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REVIEW ARTICLE

# Inhibitors of ER Aminopeptidase 1 and 2: From Design to Clinical Application

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**Abstract:** Endoplasmic Reticulum aminopeptidase 1 and 2 are two homologous enzymes that help generate peptide ligands for presentation by Major Histocompatibility Class I molecules. Their enzymatic activity influences the antigenic peptide repertoire and indirectly controls adaptive immune responses. Accumulating evidence suggests that these two enzymes are tractable targets for the regulation of immune responses with possible applications ranging from cancer immunotherapy to treating inflammatory autoimmune diseases. Here, we review the state-of-the-art in the development of inhibitors of ERAP1 and ERAP2 as well as their potential and limitations for clinical applications.

**Keywords:** Immune system, antigen, peptide, enzyme, inhibitor, cancer, autoimmunity, infection.

## 1. INTRODUCTION

### 1.1. Intracellular Antigen Processing by ERAP1 and ERAP2

Major histocompatibility complex class I molecules (MHCI, HLA in humans) present antigenic peptides to the cell surface so that they can be recognized by CD8+ cytotoxic T-lymphocytes (CTLs). When a cell is infected or otherwise aberrant, CTLs can detect the changes in presented peptides and induce cell death [1]. Antigen processing often begins in the cytosol where cellular proteins are digested by the ubiquitin-proteasome pathway [2] The proteasome determines the C-terminus of antigenic peptides but often generates N-terminally extended precursors [3] The transporter associated with antigen processing (TAP) [4] can translocate these precursors into the ER, where they are subjected to further N-terminal trimming necessary to optimize MHCI binding [5] This trimming process is catalyzed by ERAP1 and ERAP2, two interferon-γ induced aminopeptidases that reside in the ER and belong to the oxytocinase subfamily of M1 aminopeptidases [6]. ERAP1 is a critical component of MHCI antigen presentation pathway. siRNA or RNAi mediated downregulation of ERAP1 in murine cells, results in altered MHCI expression on cell surface [7-9]. Moreover, ERAP1 down-regulation has been found to enhance immunogenicity due to the presence of unstable MHC-peptide complexes [10]. Additionally, *in vivo* experiments using ERAP1 knock-out mice showed strong shifts in the hierarchy of immunodominant viral epitopes and elicited altered T cell responses [11] Despite the dominant role of ERAP1 in epitope trimming inside the ER, a concerted action of ERAP1 with ERAP2 has been also proposed to be important for optimal epitope production [12-13]. Recent immunopeptidome analysis studies have demonstrated that both ERAP1 and ERAP2 can alter the repertoire of MHCI bound peptides, suggesting that their function can complement or even override MHCI peptide selection [14-18].

### 1.2. Roles in Disease and Polymorphic Variation

ERAP1 and ERAP2 have been linked to the pathogenesis of human disease on both the genetic and functional levels [19-20] Experimental evidence for this is based on genome-wide association studies as well as studies on knock-out experimental animals or silencing in cell lines. The emerging consensus is that ERAP1

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and ERAP2 play key roles in the pathogenesis of inflammatory autoimmunity and in immune evasion by cancer or viral and bacterial pathogens. Downregulation of ERAP1 by a microRNA produced by human cytomegalovirus, facilitates immune evasion [21], while ERAP1 expression in mice can gravely affect the outcome of *Toxoplasma gondii* infection and is essential for protective immunity against the parasite [22]. Moreover, genetic silencing of ERAP1 can induce novel or enhance existing anti-tumor immune responses in several experimental models [23-25], suggesting a possible role of ERAP1 inhibition for cancer immunotherapy [26].

Single nucleotide polymorphisms (SNPs) in the genes of ERAP1 and ERAP2 have been linked to predisposition to several HLA-associated inflammatory diseases [27-29]. Their polymorphic state has been reported to affect the clinical outcome of HPV-induced cervical carcinoma [30] and their expression level can significantly vary in tumors possibly related to cancer immune evasion mechanisms [31-32]. Despite the fact that most of these polymorphic sites are located away from ERAP1 catalytic site, SNPs seem to affect ERAP1 enzymatic activity and substrate specificity [33-37]. ERAP1 haplotypes that contain the variation K528R present reduced enzymatic activity [33] which has been hypothesized to arise from changes in the conformational flexibility of the enzyme[37-38]. Another study focusing on Ankylosing Spondylitis-associated SNPs namely K528R, Q730E revealed significant alterations in ERAP1's kinetic profile [34]. Furthermore, these polymorphisms can modify ERAP1 length selectivity and alter the conformational distribution of the protein indirectly affecting its enzymatic activity [37]. In ERAP2 there are two known SNPs. The coding variant rs2248374 affects ERAP2 protein expression due to nonsense RNA mediated decay [39-40]. Homozygotes for this haplotype do not express ERAP2 and present lower levels of MHC class I proteins [40]. The second ERAP2 variant, rs2549782 encodes for N392K substitution and results in alterations in activity and specificity of the enzyme [41]. Functional alterations caused by polymorphic variations affect cellular antigen presentation as shown by analysis of human immunopeptidomes [15-16, 42].

