

Inflammatory bowel diseases: multiple benefits from therapy with dipeptidyl- and alanyl-aminopeptidase inhibitors

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Expression and function of DPIV and APN on T cell subsets
 - 3.1. DPIV and related enzymes in T lymphocyte biology
 - 3.2. Aminopeptidase N and related enzymes in /on immune cells
 - 3.3. Cellular effects of inhibitors of Dipeptidyl peptidase and Alanyl-Aminopeptidase activities
4. Expression and function of DPIV, APN and related enzymes in the gut under physiological and pathophysiological conditions
5. Effects of Dipeptidyl peptidase and Alanyl-Aminopeptidase Inhibitors on bowel inflammation in mice
6. Putative implication of DPIV or APN inhibitors in other pathogenetic aspects in colitis
 - 6.1. Augmentation of intestinal repair mechanisms by induction and stabilisation of intestinotrophic factors
 - 6.2. Putative effects of Dipeptidyl peptidase and Alanyl-Aminopeptidase inhibitors on the brain-gut axis
 - 6.3. Antibiotic effects of Alanyl-Aminopeptidase inhibitors – influence on the intestinal microflora?
7. Conclusion and Perspective
8. Acknowledgements
9. References

1. ABSTRACT

Inflammatory bowel diseases (IBD) are driven by imbalances in innate and acquired immune response. In IBD two dysregulated T cell subsets are in the focus of interest: activated effector T cells and regulatory T cells. These T cell subsets are characterized by a strong expression of the ectopeptidases dipeptidyl peptidase IV (DPIV /CD26) and aminopeptidase N (APN/CD13), which are thought to a role in the control of immune activation and in regulating cellular communication by hydrolyzing bioactive polypeptides. Since inhibitors of both enzymes were shown to be effective in limiting immune activation processes *in vitro* as well as *in vivo*, they emerged as new drug candidates for the treatment of diseases associated with an imbalanced T cell response, such as IBD. In this review we intent to throw light on the putative role of DPIV, APN and related enzymes in the regulation of immune and non-immune processes in inflammatory bowel diseases, on possible benefits from peptidase inhibitor therapy in these diseases as well on the gaps of knowledge in this field.

2. INTRODUCTION

Inflammatory bowel disease comprises two clinically distinct disorders, Crohn's disease and ulcerative colitis. Both entities are characterized by chronic inflammation of the intestinal mucosa. While Crohn's disease is a systemic condition which may affect the whole gastrointestinal tract, in ulcerative colitis inflammation is usually limited to the colon. Although the etiology of both disease entities is still unknown, it became clear that it results from a complex interplay of genetic pre-deposition, environmental, microbial and immune factors (1,2).

The classification of Crohn's disease and ulcerative colitis as autoimmune disorders is still a contentious issue. However, the appearance of autoantibodies, such as tropomyosin- or anti-neutrophil cytoplasmic antibodies (ANCA) is a common characteristic of ulcerative colitis and is partially triggered by cytokine gene polymorphisms as found more and more in ulcerative colitis patients (3-4).

Whether the occurrence of autoantibodies represents an epiphenomenon secondary to epithelial injury or neutrophil degranulation or whether the identified autoantibodies indeed are pathogenic and contribute to mucosal damage may differ depending on the identity of the autoantigen (4-6).

The immunopathogenesis of inflammatory bowel diseases involves both, imbalances in the innate as well as in the adaptive immunity. Besides macrophages and neutrophils, high numbers of activated lymphocytes infiltrate the inflamed mucosa and drive the inflammation by producing high amounts of immunostimulatory cytokines (1,2,7). Previously Crohn's disease was thought to be clearly TH1-dominated, in contrast ulcerative colitis TH2-dominated. Due to newly discovered effector pathways (e.g. TH17 effector cells) this concept is rapidly changing. However, recent data suggest that mucosal T-cell polarization varies with the course of IBD (1,8). Current opinion is that the failure of protective control mechanisms, which render the mucosa associated immune system tolerant towards intraluminal microorganisms, is a key mechanism in the pathogenesis of IBD. This break of tolerance is associated with the triggering of the whole inflammatory cascade and finally with a damage of mucosal architecture and function (1,2).

As yet, the mechanisms maintaining the immunological tolerance toward the intestinal flora as well as toward nutritional antigens are not fully understood. At least three subsets of T cells with immunosuppressive properties are believed to be involved in these processes: The transforming growth factor-beta (TGF-beta) producing TH3 cells, the inducible interleukin-10 producing T cells, and the thymus-derived natural regulatory T cells (Treg) (9). In mice, the adoptive transfer of CD4+CD25+ regulatory T cells was shown to prevent or reverse experimental colitis (10), but little is known about their role in human inflammatory bowel disease. Recent studies suggest that in humans the numbers of regulatory T cell subsets are unchanged or elevated in the colon and associated lymphoid tissues of Crohn's disease or ulcerative colitis patients and are suppressive *in vitro* (11). It is assumed that *in vivo* the inflammatory microenvironment in the mucosa may abrogate their suppressive activity (11-13). However, clinical trials exploring the possibility of restoring regulatory T-cell functions *in vivo* in IBD and other inflammatory disorders are still awaited.

Taken all facts together, in IBD two groups of dysregulated T cell subsets are in the focus of interest: activated effector T cells as well as functionally impaired regulatory T cells. These T cell subsets are characterized by an upregulated expression of the two ectopeptidases dipeptidyl peptidase IV and aminopeptidase N, which were implicated to play a crucial role in the control of immune activation processes. The role of both enzymes in inflammatory processes in general as well as in the pathophysiology of inflammatory bowel diseases and their potential as target molecules for a therapeutic intervention in these disorders will be discussed in the following chapters.

