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Regulating adaptive immune responses using small molecule modulators of aminopeptidases that process antigenic peptides

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Antigenic peptide processing by intracellular aminopeptidases has emerged recently as an important pathway that regulates adaptive immune responses. Pathogens and cancer can manipulate the activity of key enzymes of this pathway to promote immune evasion. Furthermore, the activity of these enzymes is naturally variable due to polymorphic variation, contributing to predisposition to disease, most notably autoimmunity. Here, we review recent findings that suggest that the pharmacological regulation of the activity of these aminopeptidases constitutes a valid approach for regulating human immune responses. We furthermore review the state of the art in chemical tools for inhibiting these enzymes and how these tools can be useful for the development of innovative therapeutic approaches for a variety of diseases including cancer, viral infections and autoimmunity.

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Intracellular aminopeptidases can regulate antigen presentation

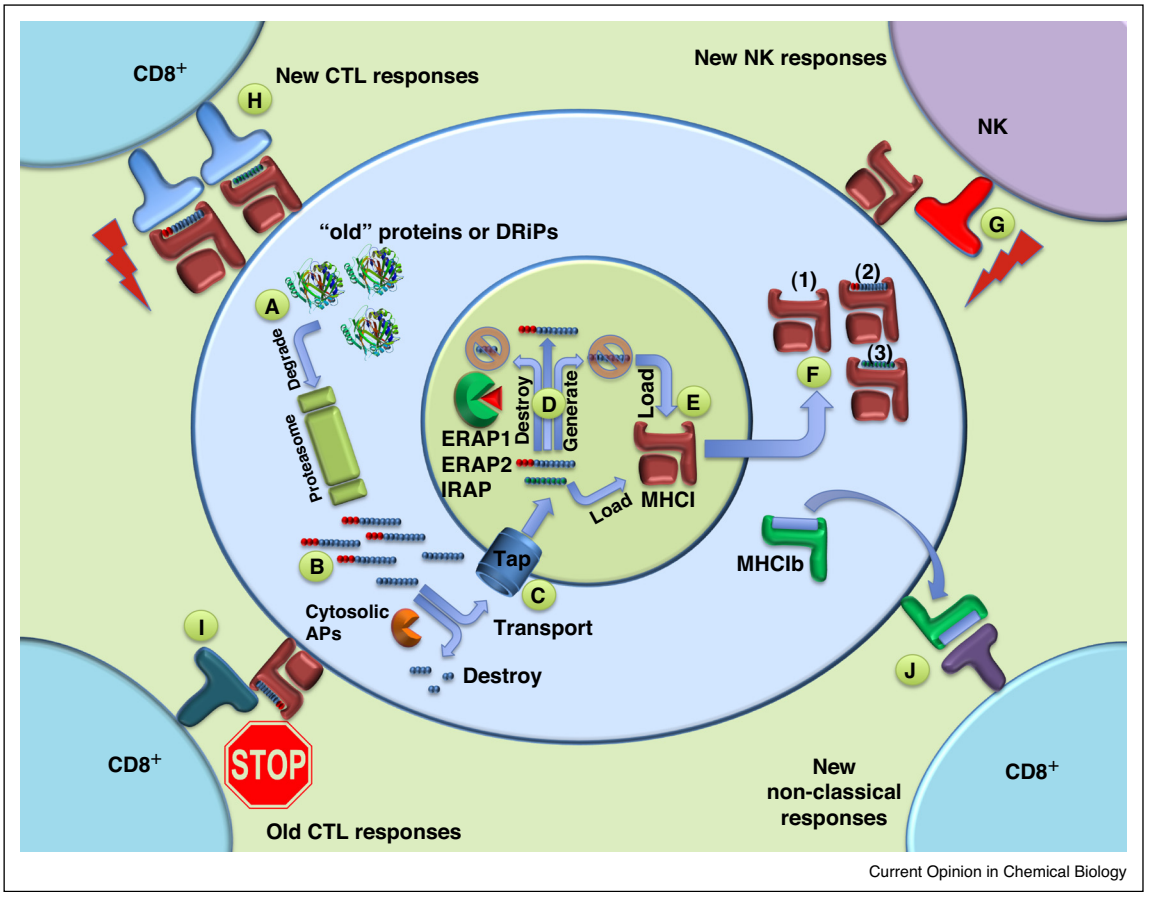
The recognition on the surface of somatic cells of short peptides bound onto specialized receptors of the Major Histocompatibility Complex (MHC) family of molecules is central to the human adaptive immune response. These peptides, called antigenic peptides, represent a sample of the protein content of the cell and are derived from the proteolytic digestion of either mature intracellular proteins or Defective Ribosomal Products (DRiPs) [1,2]. Infection or transformation of the cell results in changes in the surface presentation of antigenic peptides, changes that can be sensed by cytotoxic T-lymphocytes leading to cytotoxic responses against the diseased cell. The generation of antigenic peptides often starts at the proteasome but finishes in the ER or specialized endosomal compartments, by dedicated aminopeptidases that excise one or more N-terminal amino acids from elongated precursors of the antigenic peptides. This trimming function is non redundant for the generation of many antigenic epitopes [3,4]. This is in part due to the stringent peptide-length binding requirements of MHC class I molecules, which primarily bind peptides of 8–10 amino acids long. For the same reason, over-trimming of antigenic peptide precursors can lead to the generation of shorter fragments that can no longer bind onto MHC class I molecules, essentially destroying the antigenic peptide [5]. In this context, the aminopeptidase activity inside the ER can regulate antigen presentation, selecting which putative antigenic peptides will be presented on the cell surface and concomitant cytotoxic immune responses [6].