### 1.3. Specificity and Enzymatic Properties

In order to optimize the peptide cargo of HLA molecules, ERAP1 and ERAP2 have distinct substrate specificity and enzymatic properties. ERAP1 appears to be unique among other similar aminopeptidases as it prefers to trim longer peptides, a feature that appears consistent with its biological role [37, 43] while it shows reduced enzymatic activity for peptides that are 8-9 amino acids, the optimal length for MHCI binding [44]. Library screening of small synthetic substrates provided insight onto the S1 specificity pockets of both ERAP enzymes and revealed that ERAP1 excises hydrophobic and aromatic amino acids whereas ERAP2 prefers positively charged residues [45]. These results were further confirmed by *in vitro* studies using synthetic peptide precursors [46] and are in accordance with cellular antigen presentation assays [46] The ERAP1 N-terminal trimming rates are also affected by the internal sequence and C-terminus of the peptide with preferences for hydrophobic and positively charged residues[43, 47].

### 1.4. Overall Structure and Active Site Organization

The tertiary structures of both ERAP enzymes have been available for a few years now, and have provided many insights onto the mechanism of peptide trimming and substrate preferences [41, 48-52]. ERAP1, just like other previously characterized M1 aminopeptidases, is organized in four distinct structural domains (Fig. **1**) [48, 52]. Domain I consists of eight stranded β-sheets and docks on domain II. Domain II which adopts a thermolysin-like catalytic domain contains the active site with the GAMEN exopeptidase and zinc-binding gluzincin motifs. Domain III is a small sandwich domain between domains II and IV, that acts like a hinge following the movements of the enzyme. Domain IV of ERAP1 is composed of 16 variously sized helices arranged as eight antiparallel armadillo- or HEAT-type helix-turn-helix repeat, assembled side to side to form a concave surface facing toward the active site. ERAP1 has been crystallized in two different configurations, an open and a closed one, that differ in the orientation of domain IV relative to domains I and II [48, 52]. In the open form, domain IV is oriented away from domain II, leading to the formation of a large and solvent exposed cavity. Transition from the open to the closed conformation of ERAP1 occurs through a movement around the hinge domain III, resulting in a large central cavity between domains I, II & IV which completely excludes the enzyme's catalytic site from the solvent but can easily accommodate large antigenic peptide precursors and is lined with shallow pockets carrying mainly negative charges that could interact with peptide side chains [47-48]. The open form is postulated to be inactive due to a lack of a well-structured S1 substrate binding pocket, while the closed form has a well-structured catalytic site [38, 48, 52]. The ERAP1 active

site features a single Zn(II) ion, coordinated by two histidines and one of the glutamate residues of the HExxHx$_{18}$E motif. A nearby Tyr residue, conserved among M1 aminopeptidases has been proposed to assist in the stabilization of the tetrahedral intermediate during the catalytic cycle and to switch orientations depending on the conformational state of the enzyme. Biochemical analysis has provided evidence for an allosteric effect on ERAP1 activity by means of peptide binding to a regulatory site, located at a distance consistent with the length of precursor peptides [43, 48]. Two crystal structures of an intermolecular complex between the ERAP1 C-terminal regulatory domain and a peptide's C-terminus displayed in a fusion protein revealed a series of interactions that suggest specific peptide C-terminal recognition, supporting the proposed allosteric mechanism [49, 53].

The overall structure and domain organization of ERAP2 are similar to that of ERAP1 (Fig. **1**) [51]. The internal cavity of ERAP2 however has a different shape and is lined by generally more hydrophobic residues, something that may contribute to different substrate selectivity. The electrostatic potential of ERAP2 S1 pocket is much more negative than that of ERAP1 which is consistent with a preference for basic side chains of P1 residues [45, 54]. In all crystal structures that have been determined, ERAP2 adopts a closed conformation in which the cavity and the catalytic site have no direct access to the external solvent, making substrate capture and product release impossible without a drastic conformational transition. Co-crystallization of ERAP2 with peptidic substrates and transition state analogues revealed no evidence for specific recognition of the peptide C-terminus or for self-activation, but instead only opportunistic interactions between the substrate and the pockets and side chains along the internal lining of ERAP2's cavity [54].

### 1.5. Structure-based Inhibitor Design: Specificity Pockets and Key Pharmacophores

Most studies that describe ERAP1 and ERAP2 inhibitors up to date have focused on rational structure-based efforts, revolving around the use of specific pharmacophores that target the active site zinc(II) ion, the N-terminal recognition elements and some of the three first specificity pockets of the enzyme (S1, S1' or S2'). Particular zinc binding groups (ZBGs) that chelate the zinc(II) ion in the active site of ERAP1 or ERAP2 can be a powerful approach for designing inhibitors and for predictably orienting side-groups to occupy specificity pockets[45, 55]. The conformational plasticity however of ERAP1 and ERAP2 has created obstacles in rational design efforts since different conformations of the enzyme include changes in key structural elements of the active site that can affect both inhibitor affinity and kinetics [56-57].