3. EXPRESSION AND FUNCTION OF DPIV, APN AND RELATED ENZYMES IN T CELL SUBSETS

3.1. DPIV and related enzymes in T lymphocyte biology

Activated T helper cells were found to carry an ectopeptidase with unique enzymatic specificity, capable of catalyzing the cleavage of N-terminal dipeptides, if proline or alanine is in the second position. This highly glycosylated type II transmembrane enzyme was identified as dipeptidyl peptidase IV (EC 3.4.14.5) (14), firstly described as a kidney brush border serine proteinase (15). Functional analysis soon revealed that resting T cells express low amounts of DPIV at their surface, but the expression is strongly up-regulated upon T cell activation. Later, this enzymatic activity was linked to the previously described T cell activation antigens TA-1 in humans and THAM-1 in mice and finally clustered as CD26 (16-18). Besides T cells, B lymphocytes and natural killer cells showed an enhanced DPIV expression after activation (19,20).

Today DPIV represents the most prominent member of a growing group of proteins, called DASH for DPIV activity and structural homologues. With exception of some enzymatically inactive family members (DP6, DP10), the group is characterized by a similar substrate specificity and overlapping inhibitor profiles (21-23). Therefore, the total dipeptidyl peptidase-like activity of viable cells, cell or tissue homogenates, and biological fluids, as measured by using typical synthetic DPIV substrates, has to be considered as the net sum of several similar activities of unknown composition (including the intracellular located enzymes, since cells lacking surface DPIV show also hydrolysis of Gly-Pro-para-nitroanilide substrate). Since a quantitative assignment of activity portions to distinct members of the DASH family is difficult by the use of alternative substrates or variations of experimental conditions, the increasing availability of inhibitors highly specific for distinct DASH members will allow progress in this field. Using this approach, Maes et al. recently provided data on the expression of DP8 and DP9, in different leukocyte populations and leukocyte-derived cell lines. While these intracellularly located homologues of DPIV enzymes appear to be similarly expressed in all types of leukocytes, the expression of the lysosomal DP8 and the surface-bound DPIV was found to vary considerably (24). On mRNA level, DP8 expression was described to be elevated in activated T cells. A posttranscriptional regulation of expression has also been discussed (22). In contrast, recent quantitative PCR results from our laboratory provide no hint for an activation-dependent up-regulation of DP8 expression in isolated human CD3+ T cells stimulated by either mitogens, solid phase bound anti-CD3 mAb or anti-CD3 plus anti-CD28. In contrast, the mRNA amounts of the DP9 gene tend to be 2 to 3-fold increased upon activation in our hands (preliminary unpublished data).

The functions of cytosolic DP8, DP9 and other DASH members in physiology, pathophysiology, and in T cell activation processes in particular is still unknown. Redundant functions of several DASH members are highly

conceivable, since CD26 knock out mice were found to show an apparently normal phenotype (25, 26). This functional redundancy might also apply with respect to immune regulation since non-selective dipeptidyl peptidase inhibitors were found to be effective in reducing arthritis in DPIV deficient rodents (27). On the other hand, it has been shown that the number of lymphocytes of the CD4+ subpopulation is about 30% lower in the DPIV/CD26 knock out mice in comparison to the wildtype controls. Consistent findings were obtained in CD26 deficient F344 rat [28]. Additionally, the T cell proliferation rates in response to distinct mitogens were found to be reduced and the cytokine production seemed to be TH1-polarized in DPIV/CD26 knockouts. Moreover, in mice the DPIV/CD26 deficiency leads to an impairment of the immunoglobulin production (26, 29). In CD26 deficient rats IgE production was reduced in an ovalbumin-induced asthma model [28]. Additionally, studies in CD26 knockout mice revealed that CD26 plays a critical role in G-CSF-induced mobilization of hematopoietic progenitor cells (30).

Recently, the deletion of the DPIV/CD26 gene in mice was described to increase the susceptibility to experimental autoimmune encephalomyelitis significantly, strongly suggesting that surface-bound DPIV has a non-redundant function in controlling T cell activation. Moreover, DPIV seems to represent a key molecule in the limitation of T cell activation, since natural ligands of dipeptidyl peptidase IV induce TGF- β 1 production in T cells from wild type mice, but failed to elicit this effect in T cells from DPIV/CD26 knock out mice (31).

Based on its unique enzymatic specificity, DPIV and its activity homologues are faced with a multitude of substrate scenarios (32-36). Several chemokines, (e.g. SDF- α , RANTES), cytokines, peptide hormones as well as neuropeptides (e.g. substance P, neuropeptide Y) can be hydrolyzed with high efficiency. Not least because proline has significant impact on the three-dimensional structure of polypeptides, N-terminal truncation of these bioactive peptides affects has major impact on their biological activity (activation or inactivation, altered receptor specificities) (32). Recently published results demonstrate that the N-terminal truncation of CXCL-11 by concerted action of DPIV and APN results in a failure of CXCL-11 mediated signalling and the inhibition of lymphocyte chemotaxis but not in a loss of the desensitizing potential of the factor, suggesting that the activity of both enzymes is involved in the control of lymphocyte infiltration at the peak of inflammation (37).

