 and Box 1). For this reason, it is believed that its signaling capacity depends on its association with an 'auxiliary' protein of unknown identity [16]. Several proteins recently reported to associate with CD13, such as galectin-3 [4], galectin-4 [13], RECK (reversion-inducing cysteine-rich protein with Kazal motifs) [12] and the tumor-associated antigen L6 [17], might constitute auxiliary molecules contributing to its signaling capabilities.

Crosslinking of CD13 in monocytes has been reported to induce mitogen-activated protein kinase (MAPK) phosphorylation, Ca^{2+} fluxing, cytokine secretion and cellular adhesion [3,4]. CD13 can be co-immunoprecipitated with the adaptor molecules Grb2 and Sos in U-937 cells [4], and a dramatic increase in the levels of phosphorylation of Syk upon co-crosslinking of CD13 with Fcγ receptors, as compared with crosslinking of Fcγ receptors alone, has been found [18]. This suggests that CD13 functions as a regu-

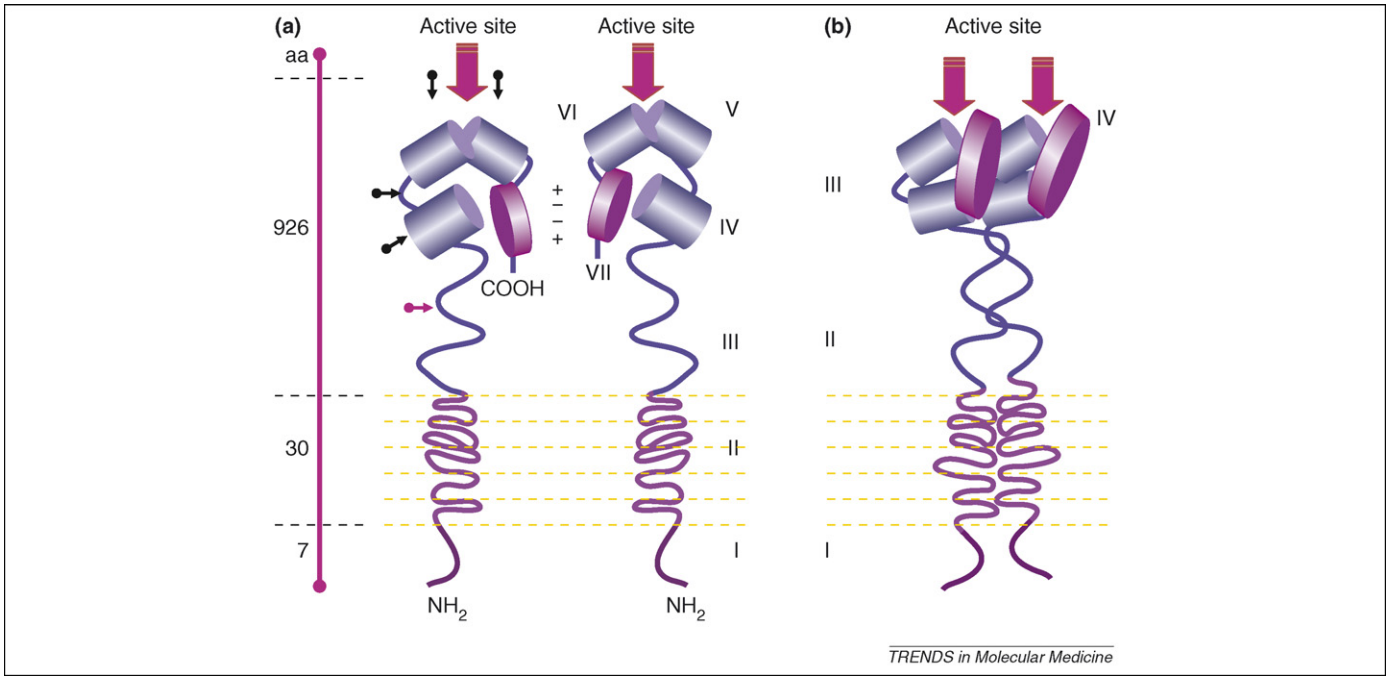

Figure 2. Hypothetical structure of human CD13. **(a)** Schematic seven domain organization of CD13. The active site is located between domains V and VI. Dimerization occurs between domains VII from each monomer, as indicated by ± signs. N-glycosylation sites are indicated with black arrows and O-glycosylation sites with pink arrows. Amino acids (aa) forming each domain are indicated on the left. Modified from Ref. [16], with kind permission of Springer Science and Business Media. According to the authors, the original model was based on electron microscopic studies of the purified enzyme, knowledge of the exon–intron organization of the gene and computer-aided structure predictions. **(b)** Schematic hypothetical four-domain model of human CD13, as proposed in Box 1. The possible conformational changes occurring upon substrate binding and the available data on the structure of other human enzymes were taken into account to modify the previous model. The active site (domain III) would be covered by the C-terminal domain (IV), and a conformational change would expose the catalytic site to allow the entrance of substrates. This process could be explained by the utilization, by substrates, of an initial recognition site such as the exopeptidase GXMEN motif, as has already been proposed for CD13 and other M_1 family members. Monomers are closely apposed to allow the predicted rollover mechanism occurring during the dynamic opening and closing of the active site, as proposed by Lendeckel *et al.* [92].

lator of signals triggered by other receptors, and as such it belongs to a group of molecules that we have termed signal regulators (SRs) [19].