Recent studies on the development of ERAPs' small-molecule inhibitors have resulted to several interesting findings and valuable structure-activity relationships. In 2013, our research efforts led to the first highly potent inhibitor of ERAPs, named DG013A (Scheme **1**) [58]. This rationally designed, transition-state analogue inhibitor displays a remarkable affinity for ERAP1 ($IC_{50}$ = 48 nM) and ERAP2 ($IC_{50}$ = 80 nM)

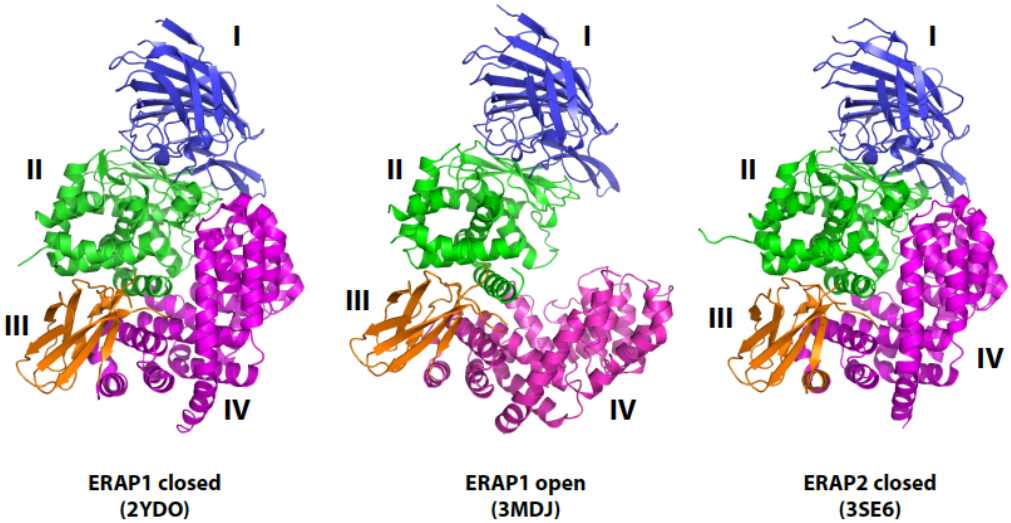
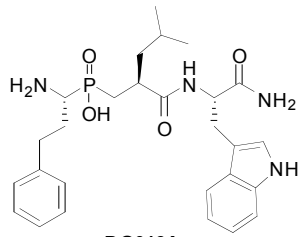



**Fig. (1).** Cartoon representations of known crystal structures of ERAP1 and ERAP2 showing domain organization (domains indicated in roman numerals). Left, the closed conformation of ERAP1; middle, the open conformation of ERAP1 and right, the closed conformation of ERAP2 [48, 51-52].

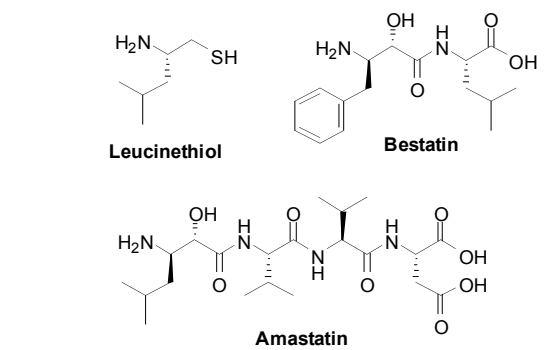
but also for IRAP ($IC_{50}$ = 57 nM)[59]. The ability of DG013A to alter antigen presentation [58, 60-61] spurred subsequent studies towards the optimization of phosphinic peptides inhibitors but also for the evaluation of other structural alternatives especially with regard to the zinc-binding group (ZBG) of candidate inhibitors.