The finding that the inactivation of the blood glucose level regulating peptide hormones is catalyzed by DPIV, led to the recent establishment of DPIV inhibitors as anti-diabetic drugs (25, 35, 38-40). Again, the involvement of other DASH members in the processing of bioactive mediators is still unknown and with respect to the intracellularly located enzymes such as DP8, DP9 and DPII questionable.

The surface-bound DPIV was reported to have additional functions probably independent from its

enzymatic activity (42,47). Since the molecule was found to interact with extracellular matrix components such as fibronectin and collagen DPIV was suggested to be directly involved in adhesion, migration and extravasation processes (42, 45). T cells highly expressing DPIV were described to pass endothelial layer without a chemokine gradient (47)

The relevance of the enzymatic activity of DPIV/CD26 for its function in T cell activation is a matter of controversial discussion with various reports supporting both the requirement of DPIV enzymatic activity for proper functioning on one side and the existence of effects that are independent thereof on the other (48, 49).

3.2. Aminopeptidase N and related enzymes in/ on immune cells

With respect to the above mentioned natural regulatory T cells, the expression of the ectopeptidase Aminopeptidase N (EC 3.4.11.2) is of particular interest. This zinc-dependent metalloprotease is identical with the myeloid lineage antigen CD13, but experimental work provided evidence that particularly CD4+CD25+foxp3+ regulatory T cells express significantly higher amounts of APN (50). Resting CD3+ T lymphocytes lack immunochemically detectable APN expression and the elevated expression of the enzyme on the surface was a matter of controversial discussion. Kunz, Wex, and Lendeckel et al. provided evidence for a T cell-associated APN expression on mRNA and protein level (51, 53). However, Riemann et al. reported an elevated surface expression of APN on activated T cells derived from local sites of inflammation or on tumor-infiltrating lymphocytes (54-56). Although the immunochemical detection of APN on the surface of lymphocytes protein by several monoclonal anti-CD13 antibodies revealed to be difficult, Western blot analysis of the plasma membrane protein fraction demonstrated the surface expression of APN on activated T cells (50, 55). However, the difficulties with the immunochemical detection of surface-bound APN are today believed to be caused by the localization of the enzyme in plasma membrane caveolae/ lipid rafts (31). Nevertheless, the enhanced APN gene transcription (mRNA) detected following TCR engagement or mitogenic stimulation correlated with the activation-dependent up-regulation of APN on protein level (57). Independent from its enzymatic activity, APN was shown to function as coronavirus receptor (58, 59).

APN is member of the M1 family of gluzincins (MA peptidase) family, which comprises at least 5 transmembrane enzymes and 4 non-transmembrane enzymes, sharing the aminopeptidase activity (60). With respect to immune functions the cytosolic alanyl aminopeptidase (cAAP; synonymous puromycin-sensitive aminopeptidase) and the leukotriene A₄-hydrolase (LTA₄H) are of particular interest and their contribution to APN-like activity always has to be taken into consideration, when aminopeptidase activities of cells and tissues are measured (50, 61, 62).

Table 1. Overview on features suggesting a co-function of the ectopeptidases DPIP and APN in immune activation

Features	DPIP/CD26	APN/CD13
Expression pattern	constitutive in kidney, liver and intestine upregulated on activated immune cells	constitutive in kidney, liver intestine and myeloid cells upregulated on activated T lymphocyte subsets
Subcellular localization	Type II transmembrane protein, partially lipid raft associated	Type II transmembrane protein plasma membrane caveolae/ lipid raft associated
Substrate specificity	X-Pro-↓X-X-X-...	X-↓ X-↓ X-↓ X-Pro-... stops if proline is in the second position; hence generation of DPIP susceptible substrates
Potential substrates	peptide hormones, neuropeptides, chemokines, cytokines	peptide hormones, neuropeptides, chemokines, cytokines
Cellular effects of ligands/ inhibitors	reduction of DNA synthesis	reduction of DNA synthesis
	reduction of the production of pro-inflammatory cytokines	reduction of the production of pro-inflammatory cytokines
	induction of TGF-beta1 production	induction of TGF- beta1 production
	modes of action probably dependent and independent from enzymatic activity	
Affected signalling pathways / molecules	MAP kinase p38, Src kinase p56 lck	wnt5a MAP kinase, Erk1/Erk2
Related enzymes	DPIP gene family members and non-members: DPIP/ QPP, DP8, DP9, FAP-alpha ¹ , Attractin	M1 family of MA clan of peptidases (gluzincins) e.g. cytosolic AAP (EC 3.4.11.3) LTA4 hydrolase ² (EC 3.3.2.6)

Abbreviations: ¹ fibroblast activating protein-alpha, ² Leukotriene A4 hydrolase

Like DPIP, aminopeptidase N and related enzymes are thought to contribute to the proteolysis of neuropeptides, angiotensins, immunomodulatory peptides and cytokines (63, 64). Since the enzyme is catalyzing the hydrolysis of neutral amino acids from the N-terminus of oligo-peptides, but stops hydrolysis if proline is in second position of the N-terminal sequence, it putatively generates DPIP-susceptible substrates. Due to their similar expression pattern, subcellular localization and their unique substrate specificities, both peptidases are believed to co-operate in the limited proteolysis of biological active proteins and thereby are assumed to synergistically modulate cellular communication. Table 1 provides a short summary of facts supporting such a co-functioning of both enzymes.