Three aminopeptidases have been identified thus far to be responsible for processing the N-terminus of antigenic peptides: ER aminopeptidases 1 and 2 (ERAP1 and ERAP2) and Insulin Regulated Aminopeptidase (IRAP) (for simplicity henceforth called Antigen Processing Aminopeptidases, APAs). All three are Zinc metallopeptidases, belong to the oxytocinase subfamily of M1-aminopeptidases, are highly homologous but have distinct substrate specificities [7,8]. ERAP1 and ERAP2 are found in the ER of all somatic cells, but can also co-localize in endosomal compartments of specialized antigen presenting cells (such as dendritic cells, DCs) performing antigenic peptide processing for cross-presented antigens. IRAP has been implicated in a distinct cross-presentation pathway of inflammatory DCs [9,10]. The activity of these three enzymes constitutes a regulatory node in antigen processing that may be exploitable for regulating antigen presentation and resulting cellular immune responses (Fig. 1).

The activity of APAs is naturally regulated

Several studies during the last few years have demonstrated that the activity of ERAP1 and ERAP2 can be regulated by pathogens as a means to evade immune responses. The expression levels of ERAP1 and ERAP2 are altered in tumor cells in a non-synchronous manner, possibly creating imbalances in the patterns of antigenic peptide processing that facilitate immune evasion [11]. Human cytomegalovirus (HCMV) expresses a specialized micro-RNA that down-regulates ERAP1 expression leading to lowered production of key antigenic epitopes and