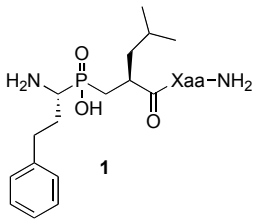

**Scheme 1.** Structure and inhibition profile of DG013A.

In principle, all ZBGs that have been utilized for the inhibition of other Zn aminopeptidases could also be recruited for the inhibition of ERAPs and potentially lead to effective inhibitors after proper optimization. Such structural alternatives have been recently reviewed in a systematic manner [62] and include, besides phosphorus-containing compounds, bestatin and analogues, hydroxamates, 2-aminothiols, boronic acids and tetralone derivatives [63-64]. So far, only phosphinic peptides have been adequately evaluated for ERAPs which implies that exploration of more ZBGs may reveal new possibilities towards the discovery of improved inhibitors in terms of both potency and selectivity. Prior to the discovery of DG013A, broad-spectrum aminopeptidase inhibitors, such as leucinethiol, bestatin and amastatin (Scheme **2**), were employed in several studies on the biological functions and structural and biochemical characterization of ERAPs, however all of them behave as weak and non-specific inhibitors [10, 23-24, 48, 52, 65]. This striking behavior, especially for leucinethiol which is a low nanomolar inhibitor of other related aminopeptidases, such as APN ($K_i$ = 22 nM) [66] and APB ($K_i$ = 130 nM) [67], implies that the development of potent inhibitors for ERAPs cannot be based exclusively on zinc deactivation (*e.g.* by a thiol group) but should also take into account a more detailed analysis of the overall topology of their active sites.

The pseudotripeptidic scaffold of DG013A constitutes an ideal template for the study of SARs of the three most proximal subsites to Zn(II) ion, namely S1, S1' and S2'. In 2016, we presented a systematic SAR study of stereochemically defined phosphinic tripeptides of the general structure **1** (Scheme **3**) that revealed several critical determinants for the effective inhibition of ERAPs [59]. The stereochemical purity of target compounds was set as an absolute requirement for drawing safe SAR conclusions, albeit synthetically challenging. From the SAR analysis of P2' position of 11 phosphinic tripeptides of type **1**, it was revealed that structural alterations of this position can lead to larger deviations of $IC_{50}$ values in the case of ERAP1 [most potent: Xaa = L-Trp (DG013A, **1a**): $IC_{50}$ = 48 nM; less potent: Xaa = L-Ser (**1b**): $IC_{50}$ = 7.5 μM) as compared to ERAP2 where the respective range of $IC_{50}$ values was much narrower [most potent: Xaa = L-Tyr (**1c**): $IC_{50}$ = 55 nM; less potent: Xaa = L-Lys (**1d**): $IC_{50}$ = 271 nM). This observation implies that ERAP2 is able to accommodate a wider variety of side-chains in its S2' position whereas ERAP1 is more insensitive to similar changes. ERAP1 cannot tolerate polar residues in its S2' subsite, a feature that can result to selective ERAP2 inhibitors such as **1b** which is 60 times more potent for ERAP2 versus ERAP1. On the contrary, there is a more pronounced correlation between the hydrophobic nature of P2' position of ERAP1 and inhibitor potency. Indeed, DG013A (**1a**) that bears a highly hydrophobic Trp in its P2' position, was the sole case of a type **1** inhibitor where a slight preference for ERAP1 over ERAP2 could be observed (Scheme **3**).




**Scheme 2.** Structures of leucinethiol, bestatin and amastatin.

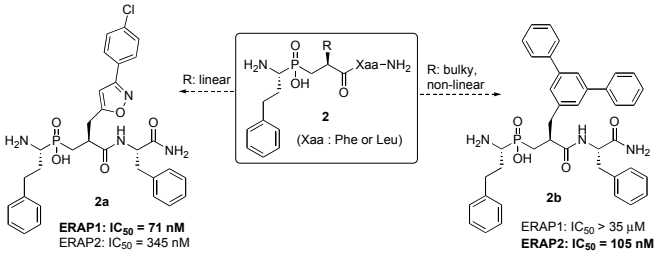



| No | Xaa | $IC_{50}$ (ERAP1) | $IC_{50}$ (ERAP2) |
|---|---|---|---|
| **a** | L-Trp | 48 nM | 80 nM |
| **b** | L-Ser | 7.5 μM | 129 nM |
| **c** | L-Tyr | 340 nM | 55 nM |
| **d** | L-Lys | 3.0 μM | 271 nM |

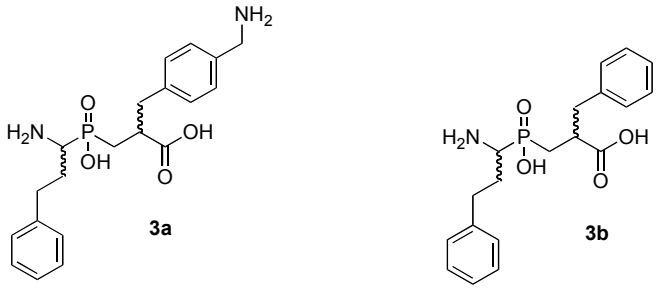
**Scheme 3.** Selected examples of type **1** phosphinic tripeptides and their inhibition profile.