A common observation when studying CD13-induced signaling is the epitope dependence of the signaling-inducing mAbs. These findings could be explained by the epitope complexity or the reported existence of distinct glycoforms of CD13 [3,4,20,21]. Also, although substrates are incapable of inducing signaling by themselves, they potentiate signaling induced by CD13 crosslinking (P. Mina-Osorio, unpublished results), implying that, when enzymatically active, CD13 becomes 'susceptible' for crosslinking by other ligands, possibly by the 'opening' of its constitutively closed conformation [22] (Figures 2,3 and Box 1).

The functions of CD13

The following discussion will mainly focus on the functions of the membrane-bound form of the human protein (referred to as hCD13). Some functional aspects of the soluble form of human CD13 (sCD13) are briefly summarized in Box 1.

Enzymatic regulation of peptides

By cleaving their N-terminus, CD13 regulates the activity of numerous peptides that participate in important biological processes, as summarized in Table 1.

Viral receptor

One of the best known functions of CD13 is its role as a viral receptor in several species. Thus, it is a receptor for human coronavirus 229E (HCoV-229E), porcine respiratory CoV, porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), feline enteric CoV (FECoV) and canine CoV (CCoV) [23]. The region of hCD13 between amino acids 288 and 295 has been identified as essential for HCoV-229E infection [24,25]. Enzymatic activity inhibitors or mutations of the active site do not affect virus binding to CD13. In most cases, CoVs of the serogroup 1 use CD13 as a receptor in a species-specific manner. However, feline CD13 serves as a receptor for all group 1 CoVs [26]. Differences in glycosylation between CD13 from the different species constitute one of the most important determinants for the species specificity of its receptor function [25].

Other reports have implicated CD13 in viral infection by human cytomegalovirus (HCMV). All mononuclear cells that are susceptible to HCMV infection *in vitro* express CD13 [27]. However, anti-CD13 antibodies block HCMV infection of CD13-negative and -positive cells, possibly by binding to a CD13-like molecule on the virion [28]. CD13 participates in the early events of HCMV infection and associates with HCMV particles during maturation, becoming immunogenic during infection. Consequently, autoantibodies against CD13 have been identified in patients with active HCMV infection and HCMV-IgG positive patients with inflammatory bowel disease (IBD) [29].

Tumor-cell invasion

There is a strong correlation between the expression and enzymatic activity of hCD13 and sCD13 and the invasive capacity of numerous tumor cell types [30,31]. Their value

Box 1. Structure, expression and their functional implications

CD13 is a heavily glycosylated, ~160 kDa, type-II membrane protein. The coding part of the human gene (*ANPEP*) is located in chromosome 15 (q25-q26) [87]. It is expressed in the renal and intestinal epithelia, in the nervous system (synaptic membranes and pericytes), in myeloid cells (monocytes, macrophages and DCs) and in fibroblast-like cells, such as synoviocytes [88,89]. Its expression in endothelial cells (ECs) has also been reported [90], although others have claimed that *in vivo*, it is only expressed by activated or angiogenic ECs [68,74]. These contradictory reports might be explained by the sensitivity of the detection techniques used (immunohistochemistry and binding of peptides) or by the presence of discrete glycoforms or conformations of the protein, which might not be recognized by certain reagents [20,72]. Indeed, via substractive proteomics, it has been found that CD13 is expressed in normal rat microvascular ECs and is upregulated in tumor vessels [91]. Additionally, by sequential immunoprecipitation and deglycosilation techniques, five different forms of CD13, corresponding to differences in oligosacharide composition, were found on ECs and monocytes [20].

Structurally, a seven domain organization of CD13 has been classically recognized (Figure 2a) [16]. Three-dimensional models of human APN/CD13 (hCD13) and the crystal structure of APN from *E. coli* have been published [92–95]. In the latter, a four domain organization was described in which the catalytic domain interacts with all other domains and the active site is inside a cavity covered by the C-terminal α-domain. This structure might not be shared by hCD13 because their sequence identity is low and hCD13 is a membrane-bound dimer. However, epitope mapping studies and mutations of the catalytic site of hCD13, as well as similar structures in other metallopeptidases of the M_1 family, support an interaction between domains and a buried active site [93,96]. A small entrance to the catalytic site would explain the restricted activity of hCD13 to short peptides (Figure 2b). With regard to the conformations that might distinguish CD13 in tumors from normal vasculature [72], distinct conformations depending on the relative positions of the monomers and their mode of interaction have been reported for rabbit and pig CD13, which are both dimeric, membrane-bound proteins [97]. Conformational changes associated with substrate binding might correspond to changes in the inter-monomeric spacing rather than on the intramolecular conformation of each monomer, and mAb crosslinking might trigger signaling because of the shortening in distance between monomers during oligomerization.