Figure 1



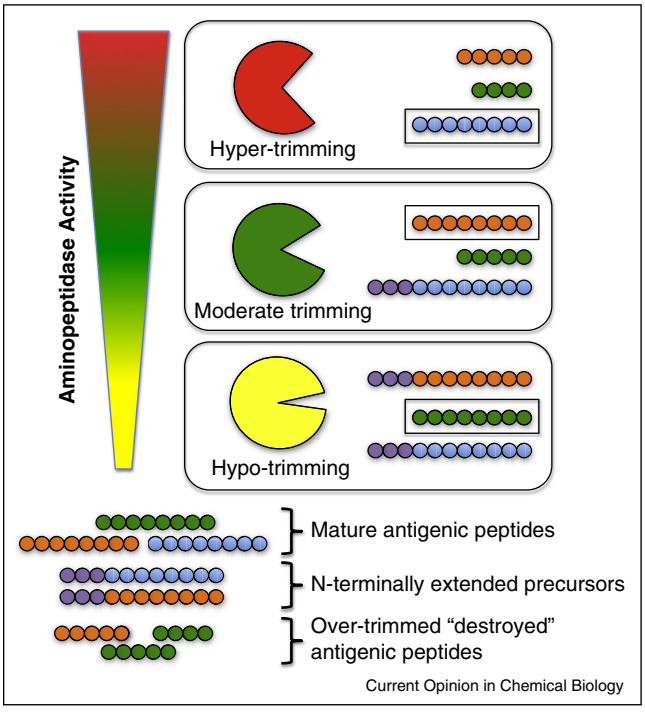
Possible effects of inhibition of APAs on antigen presentation and cellular immune responses. Proteins or Defective Ribosomal Products (DRiPs) are degraded in the cytosol (A) to antigenic peptide precursors, that often carry N-terminal extensions (B). Some of these peptides are destroyed by cytosolic aminopeptidases but some are transported by TAP into the ER (C) (or, in the case of cross-presentation, to specialized endosomes). There, the antigenic peptide precursors are further trimmed by APAs leading to the generation of correct length antigenic peptides for loading onto MHCI (D and E). Some antigenic peptides are over-trimmed and essentially destroyed. If APAs are inhibited, then processing is impaired leading to a reduced pool of antigenic peptides and sup-optimally loaded or even empty MHC class I (F, species 1) which can lead to activation of NK cells (G, upper right). Impaired ERAP processing can also spare peptides that are normally destroyed which are either of the correct length or elongated (F, species 2–3) leading to the generation of novel CTL responses (H, top left) or the abrogation of normal CTL responses (I, bottom left). Suboptimal loading can also lead to up-regulation of non-classical pathways through MHC Ib and concomitant $CD8^+$ responses (J, bottom right).

related cytotoxic responses and contributing to the virus' ability to evade the immune system and to establish long-term infections [12]. ERAP1 and to a lesser degree ERAP2 and IRAP, are naturally polymorphic and several coding single nucleotide polymorphisms (SNPs) in their genes have been associated with predisposition to disease, such as viral infections and inflammatory disease with autoimmune etiologies [13–15]. In an extreme example, one SNP in ERAP2 can lead to RNA decay and no protein expression, leaving homozygous carriers with a complete lack of ERAP2 [16]. Several studies have analyzed ERAP1 and ERAP2 SNPs and established that their presence alters enzymatic activity and sometimes selectivity and often result in dramatic alterations in antigen presentation (Fig. 2) [17,18•,19•,20,21•]. Specific patient haplotypes have been proposed to constitute either hyper-active or hypo-active versions of ERAP1 that contribute to disease pathogenesis [22••]. These studies taken together reveal that the enzymatic activity of ERAP1 and ERAP2 (and possibly IRAP) can vary greatly amongst individuals, contributing to the individual variability to fight infection, cancer and predisposition to autoimmunity. This variability of aminopeptidase activity is possibly a direct complement to the MHC class I variability and contributes to the immune variability of natural populations.

Inhibition of the function of APAs can lead to changes in immunopeptidome and elicit CTL, NK or non-classical responses

Several research groups have studied the effects of blocking ERAP1 expression. Knock-out murine models

Figure 2



Schematic representation of how alterations in APAs activity can change antigenic peptide generation and presentation. Moderate activity (middle panel) may be efficient in generating the orange colored epitope and destroy the green one, while not sufficiently processing the blue one. Hyper-trimming (top panel) can instead lead to the destruction of the orange epitope but sufficient generation of the blue one. Hypo-trimming (bottom panel) may fail to process and generate precursors of both the orange and blue epitopes while leaving intact the already mature green one. The final epitope that will be presented on the cell surface in each hypothetical case is different (indicated by a box).