3.3. Cellular effects of inhibitors of Dipeptidyl peptidase and Alanyl-Aminopeptidase activities

Targeting DPIP or APN on activated T lymphocytes from mouse and humans by dipeptidyl peptidase or alanyl-aminopeptidase inhibitors was found to result in a suppression of proliferation without affecting cellular viability (65-69). Furthermore, the production of immunostimulatory cytokines by activated immune cells is strongly decreased in the presence of the reversible (substrate-mimetic) DPIP inhibitors (66, 67) or various APN inhibitors (69). Most interestingly, both the inhibition of DPIP and APN, led to an increased transcription, biosynthesis and secretion of the most potent immunosuppressive cytokine TGF-beta1, suggesting that both enzymes play a key role in limiting and terminating immune activation (70-72). Moreover, APN inhibitors were recently found to be capable of preserving and promoting the suppressive activity of CD4⁺ CD25^{bright} T regulatory cells (73).

The molecular mechanisms of these inhibitor-induced cellular effects are poorly understood as yet, but were shown to be involved in the modulation of various signalling cascades. Kähne et al. reported that inhibitors of DPIP inhibit early phosphorylation events in T cell activation (src kinase p56lck) and to affect MAP kinase p38 (74, 75). Anti-CD26 antibodies were shown to affects the

phosphorylation of several intracellular signalling proteins, too (76-79).

In case of APN the MAP kinases Erk1/2 and Wnt-signalling pathways appear to be involved (80, 81). However, the complex molecular mechanisms of DPIP and APN inhibitor effects may include altered interaction of the ectoenzymes with costimulatory molecules as well as alterations of biological signals mediated by truncated bioactive polypeptides.

Evaluating the literature on the cellular effects of various dipeptidyl peptidase inhibitors, it becomes evident that there are no clear correlations between the potency of distinct inhibitors toward the purified enzymes and their cellular effects on immune cells (82). This is comparable with the heterogeneous results obtained by investigating the effects of various anti-CD26 antibody clones, the effectivity of which was believed to depend on the recognized epitops (76-78).

The selectivity of various dipeptidyl peptidase inhibitors toward distinct members of the enzyme family was recently discussed to determine the effects on the cellular level (82). However, the number of selective inhibitors for individual dipeptidyl peptidase family members is still very limited and most of them are not available for the scientific community. In addition to class-specific effects of inhibitors of DPIP enzyme activity there might exist substance specific properties that define the bioavailability, half-life, and thereby determine the effectivity on the cellular or tissue level. Similarly, the induction of conformational changes after formation of the enzyme-inhibitor-complex might be of particular relevance for the cellular effects. The latter phenomenon might affect functions independent from the enzymatic activity such as interactions of DPIP with CD45RO or adenosine deaminase (ADA), and which are supposed to be important for DPIP induced signalling processes (83-85). Thus, at the current state of knowledge and based on few studies only it is difficult to define precise role and contribution of distinct enzymes.

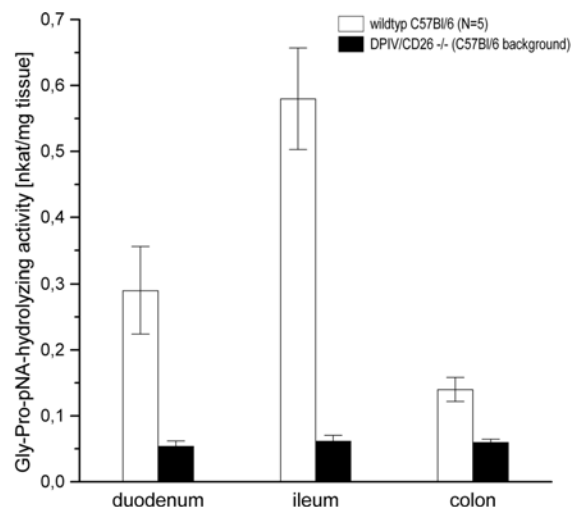


Figure 1. Intestinal tissue was obtained from 5 wildtype C57Bl/6 mice as well as 5 DPIV/CD26 (-/-) knockout mice, immediately shock-frozen and later homogenized. The specific DPIV-like activity per mg tissue homogenate was determined by using the substrate Gly-Pro-para-nitroanilide (conditions 25°C; 100mM TRIS-buffer, pH 7.4). The given data summarize the data from 5 animals (mean \pm SEM).

4. EXPRESSION AND FUNCTION OF DPIV; APN AND THEIR RELATED ENZYMES IN THE GUT UNDER PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONDITION

Among the wide variety of cell types, which express DPIV and APN constitutively, the intestinal epithelium is one with the highest expression rates. Both, DPIV and APN were identified as important brush border enzymes (86).

For several species, it has been shown that DPIV is detectable in the duodenum, strongly expressed in the jejunum and ileum, but weakly expressed or almost absent in the epithelial layer of the colon (87-89). In contrast, Young et al. described that DPIV and APN are also markers of the normal mature colonocytes in human tissue (90). Another report detected APN on epithelial cells in colon specimen from healthy human donors or in the surrounding colonic tumors, but a heterogeneous plasma membrane polarity of expression (91). On subcellular level, APN was found to be present in deep apical tubules, which have functions in membrane trafficking processes (92).

Both enzymes are believed to be constitutively expressed on intestinal epithelial cells, but several reports suggest a regulated expression. The expression of both, DPIV and APN on intestinal epithelial cells was found to be a characteristic of highly differentiated cells (87, 88, 93-94). Corresponding to this, the enzymatic activity of both enzymes was described to show an increase from the basal to the apical villus (95, 96).