A soluble form of CD13 (sCD13) has long been recognized [98]. At a concentration of ~4.6 nM in human serum, it constitutes one of various N-terminal derivatives of its membrane-bound counterpart, processed by an unknown enzyme [99]. sCD13 blocks anti-CD13 mAb-induced adhesion [79], suggesting its potential regulatory role in membrane-bound CD13 functions. However, sCD13 is fully active enzymatically and thus its main role *in vivo* might be related to the cleavage of peptides at tumor or inflammation sites, where it has been repeatedly reported to be upregulated [100].

as prognostic indicators of many types of neoplastic diseases has also been demonstrated. Among the neoplasias in which CD13 has been reported to be overexpressed or its enzymatic activity altered are: skin, ovary, thyroid, lung, stomach, colon, kidney, bone and prostate tumors [32,33].

The mechanisms by which CD13 participates in tumor-cell invasion have been inevitably linked to its enzymatic activity. However, the invasive potential of tumor cells is also blocked by mAbs that are uncapable of inhibiting enzymatic activity. Signaling mechanisms have been implicated [34].

Differentiation

The role of CD13 in cellular differentiation has been deduced from at least three types of observations. First, CD13 is differentially expressed in discrete states of differentiation of normal and neoplastic myeloid cells. This phenomenon accounts for its well-recognized utilization as a diagnostic marker of certain types of leukemia and lymphoma. For instance, CD13 is expressed on the majority of leukemic myeloblasts in acute myeloid leukemia, and although it is expressed by lymphocytes at their earliest stages of differentiation, it becomes negative as these cells mature [35]. Second, CD13 expression is regulated by differentiation agents, such as phorbol esters, transforming growth factor-β (TGF-β), interleukin (IL)-4 and IL-10 [36]. Finally, chemical inhibitors, such as bestatin and leuhistin, are known to modulate cellular differentiation *in vitro*. Although the specificity for CD13 has not always been demonstrated, in many cases blockade of the binding of bestatin by anti-CD13 antibodies results in a complete abrogation of its effect on differentiation. The phenomenon depends on the induction of the expression or secretion of growth factor or cytokine receptors, probably by inhibition of the enzymatic processing of peptides involved in their regulation [37].

Additionally, there is recent interest in studying the role of CD13 on stem-cell differentiation [38]. This is a promising field in which CD13 targeting could be of potential value.

Proliferation and apoptosis

The capacity of hCD13 inhibitors and antibodies to suppress proliferation has been described in many cell types. For example, the inhibitors bestatin and actinonin and a mAb that inhibits CD13 enzymatic activity were reported to suppress the growth of choriocarcinoma and leukemia cell lines [39]. Cells expressing antisense CD13 were also reported to have slower growth rates than cells transfected with sense sequences [40].

Other reports on the effect of bestatin in cell proliferation have shown that its effect is reversible in human promonocytic U-937 cells and that, in some cases, administration of bestatin correlates with an upregulation of the membrane levels of CD13 [41]. In this regard, it has also been reported that high levels of hCD13 correlate with leukemic-cell resistance to apoptosis. This resistance is overcome by treatment with bestatin, suggesting an involvement of the enzymatic activity [42]. However, the intracellular concentrations of bestatin appear to be important for its effect on cell proliferation, suggesting a mechanism different from inhibition of membrane-bound CD13 [43]. Furthermore, bestatin appears to exert its effect on proliferation even in cells that do not express detectable levels of CD13. Therefore, further work is necessary to clarify the role of CD13 in cell proliferation because inhibitors of its enzymatic activity might act through CD13-independent mechanisms. However, the effect of mAbs and the reported regulation of CD13 expression by cell-cycle-related transcription factors [44] support a direct participation of CD13 in this process. Regardless of the mechanism, the clinical use of these chemical inhibitors might be hindered by their effect in cell proliferation and therefore this is an aspect that requires further investigation. Additionally, it has recently been shown that bestatin enhaces the sensitivity of cervical cancer cells to radiation, underscoring the