have revealed profound changes in both the quality and quantity of generated antigenic peptides in the absence of ERAP1. Both the epitope immunodominance as well as the stability of surface MHCI molecules are affected revealing a non-dispensable role for ERAP1 in antigen generation [23,24]. Perhaps more importantly, lack of ERAP1 can lead to novel cellular immune responses manifested either as cytotoxic T-lymphocyte responses, Natural Killer cell responses or even non-classical MHC mediated responses (Fig. 1) [24,25••,26••,27•]. Interestingly, strong CTL and NK responses against cancer cells have been demonstrated in cellular and murine models in which ERAP1 expression had been down-regulated, opening up the possibility of using ERAP1 inhibition as novel approach for cancer immunotherapy [25••,26••,28]. Indeed, a newly developed potent ERAP1 inhibitor has been demonstrated to be sufficient to elicit anti-cancer CTL responses at nM dosages [29••]. In another study, ERAP1 genetic down regulation has been recently demonstrated to reduce CTL responses against a viral epitope presented by the MHC allele HLA-B27 that is associated with autoimmune reactions [30••]. Overall, inhibition of ERAP1 activity appears to be a valid approach to regulating adaptive immune responses.

ERAP1 and innate immunity

An additional function for ERAP1 has emerged the last few years that may complement its role in the adaptive immune response. Macrophages respond to stimulation by interferon-gamma and liposaccharides by secreting ERAP1 by a TLR-dependent mechanism [31,32•]. Secreted ERAP1 can in turn enhance the phagocytotic ability of these cells contributing to their inflammatory potential. Furthermore, ERAP1 knock-out mice display exaggerated innate immune responses and increased activation of NK and NKT cells [33]. These effects may contribute to the association between ERAP1 and autoimmunity and possibly extend the use of ERAP1-specific inhibitors to regulation of the innate immune responses.

Chemical inhibition of APAs for the treatment of disease

Although the down-regulation of APAs appears to have promising effects for the treatment of disease, the multitude of demonstrated effects suggests that any pharmacological approach should be performed with caution. Still, the strong effects of ERAP1 inhibition on the function of the adaptive immune response may be worth examining at the pre-clinical and clinical setting. Since the activity of ERAP1 appears to be commonly regulated by pathogens or polymorphic variation, reproducing this effect with chemical inhibitors may be a valid approach to: (i) enhance immune responses to viral infections or combat specific immune evasion mechanisms, (ii) reveal cancer cells to cytotoxic T-lymphocytes or coerce NK-cell mediated cytotoxicity and (iii) to down-regulate presentation of autoimmune-linked antigenic epitopes.

Inhibitors of APAs: state-of-the-art

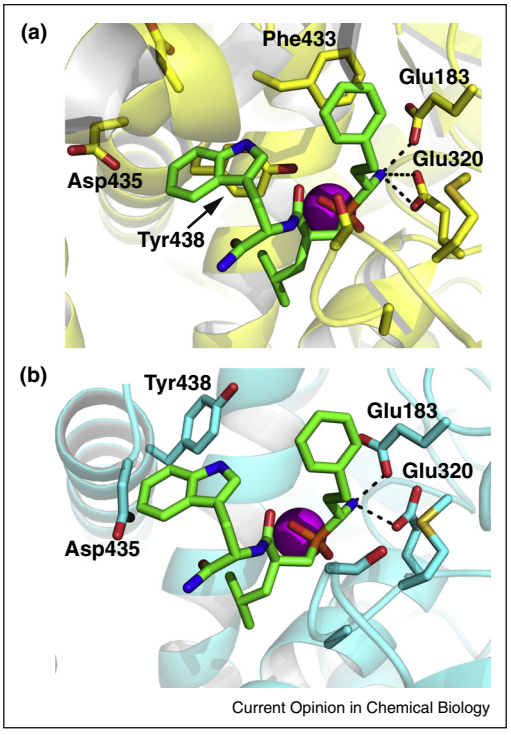
Given the importance of APAs in regulating the adaptive immune responses, it is surprising how little progress has been made in developing potent and selective inhibitors for these enzymes. The generic aminopeptidase inhibitor leucinethiol can inhibit all three APAs in the microM range but has very low selectivity and can be toxic to cells. Still, several studies have successfully used leucinethiol to reproduce effects seen by ERAP1 gene silencing [24,25••,26••]. Amastatin and bestatin, two commonly used aminopeptidase inhibitors, are poor inhibitors of ERAP1 [34]. Potent inhibitors have been developed for IRAP, but their evaluation has been limited to other biological functions of this enzyme [35,36]. Aminobenzamide derivatives were recently demonstrated as promising templates for the selective inhibition of APAs [37]. Recently, a phosphinic pseudopeptide transition analog was shown to be able to inhibit both ERAP1 and ERAP2 to nM levels and to enhance antigen presentation and