Moreover, the expression rates of DPIV and APN on intestinal epithelial cells seem to be controlled at least in part by nutrition parameters. Starvation in rats as well as

protein and proline-rich diets were demonstrated to increase the DPIV and APN expression in the intestinal brush border (97, 98). Other data showed that the high protein intake evoked an increase in aminopeptidase activity, with a concomitant increase in the amount of immunoreactive protein (99).

Changes in the expression of both ectopeptidases in inflamed intestinal tissues from colitis patients were not reported as yet. Interestingly, in celiac disease as well as in patients suffering from malabsorption syndrome DPIV activity in the small intestine correlated inversely with the grade of mucosal damage (100). In the trinitrobenzene sulphonic acid (TNBS)-induced colitis model in rats, a reduced aminopeptidase activity in the jejunum and ileum was reported to indicate alterations in mucosal function in these intestinal segments (101).

Interestingly, some APN and DPIV-related changes in body fluids were reported for colitis patients. Hildebrandt et al. reported a reduced serum DPIV activity in colitis patients (102). APN and DPIV activities were found to be elevated in the urine of colitis patients probably indicating an extra-intestinal disease manifestation (103). Moreover, in the serum of colitis patients cytotoxic anti-APN/ CD13 auto-antibodies were detected (104).

To date, almost nothing is known about the expression and function of the other DASH family members in the intestine under physiological and pathophysiological conditions. The same holds true for other members of the gluzincin family with aminopeptidase activity. A recent comparison of Gly-Pro-pNA hydrolyzing activities in intestinal tissues of healthy C57Bl/6 wild type mice and DPIV/ CD26 knockout mice in our laboratory revealed that the total activity per mg tissue is reduced by more than 80% in the duodenum and ileum of DPIV knockout mice, suggesting that in these tissues the majority of the total dipeptidyl peptidase-like activity is assigned to the surface associated DPIV. In the colon of the DPIV knockout mice the total dipeptidyl peptidase-like activity is 50% lower than in same tissue from wild type mice (Figure 1).

Although the strong expression of DPIV and APN on intestinal epithelial cells is known since more than 30 years, the understanding of their function in the intestinal tissue is still limited. At the mucosal surface of the gut both exopeptidases are thought to play an important role in the final stages of protein digestion, in case of DPIV exclusively in the digestion of proline-containing peptides (105-107). Other functions of enzymatically active DPIV, APN or related enzymes on intestinal epithelial cell functions apart from this nutritional role have not been elucidated in detail as yet. Nevertheless, several studies suggest that both enzymes may have a regulatory role in the intestinal physiology and pathophysiology, too. Since many bioactive polypeptides including incretins represent substrates of DPIV and APN, both brush border enzymes are thought to be implicated in the regulation of food intake and metabolism (32, 33, 35, 108).

Independent from its enzymatic activity and contribution to the catabolic degradation of proteins, both enzymes involved in transcytotic transportation processes (106, 107). APN was suggested to be involved in cholesterol absorption via intracellular trafficking routes as well (108).

Furthermore, the DPIV and APN expressing intestinal epithelial cells hold a strategic position between the intestinal lumen and the mucosa, the importance of these cells for the mucosal immunity is widely unknown and a putative role of DPIV and APN in the regulation of mucosal immunity is a matter of speculation as yet. In vitro studies suggested a role of DPIV/ CD26 in the interaction between epithelial cells and mucosal lymphocytes. It was shown that the adhesion between intestinal epithelial cell lines such as Caco-2 or SKW6.4 and lymphocytes correlates with the DPIV expression level. The DPIV binding enzyme ADA seemed to be involved in the adhesion process (109). Additionally, the contact of T cells and intestinal epithelial cells via DPIV was shown to lead to signalling events and to the upregulation of integrins.

Moreover, the fact that proline-containing peptides, as generated in high amounts in the gut, may represent competitive inhibitors of DPIV activity, raises the interesting question whether DPIV activity and engagement in the gut could be involved in the maintenance of tolerance.

5. EFFECTS OF DIPEPTIDYL PEPTIDASE AND ALANYL-AMINOPEPTIDASE INHIBITORS ON BOWEL INFLAMMATION IN MICE

In 2006 a first study investigating the effects of dipeptidyl peptidase inhibitors on colitis was published by an Australian group (110). They could demonstrate that the DPIV inhibitors Ile-cyano-pyrrolidid and Ile-thiazolidid, administrated daily from experimental day 0 to 14, significantly decreased the colitis disease activity and helped to maintain intestinal integrity. However, both inhibitors were not capable of fully preventing the onset of colitis symptoms and showed a different potency to attenuate the colitis strength. Since DPIV/ CD26 knockout mice showed no change in susceptibility toward the chemically induced model of acute colitis (dextrane sulphate sodium; DSS), it has been proposed that other DASH members elicit a redundant role in this colitis model (111).