Exploration of the nature of P1' side-chain of phosphinic tripeptides **2** (Scheme 4) showed that the overall shape of the P1' ligand may significantly affect the selectivity pattern [59]. Linear hydrophobic groups

fit well to both ERAP1 and ERAP2 affording in all reported cases moderate to low nanomolar inhibitors. This structural characteristic is particularly favorable for ERAP1, as shown in the case of **2a** which displays a 5-fold selectivity for ERAP1 ($IC_{50}$ = 48 nM) over ERAP2 ($IC_{50}$ = 345 nM). Indeed, compound **2a** is the most potent and selective inhibitor of ERAP1 versus ERAP2 described in this report. Most probably, in those cases where the shape of P1' ligand is linear, inhibition of ERAP1 is less susceptible to structural alterations of P1' substituents than ERAP2, as long as these alterations do not increase the *overall* bulk of the P1' side chain. A similar observation was reported by Mucha and co-workers who screened a library of diastereoisomeric mixtures of phosphinic dipeptides initially developed as inhibitors of APNs (Scheme **5**) [68]. Among the tested compounds, phosphinic derivative **3a** displayed a higher affinity for ERAP1 ($K_i$ = 3.73 μM) than for ERAP2 ($K_i$ = >250 μM). Obviously, an amine substituent cannot be well-tolerated by ERAP2, however ERAP1 is not significantly affected (compare inhibition profiles of **3a** and **3b** where a reversal in ERAP1/ERAP2 selectivity trend can be detected).



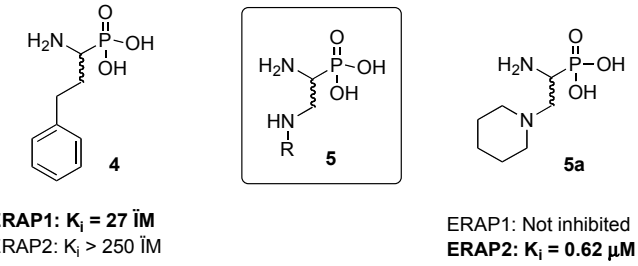

**Scheme 4.** Selected examples of type **2** phosphinic tripeptides and their inhibition profile.




**Scheme 5.** Inhibition profiles of phosphinic dipeptides **3a** and **3b**.

On the contrary, introduction of bulky, non-linear side chains in P1' position could be an effective way to achieve selectivity for ERAP2 over ERAP1 (Scheme **4**). We observed that increasing the bulk of P1' hydrophobic ligands, primarily affects affinity for ERAP1 and to a lesser extent for ERAP2. This is obvious in the case of compound **2b** which is the most potent and selective inhibitor of ERAP2 ($IC_{50}$ = 105 nM) over ERAP1 ($IC_{50}$ > 35 μM) identified in this study. However, it must be taken into account that such a strategy is not suitable for discrimination of ERAPs from IRAP which is able to easily accommodate both linear and non-linear bulky hydrophobic groups in its S1' subsite due to the increased plasticity of its active site, as it was recently described [69].

Mucha and co-workers have also explored a series of racemic aminophosphonic derivatives which lack a primed side and could reveal important SARs for P1/S1 interactions (Scheme **6**) [68]. Although the potency of such molecules is generally low, it is interesting that a switch in selectivity can be achieved by introducing an additional amine group in their β-position. In particular, compound **4** is a moderate inhibitor of ERAP1 but it is inactive towards ERAP2, whereas compounds of type **5** are inactive towards ERAP1 but, in general, they display moderate potency for ERAP2 with the piperidine analogue **5a** being the most potent ERAP2 inhibitor of the series. Consequently, the inability of ERAP1 to accommodate amine groups in its S1 subsite is a feature that must be taken into consideration during efforts to develop potent ERAP1 inhibitors.




**Scheme 6.** Inhibition profiles of aminophosphonic derivatives **4** and **5a**.

Inhibitors of moderate potency but with promising selectivity profiles were identified after a wide screening of 3,4-diaminobenzoic (DABA) derivatives bearing natural and unnatural aminoacid substituents (Scheme **7**) [70-71]. As it was recently demonstrated by X-ray crystallographic analysis [55] and in accordance with previous docking experiments [71], this binding motif chelates zinc ion weakly through the carbonyl oxygen of DABA core (Fig. **2**). A striking feature of these inhibitors is that 3-amino substituted DABA derivatives exhibit different inhibition profiles than the corresponding 4-amino substituted regioisomers which implies that the orientation of aminoacid side-chains is highly regulated by the different substitution patterns. The most potent inhibitor of ERAP1 (Scheme **7**, com-