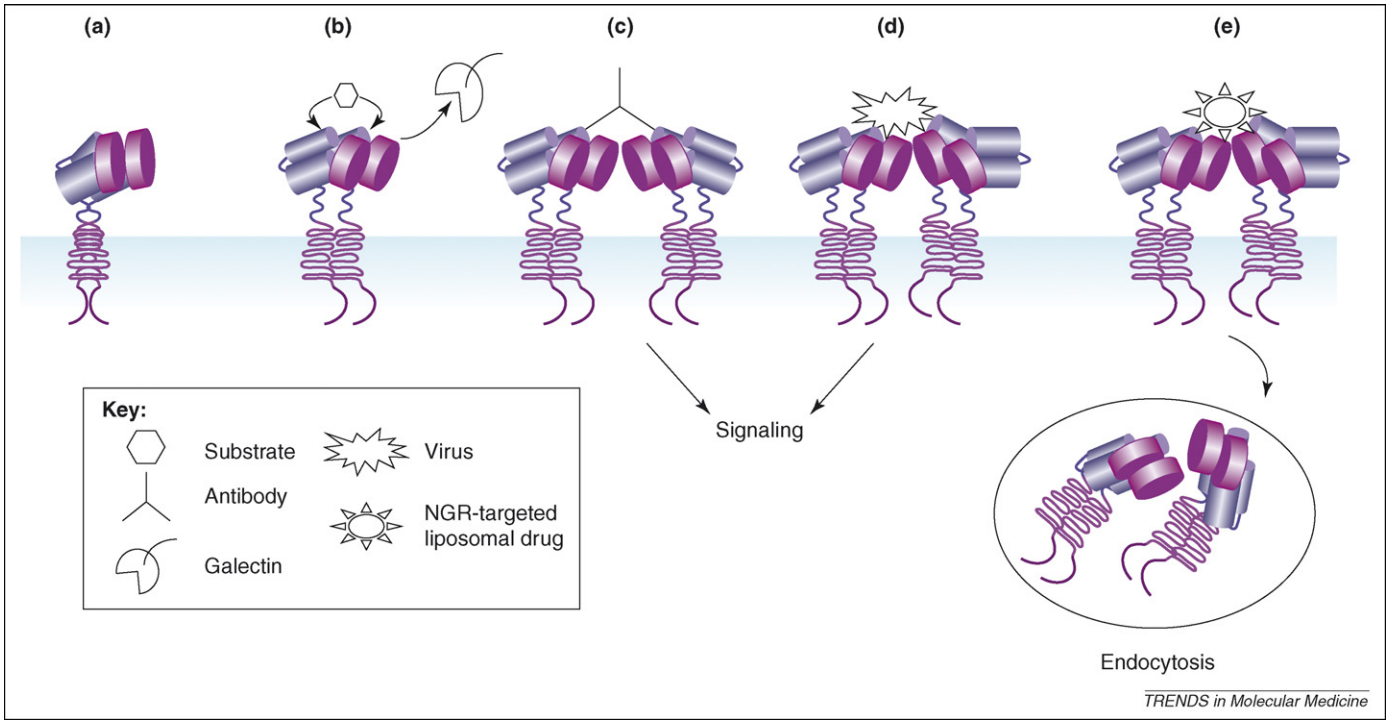

Figure 3. Possible structural modifications occurring upon ligation of CD13. **(a)** In the resting state, the CD13 enzymatic active site is cryptic (hidden). **(b)** Substrate or inhibitor binding to the initial contact site induces a conformational change that exposes the cryptic active site as well as other cryptic epitopes that lie close to it, resulting in activation or inhibition of the enzymatic activity [22]. This conformational change might also involve the release of galectins and, consequently, the concomitant unmasking of the carbohydrate moieties involved in ligand binding, which could explain the potentiation of signaling functions by substrates [78]. Upon the exposure of cryptic epitopes, anti-CD13 autoantibodies or ligands **(c,d)** bind to and crosslink CD13, resulting in oligomerization and signaling. In the absence of a pre-bound substrate, oligomerization by multivalent ligands, such as antibodies and viruses, might be sufficient to trigger signaling or internalization [3,4]. **(e)** Additionally, substrate, antibody or virus-associated proteins regulate the endocytosis of CD13 [4,11,12], which might constitute a mechanism of signaling and inhibition of its enzymatic activity and could be responsible for its dissociation from galectins and adaptor molecules. This would explain some of the functions modulated by mAbs that do not block the enzymatic activity.

potential importance of the regulation of proliferation and death-related phenomena by CD13 inhibitors [45].

Motility

CD13 is known to participate in tumor cell motility and in spermatozoid motility. In the first case, anti-CD13 mAbs significantly inhibit the migration of cancer cells such as lung cancer cells [17]. This effect correlates with the capacity of mAbs to crosslink CD13. Stable expression of membrane-bound CD13 on CL1-0 tumor cells or incubation with sCD13 greatly increases their migratory capacity. Cells expressing an enzymatically inactive mutant of CD13 also show increased migration, although they show less migration than cells expressing the enzymatically active form. CD13-targeting siRNA and highly specific chemical inhibitors block both the migration of endothelial cells (ECs) through matrigel and their motility during wound healing assays [5]. An anti-CD13 mAb was identified among mAbs raised against human fibrosarcoma cells and screened for inhibition of cell motility. It inhibits motility of human colon carcinoma, human lung carcinoma and human fibrosarcoma cells [46,47].

hCD13 has also been implicated in spermatozoid motility. CD13 accounts for 0.5% to 1% of the total protein in human seminal plasma [48]. Its highest levels were found in prostasomes, membranous vesicles that enhance motility of the ejaculated spermatozoa. A role for CD13 in motility was proposed because its catalytic activity on seminal cytokines and enkephalins, which are known to play a role in sperm motility, is altered in male infertility [49]. Additionally, it was reported that the aminopeptidase activity in sperm correlates with fertility and that the percentage of immobile spermatozoa negatively correlates with CD13 activity in sperm and prostasomes [50].