As already mentioned above, DPIV and APN (and/or their family members) are believed to co-operate in immune regulation (72). Based on the finding that synthetic inhibitors of both enzymes elicit additive to super-additive suppressing effects on DNA synthesis and proinflammatory cytokine production particularly at low inhibitor concentrations *in vitro*, a novel therapeutic strategy was proposed, which was termed Peptidase-targeted Immunoregulation (PETIR™). The hypothesized mechanism is the restoration and maintenance of immune balance directly by limiting T effector cells activation, and indirectly, by the induction of powerful endogenous

immunosuppressive mechanisms, such as TGF-beta1 and regulatory T cells (70, 73, 112). We were recently able to demonstrate that a daily intraperitoneal or oral application of DPIV and APN inhibitors after the onset of colitis symptoms (day 4 to 7) reduces disease progression in the DSS colitis model (113). While the dipeptidyl peptidase inhibitor Lys-Z-nitro-pyrrolidide or the aminopeptidase actinonin alone had marked, but non-significant therapeutic effects on the colitis activity, the combined inhibition of both activities elicited a significant improvement of the DSS-induced disease. A newly developed inhibitor with inhibitory capacity toward both enzyme activities significantly attenuated the clinical manifestation of colitis in the mice. In the colonic tissue of peptidase-inhibitor treated animal, the production of TGF-beta1 and foxp3 (on mRNA level) was found to be enhanced. With respect to a restoration of the normal mucosal tolerance, TGF- beta1 seems to be a key factor, since TGF- beta1 related gene defects result in an increased susceptibility to the onset of colitis in mice (114, 115). Moreover, TGF- beta1 augments the production of immunoglobulin (Ig) A, and decreases the production of IgG, IgM, and IgE (116).

The plasma levels of proinflammatory cytokines tend to be reduced in peptidase inhibitor- treated animals in comparison to vehicle treated control mice (no significance because of large intra-individual deviations and low animal numbers per group). Most interestingly, the plasma levels of IL-6 and IL-17 were detected to be significantly lower, indicating that the activity of detrimental TH17 cells can be attenuated by administration of the peptidase inhibitors. Moreover, the relative and absolute numbers of polymorphonuclear neutrophils in the circulation are normalized in peptidase inhibitor treated animals. Thus, the reduced inflammation in the gut is reflected by systemic inflammatory parameters, too. Since the systemic bioavailability of these new inhibitor molecules is limited, it has been hypothesized that the inhibitors act in a topical manner in the gut.

Recently, the stabilizing effect of alanyl-aminopeptidase inhibitors on the suppressive function of regulatory T cells could be also demonstrated in the DSS induced model of colitis in mice (117). The transfer of CD4+CD25+foxp3+ regulatory T cells isolated from a syngenic donor animal and treated for a short time with the alanyl-aminopeptidase inhibitor phebestin *ex vivo* was found to significantly reduce clinical symptoms in colitis mice. A single administration of the inhibitor alone had slight but not significant effects on the progression of the DSS colitis.

However, the chemically induced colitis models such as DSS and TNBS were controversially discussed with respect to the pathogenic role of T lymphocytes for the disease onset and progression. Although the intestinal inflammation in these models is associated with T cell activation secondary to the chemically induced epithelial cell damage, other animal models are discussed to be more suitable for investigating T cell related effects (118, 119). Further studies, for instance in the colitis model in IL-10

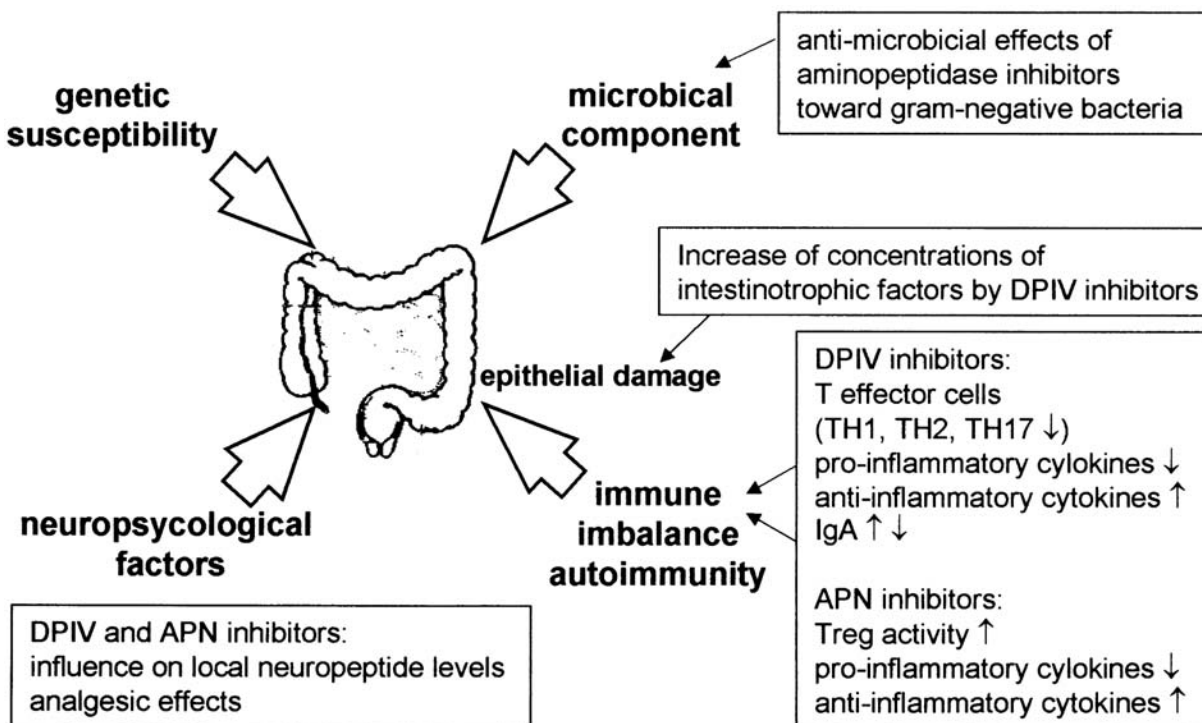


Figure 2. Overview on multiple effects of dipeptidyl peptidase inhibitors and alanyl-aminopeptidase inhibitors on various aspects of IBD pathogenesis.

knockout mice (120-122) are expected to provide more information on the potency of dipeptidyl peptidase and alanyl-aminopeptidase inhibitors as therapeutics for colitis patients.