cytotoxic T-lymphocyte responses towards cancer cells, encouraging future studies of such inhibitors as tools for cancer immunotherapy [29••]. These compounds are good starting tools to help study the possible applications of pharmacological regulation of APAs activities on disease models.

Two conformations make inhibitor design a complicated matter

ERAP1 has been shown to be able to adopt at least two distinct conformational states that involve large inter-domain motions and expose a large internal peptide-binding cavity to the solvent [38–40]. The two conformations, termed 'open' and 'closed' also differ in the organization of key active site elements that influence enzymatic activity. In the closed conformation, a key active site residue is re-oriented to facilitate catalysis and specificity residues organize to form the S1 specificity pocket. In contrast, the open conformation is necessary to allow access of large substrates to the active site. Cycling between the two conformations is necessary for performing trimming of large peptides [39,40]. The conformational plasticity and lack of a well-formed S1 specificity pocket in the open conformation, make inhibitor development difficult (Fig. 3). Targeting the S1 specificity pocket is a common strategy for achieving both potency and selectivity when inhibiting aminopeptidases, and the poorly formed S1 pocket in the open conformation of ERAP1 could make targeting difficult especially compared to off-target aminopeptidases that have a pre-formed S1 pocket. Optimizing interactions so that inhibitors can promote S1 pocket formation or avoiding the S1 pocket altogether may be necessary for optimizing inhibitor potency and selectivity. Although ERAP2 has only been crystallized in an apparently closed conformation, complete lack of active site access to the external solvent in that conformation suggests that ERAP2 may also have an open conformation and thus present similar inhibitor development problems as ERAP1 [41•].

Figure 3



Molecular model of the phosphinic pseudopeptide inhibitor DG013A (in green, taken from the structure with PDB code 4JBS) on the two known conformations of ERAP1 (PDB codes: 3MDJ and 2YD0) reveal changes in the enzyme's active site that complicate inhibitor binding and design strategies. Two key residues for substrate recognition and catalysis that should interact with the inhibitor in the closed state of ERAP1 (panel A, residues Tyr438 and Phe433) are either disordered or dislocated in the open state of ERAP1 (panel B). Another nearby residue, Asp435 is re-oriented so that it may interfere with inhibitor binding. Still, the open state of ERAP1 has to be taken into account since it may be the ERAP1 conformation first encountered by the inhibitor.

Issues with selectivity

Selectivity of inhibition outside and within the APAs may be a crucial component in the effectiveness of compounds in regulating immune responses. The non-redundant roles of ERAP1, ERAP2 and IRAP may necessitate the development of highly selective inhibitors that can manipulate targeted functions in antigen processing and have fewer off-target effects. Selective inhibitors for IRAP have been described [35] but not evaluated for their effects on immune responses. The most potent ERAP1 inhibitor described is also a potent ERAP2 and IRAP inhibitor [29••]. A different approach for designing inhibitors of APAs has shown promise in selectivity but currently lacks potency [37]. The high homology between the three APAs could be a significant hurdle to overcome; still, selectively inhibiting only a subset of APAs could be a potent approach in limiting dangerous side-effects linked to dramatic changes in antigen presentation.

Inhibiting cytosolic peptidases

Cytosolic production of antigenic epitopes or elongated precursors exposes them to a slew of aminopeptidase activities that can in theory contribute to mature epitope production or destroy the epitope altogether. Surprisingly, genetic studies have shown a sporadic role for cytosolic peptidases in antigen generation [42–44]. The generation of N-terminally extended versions of mature antigenic peptides by the immunoproteasome has been hypothesized to aim to enhance the longevity of the precursors in the cytosol before they can be transferred into the ER [45]. Still off-target effects on cytosolic aminopeptidases have to be carefully assessed when trying to control antigen generation by inhibiting ERAP1 or ERAP2. Despite the limited role of cytosolic aminopeptidases on antigen processing, down-regulating their

activity can also have unwanted off-target effects on the health of the cell and should be avoided if possible.