Chemotaxis

A correlation between the numbers of T lymphocytes and aminopeptidase activity in the bronchoalveolar fluid of patients with sarcoidosis has been reported. The levels of expression of CD13 are higher in alveolar macrophages from these patients than in control individuals, and purified CD13 induces lymphocyte chemotaxis *in vitro*, a phenomenon that can be inhibited by bestatin [51]. Similarly, CD13 participates in lymphocytic alveolitis after thoracic irradiation in rats, as well as in lymphocytic infiltration into the synovial fluid of patients with rheumatoid arthritis [52]. Importantly, CD13 degrades the chemokine CXCL11, downregulates CXCR4 and modulates stromal cell-derived factor-1 (SDF-1, also known as CXCL12)-induced migration [53,54]. Additionally, signaling triggered by CD13 crosslinking results in CXCL8 secretion, suggesting that enzymatic activity is not the only mechanism involved [3].

Antigen presentation

CD13 is frequently cited as an antigen-processing enzyme mainly because of early studies that implicated it in extracellular peptide degradation of synthetic class I peptides by

Table 1. Natural substrates and inhibitors

Substrate	General comments
Enkephalins	CD13 and, to a lesser degree, CD10 participate in the metabolism of enkephalins. CD13 hydrolyses the N-terminal Tyr-Gly bond of Leu-enkephalin and Met-enkephalin, which might account for the short half-life of these peptides *in vivo*. Although resistant to hydrolysis by CD13, the neuropeptide substance P inhibits enkephalin cleavage by CD13 [101].
Angiotensins	CD13 hydrolyzes the N-terminal Arg of angiotensin III to generate angiotensin IV. In normotensive and hypertensive rats, infusion of CD13 into the third ventricle causes lowering of blood pressure. Aminopeptidase inhibitors block this drop in blood pressure and increase vasopressin release as a result of the subsequent increase in the half-life of angiotensin III. Dietary salt regulates the expression of CD13 in the kidneys, and CD13 is more highly expressed in the kidneys of Dahl salt-resistant rats than in those of Dahl salt-sensitive rats. CD13 reduces basolateral Na^+/K^+ ATPase levels via angiotensin IV receptor signaling [86].
Tuftsin	The first peptidic bond of this immunoregulatory tetrapeptide is hydrolyzed by CD13, generating an antagonist that competes for receptor binding and thus regulating tuftsin functions [101].
Kinins	Both kallidin (lysyl-bradykinin) and its derivative Lys-des-Arg^9-bradykinin are CD13 substrates. The latter generates bradykinin upon hydrolisis [102]. Bradykinin is resistant to hydrolysis by CD13, acting rather as a natural inhibitor [101]. Intriguingly, the bradykinin inhibitor Icatibant is a CD13 inhibitor [103], and as such there is the danger of possible crosstalk between systems in which bradykinin-receptor targeting can result in an interfering CD13 inhibition.
Hemorphins	Because these peptides, derived from the β-chain of hemoglobin, share their N-terminal sequence with angiotensin IV (i.e. VY), they compete with it for the catalytic site of CD13, resulting in an inhibitory effect of CD13 activity on angiotensin IV [104].
Cytokines or chemokines	*In vitro*, CD13 hydrolyzes synthetic oligopeptides corresponding to the N terminus of IL-1α, IL-1β and IL-2, although natural IL-2 and G-CSF are resistant to hydrolysis [105]. Because hCD13 is believed to cleave small peptides only, its activity on cytokines or chemokines would require 'auxiliary' enzymes, as in the case of CXCL11, where initial removal of the NH_2-terminal dipeptide by CD26 allows for further truncation by CD13 [53].
Extracellular matrix proteins	Their high molecular weight makes it unlikely that CD13 alone is capable of degrading them. Nevertheless, at least two proteins have been proposed to be cleaved by CD13: entactin (nidogen) and type IV collagen [76,106]. Due to the potential relevance of this type of cleavage in tumor cell invasion, this assumption needs to be confirmed.
Other substrates	*In vitro,* CD13 participates in the metabolism of glutathione, somatostatin, thymopentin, neurokinin A, splenopentin, nociceptin (orphanin) FQ and peptides derived from the thrombin receptor [107,108].

dendritic cells (DCs) [55]. However, no direct evidence of CD13 involvement was presented.

Later studies have supported a role for CD13 in the extracellular processing (trimming) of antigenic peptides protruding from MHC class II molecules. Both peptidase inhibitors and anti-CD13 antibodies that block enzymatic activity impair antigen presentation. Intriguingly, antibodies that recognize an epitope different from the catalytic site are also effective [56]. The capacity of CD13 to degrade synthetic peptides on DCs, thus reducing their antigenic activity, was also shown [57]. In another report, pre-incubation of DCs with three different anti-CD13 mAbs resulted in inhibition of T-cell proliferation. At least one of these mAbs does not inhibit enzymatic activity [58], suggesting either that a different mechanism is involved or that enzymatic activity is modulated by mAb-induced endocytosis of CD13, as proposed above and in Figure 3. Further work is necessary to support a direct role for CD13 in antigen presentation.