Moreover, there is no information available yet on how potentially inhibitors that are selective for individual members of the DASH family perform in colitis models *in vivo*. All dipeptidyl peptidase inhibitors used so far in colitis animal experiments were found to be non-selective for DPIV, DP8, DP9 and other members of the DASH family (82). The same is true for the most alanyl-aminopeptidase inhibitors which are known to inhibit various alanyl aminopeptidase-like activities.

6. PUTATIVE IMPLICATION OF DPIV OR APN INHIBITORS IN OTHER PATHOGENIC ASPECTS IN COLITIS

As complex the pathogenesis of inflammatory bowel diseases is, as complex the conceivable effects of peptidase inhibitors in the therapy of colitis could be. Due to the demonstrated multiple effects of both ectopeptidases on cellular function and the bioactivity of chemokines, cytokines and peptide hormones *in vitro*, the effects of orally applied inhibitors in the gut might be manifold.

Since “hard” data on the molecular consequences of the administration of dipeptidyl- or alanyl-aminopeptidase inhibitors in colitis are rare, it might be presumptuous, to speculate in this field. However, the current literature provides first pieces of the puzzle and

gives an idea, which regulatory pathways apart from the immune response itself might be affected by the peptidase inhibitors. This information is of interest with respect to possible side effects of the peptidase inhibitors. The following short sections and the scheme (Figure 2) illustrate some of these putative mechanisms.

6.1 Augmentation of intestinal repair mechanisms by induction and stabilisation of intestinotrophic factors

Since the intestinal epithelium has an important barrier function an augmentation of intestinal repair mechanisms could be another approach for the treatment of patients with IBD.

As already known, both dipeptidyl peptidase and alanyl-aminopeptidase inhibitors and ligands were found to induce the production and secretion of TGF-beta1. Besides its immunosuppressive function, TGF-beta1 is supposed to play an important role in reconstituting of epithelial integrity after mucosal injury. Sakuraba et al. had shown that TGF-beta1 is capable of reducing the rate of intestinal epithelial cell apoptosis (123). Moreover, TGF-beta1 seems to regulate the migration of intestinal epithelial cells, since it promoted rapid “healing” of the epithelial cell monolayers through stimulation of migration of cells across the wound margin *in vivo* (125). The administration of neutralizing anti-TGF-beta1 antibodies was reported to exacerbate the DSS-induced colitis and to accelerate mucosal destruction *in vivo* (127).

Another intestinotrophic factor of interest is glucagon like-peptide (GLP)-2 which is produced by L-

cells in the intestinal mucosa (127,128). This peptide hormone consisting of 33 amino acids is known to represent a substrate of DPIV and its N-terminal truncation results in a loss of its growth-promoting activity. The administration of GLP-2 alone was reported to reduce the severity of colitis in rodents (129), but data from Hartmann et al. suggested that the administration of dipeptidyl peptidase inhibitors may enhance the intestinotrophic effects of GLP-2 (130). There are hints that various members of the DASH family may be involved in this mechanism (111, 112). Interestingly, the GLP-2-induced improvement of the intestinal wound healing seems to involve TGF- β 1 mediated effects (131).

A third example is Peptide YY (1-36) which has also intestinotrophic function (132). The N-terminal truncation by DPIV alters the bioactivity profile toward the inhibition of secretion (133), thus DPIV inhibitors should favour the intestinotrophic properties of untruncated peptide.

GLP-1 and GIP (gastric inhibitory peptide) are other intestinotrophic factors which carry the dipeptidyl peptidase susceptible N-terminal sequence (33, 134). Although both polypeptides were intensively studied since their blood glucose level regulating activity led to the development of anti-diabetic drugs, their relevance in intestinal inflammation is unknown as yet.

In conclusion, the administration of dipeptidyl peptidase inhibitors might become one of the therapeutic approaches which are directed at the improvement of intestinal epithelial functions in colitis patients.

6.2. Putative effects of Dipeptidyl peptidase and Alanyl-Amino peptidase inhibitors on the brain-gut axis

Psychological factors are today considered to be contributory, but not causative to the onset and the exacerbation of intestinal inflammation in humans and animals. A variety of neuropeptides, cytokines and hormones is discussed to be involved in the cross-talk between brain, enteric nervous system, intestinal mucosa and immune cells (135, 136) – many of them being recognized as potential substrates of DPIV and/ or APN (32, 33, 63). Since most of the key mediators involved in stress reactions, such as substance P, neuropeptide Y, vasoactive intestinal polypeptide (VIP) elicit both neuronal and immunological effects (135, 136), the possible consequences of changes in their bioactivity are difficult to assess.

Interestingly, the levels of substance P and VIP were found to be significantly diminished in biopsy specimen of patients with ulcerative colitis (137). VIP evokes a wide spectrum of biological activities relevant for the pathogenesis of colitis: It is involved in the regulation of the water and electrolyte secretion as well as in the glucose metabolism, controls the peristaltic reflex, and has stress-attenuating and immunosuppressive properties (138). Since the N-terminus of VIP is crucial for the VIP binding and a decreased VIP binding was found in the colon of colitis patients (139), inhibitors of dipeptidyl peptidases might be

helpful to prolong its half life and stabilize its protective effects on mucosal function and inflammation. The relevance of such mechanisms *in vivo* remains to be elucidated.