Partial inhibition of the activity of APAs may be safer

The wide possible applicability of APAs inhibition in the treatment of disease may carry significant risks. Any effort in modulating antigen presentation by regulating intracellular antigen processing may also have dangerous side effects. For example, complete inhibition of ERAP1 activity may effectively reveal tumor epitopes to CTLs helping to eradicate cancer, but may also enhance the presentation of autoimmunity-promoting epitopes in bystander cells. Partial inhibition or targeted delivery may ameliorate such side effects. However, the natural variability of ERAP1 activity may hold the key to therapeutic approaches. Since lowered ERAP1 activity can be tolerated in the population, partial inhibition of ERAP1 may still generate the desired changes in CTL or NK responses without strong bystander effects.

Activation instead of inhibition

Given the pre-existing experience of the scientific community in developing metalloprotease inhibitors and the well characterized biological effects of genetic deletion of APAs it is reasonable that initial efforts have been focused in inhibitor development. The variability of naturally occurring activities due to polymorphic variation and the down-regulation of APAs expression levels by pathogens attempting to evade immune responses, suggests however that inhibition may not always be desirable. A fundamental property of ERAP1, to be self-activated by larger peptides, may provide an alternative route to control antigen processing [39]. Unfortunately, peptide-mediated ERAP1 activation has only been demonstrated for small model substrates and is therefore not useful for the enhancement of large antigenic peptide trimming. To solve this problem, our group recently identified a small molecular weight compound that can enhance ERAP1 activity for trimming large antigenic peptides [46]. Such activators, used in complementation with inhibitors may be useful for fine-tuning ERAP1 activity to correct over-active or under-active phenotypes related to disease (Fig. 2).

Immunopeptidomics and inhibitors of APAs

During the recent years, proteomic technology has sufficiently matured to allow the detailed analysis of the sum of peptides bound onto MHC, collectively called the immunopeptidome [47]. This approach has proven powerful in highlighting the immunopeptidome as a key marker of the cellular state and its environment as well as helping decipher disease mechanisms [48]. Significant changes in the cell-surface immunopeptidome due to ERAP1 knock-out have been described and include the presence of previously absent antigenic peptides, the eradication of normally presented peptides as well as the presentation of unstable MHC class I complexes [24,49]. Such analysis may be highly relevant for gauging the effects of APAs inhibitors or activators. Titration of inhibitors may reveal gradual changes to the immunopeptidome that can be exploited for therapeutic approaches.

Inhibiting APAs activity is a personal matter

The natural variability in ERAP1 and ERAP2 activity due to polymorphic variation necessitates that any pharmacological approaches to regulate their activity to 'correct' the immunopeptidome, are evaluated on a personalized medicine level. Functional analyses have indicated that different ERAP1/ERAP2 haplotypes vary in both function and in their ability to predispose individuals to disease [13,14,22••,50]. For example, inhibition of ERAP1 or ERAP2 activity could be beneficial for therapeutic purposes but only if the individual carries hyperactive alleles. In the case of individuals carrying hypoactive alleles, using an activator would be necessary. As a result, any attempt to regulate aminopeptidase mediated antigen processing would have to take into account the underlying genetic haplotype. This is reinforced by the recent finding that even the affinity of known inhibitors can be affected by the polymorphic state of these enzymes ([18•] and unpublished data).

Concluding remarks

The pharmacological regulation of the enzymatic activity of APAs is a rapidly emerging field with promising therapeutic applications. During the last few years, the field has sufficiently matured to allow for the development of the first generation chemical tools that are now starting to be evaluated on disease models. Although progress has to proceed with great caution due to possible side-effects, the development of therapies that rely on manipulating antigen processing to either coax the immune system to better fight pathogens and cancer or to reduce exposure of autoimmunity-related antigens may prove to be a novel paradigm in immunotherapy.

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