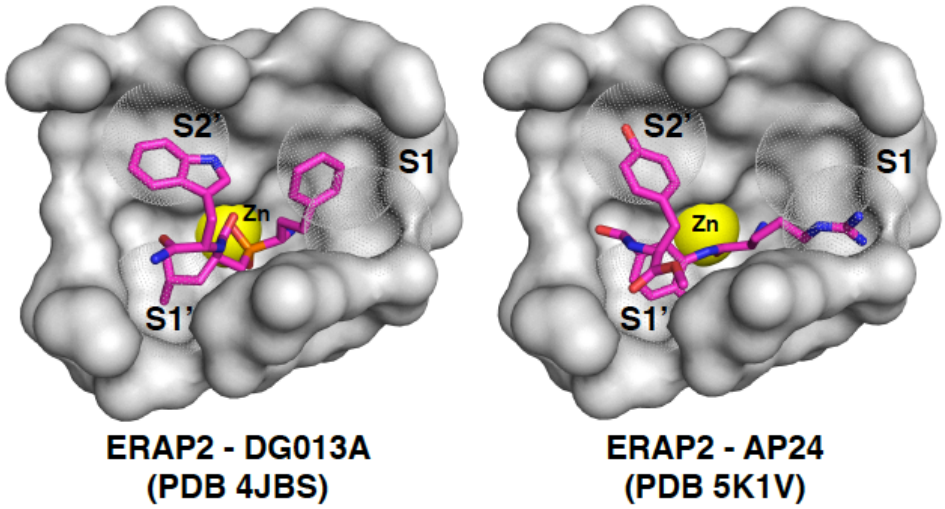
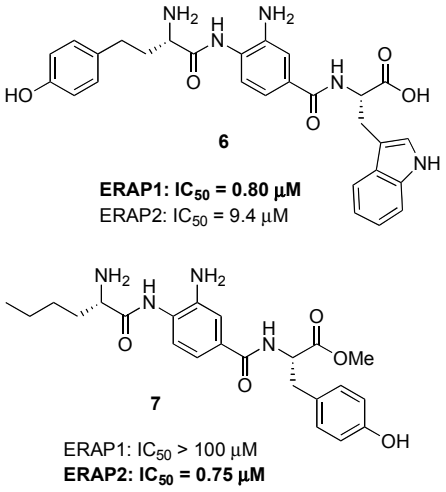


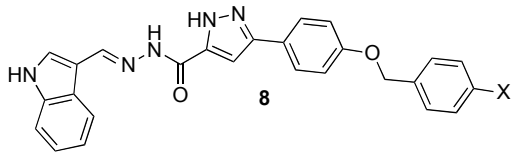

**Fig. (2).** Schematic representation of known crystal structures of ERAP2 with inhibitors DG013A and AP-24 [55, 58]. The active site of the enzyme is shown in surface representation and the inhibitors as sticks. The (active site) Zn(II) ion is shown as a sphere. The three specificity pockets of ERAP2 (S1, S1' and S2') are indicated as dotted spheres.

pound **6**) identified in this study displayed also a good selectivity over ERAP2 which is a promising observation considering that this selectivity is almost twice as high as the selectivity of the most ERAP1 selective phosphinic inhibitor **2a** (Scheme **4**). This is in perfect agreement with previous studies that emphasize how advantageous for improving selectivity can the weakening or in certain cases elimination of the ZBG (as in the paradigm of MMP13 non zinc-chelating inhibitors[72-73]) be, of course in expense of potency. The high affinity of **6** for ERAP1 verifies its preference for hydrophobic residues in the P2' position of inhibitors, as it was already discussed for DG013A. In addition compound **7** is a high nanomolar inhibitor of ERAP2 that exhibits a remarkable selectivity for ERAP2 over ERAP1. Although this trend is not general for all similar tyrosine derivatives examined in this study, it is assumed that the conformation of Tyr in the primed subsite of ERAP2 allows the formation of a hydrogen bond between the –OH group of Tyr and R345 and makes hydrophobic interactions with W363 which is replaced by G346 in the case of ERAP1.

Evidently, the design of potent and selective inhibitors of ERAPs is a challenging task that requires more detailed and accurate SAR data in order to rationalize the discovery of new drugs with improved properties. However, except from the rational design approach, other strategies such as high-throughput screening or virtual screening of compound libraries may also lead to interesting results much faster and probably more efficiently. In such a case, virtual screening of drug databases resulted to the discovery of moderate ERAP1 inhibitors based on the (*E*)-*N*'-((1*H*-indol-3-yl)methylene)-1*H*-pyrazole-5-carbohydrazide scaffold (structures of type **8**, Scheme **8**)[74]. A targeted functional screen for novel zinc-binding groups generated the unexpected finding that thimerosal (**9**), a known pharmaceutical agent that has been long-used as a vaccine preservative, is a high nanomolar inhibitor of ERAP1 ($IC_{50}$ = 240 nM) and inactive for ERAP2, IRAP and LAP up to 50 μM of inhibitor concentration (Scheme **8**) [74]. It was proposed that coordination of the Hg(II) atom with the hydroxyl groups of two non-conserved Ser residues (Ser869 and Ser316) of ERAP1 may account for this remarkable preference of thimerosal for ERAP1 although other non-specific mechanisms are also possible but have not been investigated yet.