Substance P, which carries also a proline in second position of its N-terminal sequence, was shown to have a shortened half-life time after truncation of the N-terminal dipeptide (140). Substance P is a common neurotransmitter in the enteric nervous system. With respect to intestinal inflammation, substance P might have double-edged effects: On one hand, this neuropeptide has proinflammatory properties (141) and the administration of substance P to mice suffering from dinitrobenzenesulphonic acid (DNBS)-induced colitis was found to increase the number of activated lymphocytes in the lamina propria (142). Moreover, antagonists of the Substance P receptor NK (neurokinin)-1 receptor reduced the colonic inflammation in rats (143). On the other hand, substance P was recently proposed to be protective in colitis by inducing the intestinal wound healing via TGF- β dependent effects (144).

Remarkably, both dipeptidyl peptidase activity as well as alanyl-amino peptidase activity have been implicated in the regulation of pain via the inactivation of peptide mediators with analgesic/ anti-nociceptive effects such as endomorphin-2 (potential substrate of DPIV) or enkephalins (substrate of APN) (145-147). *In vitro* studies and *in vivo* experiments in rodents provided data on the impact of the ectopeptidase inhibitors on the analgesic effects of these mediators (148, 149). For instance, the alanyl-amino peptidase inhibitors actinonin has been described to elicit analgesic effects via the inhibition of enkephalin degrading activity of APN (150).

6.3. Antibiotic effects of Alanyl- amino peptidase inhibitors – influence on the intestinal microflora?

The gastrointestinal tract contains numerous species of intestinal bacteria, mediating detrimental or beneficial effects. Although the normal resident gastrointestinal microflora plays an essential role in the well-being of the host, abundant evidence indicates that the intestinal microflora has a role in the pathogenesis of inflammatory bowel disease (151-153). Pathogenic as well as commensal bacteria might represent drivers of dysregulated immunity and IBD. The composition of the gut microflora was found to be altered in IBD patients with increased numbers of aggressive pathogenic bacteria and decreased protective bifidobacteria and lactobacilli (154, 155). Therefore, the administration of antibiotics and introduction of probiotic species is successfully used in order to change the bacterial composition in the gut (156, 157).

A number of enteropathic bacteria strains (*Helicobacter spec.*, *Salmonella*, adherent/invasive *Escherichia coli*, *Bacteriodes*) belong to the gram-negative bacteria type (151, 153, 158-161). Gram-negative bacteria are characterized by a high expression of amino peptidase activity (162). The enzymatic activity of gram-negative bacteria is exploited for differentiating the bacteria types

since many years (163). In contrast, the Lactobacilli and Bifidobacteria which have been shown to have protective properties are gram-positive and lack aminopeptidase activity.

Interestingly, various well-characterized alanyl-aminopeptidase inhibitors such as actinonin, amastatin, bestatin, probestin and phebestin were originally discovered as antibiotics (164-169). In light of this data, it is highly conceivable that microbial effects of alanyl-aminopeptidase inhibitors could contribute to the attenuation of the bacterial overgrowth and dysbiosis observed in colitis patients by selectively inhibiting gram-negative pathogens and sparing the gram-positive strains with probiotic activity. However, the results of ongoing animal studies in several colitis models in our laboratory will provide further insights whether this beneficial "side" effect of peptidase inhibitors might gain relevance *in vivo*.

7. CONCLUSION AND PERSPECTIVE

The data obtained from experimental colitis models in mice suggest that DPIV and APN indeed represent interesting target molecules for a pharmaceutical intervention in intestinal inflammation. Besides the well-known direct effects on immune cells, several *in vitro* and *in vivo* studies provided conclusive evidence that inhibitors of both peptidases activities may provide further beneficial effects in inflammatory bowel diseases. Whether those mechanisms are of *in vivo*-relevance remains to be elucidated.

However, there remain gaps in the knowledge on the functional role of both ectopeptidases and the members of their growing families of related enzymes in the immune system as well in the intestinal pathophysiology. Further studies are necessary to better understand the contribution of distinct enzymes of the peptidase families in immune activation and other biological processes. Moreover, it has to be proven whether dipeptidyl peptidase and alanyl-alanyl-aminopeptidase inhibitors are also effective in other colitis models in rodents and whether the results are finally reproducible in human disease. Also unsolved is, whether dipeptidyl peptidase or alanyl-aminopeptidase inhibitors significantly affect the physiological functions of the brush border enzymes DPIV and APN. However, the development of competitive and reversible inhibitors as well as the use of intelligent drug release systems might limit the impact of the physiological function of these enzymes.

Moreover, the entry of the first DPIV inhibitors sitagliptin and vildagliptin into clinic now offers the opportunity to get first insight into the relative risk/benefit profile of DPIV inhibitors as drugs.

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Abbreviations: APN: Aminopeptidase N; CD: cluster of differentiation; DASH: DPIV activity and structure homologues; DPIV: Dipeptidyl peptidase IV; GIP: gastrointestinal peptide; GLP: glucagone-like peptide; IBD: inflammatory bowel diseases; IL: interleukin; TGF-beta: transforming growth factor-beta; TH: T helper; Treg: regulatory T cells; VIP: vasoactive intestinal peptide

Key Words: Dipeptidyl peptidase IV, CD26, Aminopeptidase N, CD13, Peptidase Inhibitors, Inflammatory Bowel Disease, Colitis, Review

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