Cholesterol crystallization

By isolating biliary vesicles from patients with cholesterol gallstones, a 130 kDa protein, subsequently identified as CD13, was shown to promote cholesterol crystallization *in vitro* [59]. Later, CD13 was identified as the major concanavalin-A-associated protein in the bile (concanavalin-A-associated proteins represent the major source of cholesterol crystallization agents) [60]. However, when using similar methods, other authors did not find such an activity for CD13, suggesting that the protein was misidentified as CD13 or that the attribution of crystallization-promoting activity to CD13 was incorrect. Nevertheless, the protein present in sera of patients with cholestatic diseases was later found to be a high molecular isoform of CD13 (260 kDa), which originates in the liver [61]. Later work has suggested that the role of cholesterol crystallization promoters *in vivo* is not as crucial in the development of cholesterol gallstones as other factors, thus undermining the possible relevance of CD13 in the pathogenesis of gallstone disease. However, its diagnostic value is still undeniable.

Cholesterol uptake

The function of CD13 in cholesterol uptake has stronger experimental support. It has been reported that CD13 is a target for Ezetimibe (Merck/Schering-Plough Pharmaceuticals), an inhibitor of cholesterol intestinal absorption [62]. Although another protein, Niemann-Pick C1-like 1 (NPC1L1), was proposed to be the main target for the drug [63], it has recently been reported that Ezetimibe is equally capable of reducing cholesterol uptake in NPC1L1-deficient and wild-type mice [64]. Binding of photoreactive Ezetimibe to a 145 kDa protein on enterocyte membranes was shown to be sufficient for the effect of the drug, and purification of this protein identified it as CD13. Inhibition of cholesterol absorption by binding of Ezetimibe analogs to CD13 does not affect its enzymatic activity. Instead, it has been proposed that CD13 participates in the endocytosis of cholesterol by enterocytes and that binding of Ezetimibe to CD13 could: (i) affect its association with lipid rafts, inducing its internalization; (ii) downregulate the expression of raft-associated proteins implicated in cholesterol uptake (e.g. Fcγ receptors and CD36); and/or (iii) interrupt the association of CD13 with Fcγ receptors or CD36 by inducing a conformational change [65]. Because a functional cooperation between CD13 and Fc receptors has been reported [18], a link between the role of CD13 on cholesterol uptake and Fcγ receptor function can be proposed. It is known that lipoproteins regulate Fcγ receptor expression and Fcγ-receptor-mediated phagocytosis. Thus, given the importance of cholesterol uptake by phagocytes in atherosclerosis and the fact that Ezetimibe blocks

low-density lipoprotein (LDL) uptake in macrophages, determining whether CD13 is a target for Ezetimibe in these cells is an interesting question, which is currently being addressed.

Phagocytosis

It was reported that the most actively phagocytic cells in the monocytic gate of peripheral blood mononuclear cells express more than twice the amount of CD13 as less phagocytic cells [66]. Moreover, induction of differentiation of these cells with 1,25-dyhydroxyvitamin D3, which increases the phagocytic capacity of monocytes, diminishes the expression of CD13 in cells with low phagocytic capacity but not in those with high phagocytic capacity.

It was later found that CD13 actively participates in phagocytosis [18]. It spontaneously redistributes to the zones of phagocyte–target interaction and is internalized into the phagosomes. Particles that simultaneously crosslink Fcγ receptors and CD13 are phagocytosed more efficiently than particles that crosslink Fcγ receptors alone. Interestingly, particles crosslinking CD13 alone are also efficiently phagocytosed, although saturating antibody concentrations are necessary, raising doubts about the potential *in vivo* relevance of CD13-mediated phagocytosis. This functional cooperation between a viral receptor (CD13) and the Fcγ receptors has important implications because Fc receptors participate in the entry of many viruses via antibody-dependent enhancement of infection.

Angiogenesis

Extracellular peptidases participate in angiogenesis by at least three mechanisms: (i) degradation of matrix proteins; (ii) generation of peptides with angiogenic and/or antiangiogenic properties; and (iii) modulation of signaling by growth and/or angiogenic factors [30]. CD13 is not an exception. Its role in the process of capillary tube formation and as a marker of angiogenic vessels has been extensively investigated. Studies using phage display technologies to identify tumor-homing peptides resulted in the discovery of the asparagine-glycine-arginine (NGR) peptide, which resembles the integrin-binding peptide arginine-glycine-aspartic acid (RGD) [67]. Coupling antitumor drugs to the peptides RGD-4C or CNGRC (Cys-Asn-Gly-Arg-Cys) resulted in increased efficacy and lower toxicity. RGD peptides bind to αv-integrins. However, the RGD-4C peptide does not compete for integrin binding with phages displaying the NGR motif, which suggested the existence of a different receptor for NGR. The sequence $W^{N}/_{D}$DGWL was identified as a peptide mimic of the binding site of the RGD peptide on integrins. The fact that this sequence is present in the extracellular domain of CD13 (amino acid 404) led to the discovery that CD13 functions as a receptor for NGR in tumor blood vessels and other vessels undergoing angiogenesis [67,68]. Several studies have demonstrated the dramatically improved capacity of various drugs to target tumor angiogenic vessels *in vivo* when coupled to NGR or NGR-peptide liposomes [69–71].