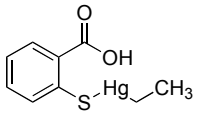



**Scheme 7.** Inhibition profiles of 3,4-diaminobenzoic (DABA) derivatives **6** and **7**.



**8**

**8a** (X = H)**:** $IC_{50}$ (ERAP1) = 43 μM
**8b** (X = Me)**:** $IC_{50}$ (ERAP1) = 27 μM
**8c** (X = Cl)**:** $IC_{50}$ (ERAP1) = 18 μM



**thimerosal (9)**

**ERAP1: $IC_{50}$ = 0.24 ïM**
ERAP2 / IRAP / LAP: $IC_{50}$ > 50 μM

**Scheme 8.** Inhibition profiles of derivatives of type **8** and thimerosal (**9**).

### 1.6. Selectivity

Clinical development of enzyme inhibitors hinges around both potency and selectivity. Although for ERAP1 and ERAP2 sufficient potency has been already achieved in several cases, selectivity has been more challenging or at least much less studied till now. Most selectivity studies or rationally designed ERAP1 and ERAP2 inhibitors have been limited to same subfamily homologous aminopeptidases that are the most likely off-targets [59, 68, 71]. However selectivity towards aminopeptidases outside the family is important to address since it can result in unexpected off-target effects affecting both antigen presentation and other cellular functions. In this context, mechanism-inspired inhibitors and transition-state analogues although invaluable in designing high-potency inhibitors for target enzymes, may also inhibit non-homologous enzymes utilizing identical catalytic mechanisms [75]. Indeed, phosphinic pseudopeptides have been described to inhibit other aminopeptidases and as a result selectivity can only be achieved by careful fine-tuning of specificity pocket occupancy [76]. Furthermore, strong Zn(II) chelating groups may also affect other metalloenzymes, something previously reported for hydroxamic acid derivatives [77]. These potential pitfalls necessitate further testing of developed inhibitors in order to predict and avoid unwanted off-target effects during pre-clinical and clinical development stages.

An additional potential hurdle to be overcome regarding the development of ERAP1 and ERAP2 inhibitors is selectivity versus the highly homologous IRAP. IRAP is a transmembrane protein with an aminopeptidase extracellular domain that is highly homologous to ERAP1 [50]. It has a role in antigen presentation and more specifically in cross-presentation by dendritic cells [78-79]. However, IRAP also has several other important biological functions including the regulation of trafficking of the glucose transporter type 4, the control of oxytocin levels in pregnancy and the regulation of brain oxytocin and vasopressin levels [29]. Indeed, IRAP inhibitors have been developed in an effort to modulate some of these biological functions [80-81]. It may be highly desirable for the clinical application of ERAP1 and ERAP2 inhibitors, that they are selective against IRAP so as to avoid side effects. The structural and functional similarities between these enzymes however make this challenging and most of the ERAP1 inhibitors in the literature suffer from low selectivity against IRAP [58-59, 71]. Curiously, the reverse has been easily achievable and several of the published IRAP inhibitors do not target ERAP1 [59, 80-81]. This has been recently hypothesized to arise from some unique plasticity of the IRAP active site that can reorganize around bound inhibitors [69]. Further SAR optimization appears necessary to generate inhibitors that overcome this potential limitation.

### 1.7. Cell-based Models and Inhibitor Efficacy

Existing ERAP1 and ERAP2 inhibitors have been tested in several cell based models for activity in regulating antigen presentation and immune responses. Phosphinic pseudotripeptides have been found to be able to enhance HLA-B27 surface presentation on HeLa cells and to enhance anti-GSW11 epitope presentation on CT26 colon carcinoma cells [58]. Furthermore DG013A was found to down-regulate innate immune responses such as activation of macrophage phagocytosis and NK cell activation after LPS treatment and to also suppress of Th17 responses *in vitro* [60-61]. 3,4-Diaminobenzoic acid derivatives were also found to be active in reducing activation of macrophage phagocytosis after IFN-γ and LPS treatment[71]. Finally, thimerosal (Scheme **8**), was found to be able to down-regulate IL-2 secretion by transgenic OT-I CD8+ T cells incubated with OVA loaded- BMDC [74].

### 1.8. Potential Clinical Applications

Given the importance of ERAP1 and ERAP2 in generating antigenic epitopes and the ongoing development of small-molecular weight inhibitors that can be used to regulate their enzymatic activity, potential clinical applications of such inhibitors are gradually emerging. Perhaps the most promising clinical application of ERAP inhibitors lies in the field of cancer immunotherapy. ERAP1 down-regulation by genetic or