One potential caveat for the use of the NGR peptide is the fact that myeloid cells express high levels of CD13 and could potentially bind NGR. However, it was demonstrated that NGR–tumor necrosis factor (TNF) and certain CD13-specific antibodies bind exclusively to CD13 expressed in tumors and not to CD13 expressed in normal tissues. This suggested that a different isoform of CD13 is expressed in tumors, further underscoring the potential of the NGR peptide for tumor targeting [72]. Others have shown that although NGR–TNF does have some cytolytic activity towards CD13-expressing myeloid cell lines, this activity might depend on its binding to TNF receptors and not to CD13. However, the dose of peptide is critical. Thus, low doses of the drug permit efficient targeting without TNF-receptor-dependent toxicity [69]. Finally, the usefulness of NGR peptides as tools for detecting cardiac angiogenesis provides further evidence of the specificity of NGR peptides for angiogenic vasculature and expands their use to diagnostic applications [73].

Other groups have reported that hypoxia and angiogenic factors induce CD13 expression in ECs and that enzymatic activity inhibitors impair angiogenesis *in vitro* [74]. However, high doses of bestatin and antibodies were required, raising concerns over the potential therapeutic use of these tools. A recent publication addresses this concern, showing that continuous treatment with therapeutic doses of bestatin, but not other aminopeptidase inhibitors, results in abrogation of capillary tube formation, suggesting that the effect of bestatin is not specific for CD13 [6]. Nevertheless, many other studies have demonstrated the capacity of both bestatin and anti-CD13 antibodies to block angiogenesis *in vivo*, as well as the potential prognostic significance of CD13 in relation to angiogenic properties in some types of cancer [46]. Finally, CD13-null mice have unequivocally proven the importance of this molecule in angiogenesis [7]. These mice have dramatically decreased retinal neovascularization under hypoxic conditions, as well as reduced angiogenic responses to growth factors. One of the mechanisms involved appears to relate to the association of CD13 with galectin-3 in ECs [75]. Galectin-3 has been proposed as a therapeutic target for tumors originating from malignant endothelia. Therefore, the functional association between CD13 and galectin-3 underlines their importance for anti-angiogenic therapies.

Adhesion

During the characterization of an anti-CD13 mAb, it was observed that CD13 appeared diffusely expressed in cells growing independently but relocated to the zones of cell–cell contact at confluency [76]. Later, it was found that cell–cell contact induces the expression of CD13 in lymphocytes [77]. More recently, it was found that crosslinking of CD13 on human monocytes results in a strong homotypic aggregation (HA) phenomenon that is signal-transduction-dependent but independent of enzymatic activity. Crosslinking results in CD13 polarization to the leading edge of monocytes migrating towards the cells forming aggregates and its subsequent accumulation at the zones of cell–cell contact [4].

Expanding this work, it was found that CD13 is constitutively associated with galectin-3 in monocytes and dissociates from it during HA [78]. Ligation of the N terminus of galectin-3 results in a complete abrogation of CD13-induced HA. Additionally, by studying the role of

Table 2. CD13-targeting agents

Type	Examples	Major characteristics	Refs
Natural inhibitors	Bestatin (ubenimex), probestin, amastatin, phebestin, leuhistin, actinonin, curcumin, puromycin, betulinic acid	- Low toxicity - Broad spectrum - Poor tissue specificity - Poorly characterized effect on enzymatic activity-independent functions of CD13	[8,109]
Synthetic peptidomimetic inhibitors	β-Amino-thiols, α-aminophosphonates	- Low bioavailability and poor stability *in vivo*	[110]
Synthetic non-peptide inhibitors	Flavone-8-acetic acid (FAA) derivatives (e.g. 2′, 3-dinitroflavone-8-acetic acid)	- High specificity and potency - Low cytotoxicity to human cells in culture - FAA has already demonstrated poor antitumor activity in human tumors	[111]
Tumor-homing peptides	NGR	- Highly specific for tumor vasculature - Low immunogenicity	[72,85]
Cholesterol-lowering drugs	Ezetimibe	- Reduces intestinal absorption of cholesterol by an unknown mechanism	[62]
Recombinant antibodies	Single-chain Fv antibody fragments	- Specific binding without crosslinking - Targeting of hematological malignancies expressing CD13	[112]
Monoclonal antibodies	Enzymatic activity inhibitors (e.g. WM15), coronavirus infection inhibitors (e.g. Y2-K), adhesion/signaling-inducing Abs (e.g. 452)	- Binding to discrete glycoforms of the protein could be used for tissue specific targeting or for the design of recombinant antibodies - Some antibody clones could be used for signaling modulation or inhibition of viral infection	[20]

CD13 in adhesion of monocytes to ECs, it was found that both monocytic and EC CD13 are in the same complexes during adhesion and that purified recombinant CD13 mediates adhesion of monocytes [79]. Importantly, HCoV-229E induces similar effects as mAbs that block viral infection. Experiments treating mice with anti-CD13 mAbs in a peritonitis model demonstrate impaired transendothelial migration of leukocytes to the peritoneum [79], supporting a role for CD13 in leukocyte adhesion *in vivo*, as proposed for other ectoenzymes [80].

Concluding remarks

As discussed in this review and summarized in Figure 1, the functions of CD13 can be divided into three groups according to the mechanism of action involved: enzymatic cleavage of peptides, endocytosis or signal transduction. However, more than one mechanism is likely to be required for most of the functions. Additionally, there is evidence to suggest that binding of substrates, autoantibodies or viruses induces conformational changes and/or clustering of CD13 [22,79] that trigger signal transduction cascades and that might downregulate the enzymatic activity via endocytosis [4].

The multifunctionality of CD13 can be responsible for unexpected clinical complications, poor efficacy of CD13-targeting agents (especially chemical inhibitors) and misleading CD13-dependent biological phenomena *in vitro* because of: (i) modulation of signaling-dependent functions by enzymatic activity inhibitory compounds; (ii) undesired modulation of poorly characterized or unknown functions of the molecule; (iii) differential modulation of the same functions in different tissues; (iv) simultaneous modulation of different functions in discrete tissues; (v) requirement for simultaneous inhibition of other 'auxiliary' enzymes to obtain a significant effect; and (vi) undesired off-target effects because of binding to discrete isoforms of CD13 expressed in different cell types. All possible isoforms of the molecule that are likely to be differentially targeted by some agents have not been characterized exhaustively in humans. Finally, in some cases, the interplay between multiple functions of CD13 could result in an enhancement of the therapeutic activity of certain agents. For instance, CD13-dependent induction of apoptosis of tumor cells targeted with the NGR peptide coupled to a chemotherapeutic drug. Therefore, some of the functions of CD13 that could positively or negatively alter the outcome of its therapeutic targeting require further investigation. A summary of the major characteristics of some CD13-targeting drugs is presented in Table 2.

For these reasons, specific mAbs should be used along with chemical inhibitors for the study of CD13 functions *in vitro*. Several mAbs available are known to block virus binding [24], to recognize the isoform of CD13 expressed in tumors [72], to discriminate between some of the distinct glycoforms [22], to compete for binding with naturally occurring autoantibodies [29] and/or to trigger signaling [3,4], underlining their usefulness as ligand-mimicking tools. The expanding list of autoimmune diseases in which anti-CD13 autoantibodies and/or high levels of aminopeptidase activity are present [29,81,82] suggests their participation in the pathogenesis of these diseases. IBD and rheumatoid arthritis, for instance, could be interesting models in which to test the efficacy of anti-CD13 targeting tools, especially in view of the recent reports on anti-inflammatory effects of combined CD13/CD26 blockade *in vivo* [9,83].

For the time being, it appears straightforward to predict that the most promising approaches are those using CD13 to target drugs into tumor vessels instead of those directly modulating CD13 functions. The NGR peptide, when coupled to TNF-α, melphalan, doxorubicin, interferon-α and endothelial-monocyte-activating polypeptide II, has already been demonstrated to improve their efficacy *in vivo* [84,85]. However, although the current reports are

Box 2. Outstanding questions

- What is the structure of human CD13?
- What is the expression profile of human CD13 in normal versus diseased tissues?
- Are there distinguishably different glycoforms or conformations of CD13 in normal versus diseased tissue that can be used to improve the targeting properties of the NGR peptide or to design new targeting peptides?
- Does virus binding to CD13 trigger signaling cascades that modify the migratory or adhesive properties of the infected cells? Is this a common mechanism of coronavirus or other viral infections?
- Do CD13-specific autoantibodies participate in the pathogenesis of autoimmune diseases?
- How is the trafficking of CD13 regulated? Is ligand-induced endocytosis a mechanism of regulation of its enzymatic activity?
- What is the importance of the association between CD13 and proteins such as galectin-3 and galectin-4 *in vivo*?
- Does CD13 participate in the physiological uptake of cholesterol? Is the cooperation between CD13 and Fc receptors relevant for cholesterol uptake by monocytes in the atherosclerotic plaque?

promising both with regard to the low antigenicity of the peptide and to its specificity for tumor vessels, the potential of targeting other cell types, such as myeloid cells, has not been completely ruled out. New insights into these issues will hopefully result from basic research and clinical trials currently under development, in which understanding the mechanism of action of the drug will be a major goal.

Finally, except for some studies on the regulation of blood pressure and nociception by CD13 in rodents [86], many of the functions discussed here have been observed *in vitro* exclusively. Only recently have animal models of diseases, such as IBD and experimental autoimmune encephalitis, been utilized [83]. Therefore, given the importance of some of the functions discussed and the dramatic effect of CD13 manipulation *in vitro*, the next challenge for translational progress in the field will be to take these studies into *in vivo* systems to allow a better understanding of their real pathophysiological relevance, as well as more rational design of CD13-targeting drugs. Two of the most critical aspects yet to be studied (Box 2) are the three-dimensional structure of the human protein and the expression profile of the different glycoforms or conformations of the protein in normal and diseased human tissues.

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