chemical means has been shown to enhance anti-tumor CD8+ and NK responses in at least two distinct cellular systems and to inhibit tumor growth and enhance survival in two syngeneic mouse cancer models [23-24, 82]. Unfortunately these effects have only been demonstrated with the non-selective aminopeptidase inhibitor leucinethiol (Scheme **2**), a molecule that has the potential to also interfere with the function of many other metalloproteinases [83] and is unlikely to be further developed pharmacologically. As a result, reproducing these effects with more selective ERAP inhibitors, using the same or different cancer models is necessary to confirm the potential of ERAP inhibitors for clinical development. Furthermore, the ability of ERAP inhibition to enhance anti-tumor responses has been till now attributed to either the upregulation of specific tumor-associated antigenic peptides [23, 25] or to the production of poorly folded immunogenic MHC class I molecules in specific cell lines [10, 24, 82], both mechanisms that may have limitations on how well they are translated to cancer immunotherapy in humans. Regardless however, there is significant evidence that affecting ERAP1 activity may hold promise as a more general tool to enhancing tumor-antigenicity. In particular, ERAP1 down-regulation has been shown to increase presentation by non-classical MHCI and result in enhanced cytotoxic responses [84-85]. Similarly, a recent study found that ERAP1 was consistently overexpressed in HPV16+ tumor cell lines as an immune evasion mechanism and that ERAP1 down-regulation resulted in enhanced killing of cancer cells [31]. Additionally, p53 has been shown to increase surface MHC class I expression by up-regulating ERAP1 suggesting an additional evidence that may implicate ERAP1-dependent antigen processing to tumor immune evasion [32, 86]. In a recent study, ERAP2 overexpression was found to be involved in the metastasis potential of oral cavity squamous cell carcinoma [87] Furthermore, recent work has revealed that the potency of immune-checkpoint inhibitor therapies depends largely on the generation of cancer-specific neoantigens by cancer cells [88-90]. Since neoantigens drive tumor immunogenicity largely through CD8+ responses against antigenic peptides derived from neoantigens, intracellular antigen processing by enzymes such as ERAPs is bound to be a powerful tool for fine-tuning neoantigen presentation in order to enhance tumor immugenicity. Indeed, ERAP1 and ERAP2 activity has been shown to regulate the cellular immunopeptidome in cell lines [91-92] and global "antigenic signature" shifts in cancer cells have been proposed to be a more reliable predictor of the efficacy of adaptive immune responses against cancer [93]. As a result, pharmacological ERAP inhibition may be applicable to cancer immunotherapy either as a stand-alone approach or as a potentiator of immune-checkpoint inhibitor therapy. Further investigations on the effects of ERAP inhibitors on the immunopeptidome and antigenicity of different tumor models will be necessary to establish the generality of this approach for cancer immunotherapy.

### 1.9. Autoimmunity

A very large number of genetic and population studies have linked ERAP1 and ERAP2 polymorphic variation to predisposition to HLA-associated autoimmunity (reviewed in [20, 28]). Furthermore, these associations have frequently demonstrated epistasis between ERAPs and HLA [94-95]. These findings, in combination with established effects of ERAP1 and ERAP2 SNPs on the activity and selectivity of these enzymes [34, 36, 41] suggest a causative relationship between the activity of ERAPs and the development of HLA-associated autoimmunity. This notion has significant support from several studies of the changes in the immunopeptidome of cells carrying the HLA-A29, HLA-B27 and HLA-B51 alleles depending on the polymorphic state of expressed ERAP1 or ERAP2 [14, 91-92, 96-97]. Furthermore, expression of a human ERAP1 variant in mice was recently demonstrated to alter T-cell and NK responses and lead to increased in utero and perinatal mortality [98]. Perhaps related to this, a hyper-active variant of ERAP2 has been associated to the risk for preeclampsia [39]. As a result, it is tempting to hypothesize that modulating ERAP activity may have therapeutic value in the treatment of inflammatory diseases that carry HLA-associated autoimmune etiologies. Indeed, in a recent study inhibition of ERAP1 by a potent inhibitor resulted in suppression of Th17 responses *in vitro* [61]. It should be noted however that genetic differences that lead to altered ERAP1 or ERAP2 activity, exist during formation of immunological tolerance and as a result modulating ERAP activity post-tolerance may not have the desired results. As a result, the question of whether current experimental animal models of inflammatory autoimmune disease could serve as an appropriate tool for testing the effect of ERAP inhibitors, remains open. Still, the effect of antigenic shifts on the severity of autoimmune inflammatory responses may hold therapeutic promise and needs to be evaluated.

### 1.10. Infections

Since the activity of ERAP1 and ERAP2 has also been linked to proper immune responses to infections,

and their expression levels and activity can be manipulated by pathogens presumably to assist with immune evasion, inhibitors or activators for these enzymes may find applications to boosting immune responses against pathogens. More specifically, a microRNA produced by human cytomegalovirus can down regulate the expression levels of ERAP1 and reduce cytotoxic responses to infected cells due to reduction in the generation of particular antigenic epitopes [21]. As a result, activation of ERAP1 may be beneficial in treating HCMV. Similarly, the allelic state of ERAP1 (and presumably its activity) has been found to affect the persistence of HPV infection and the clinical outcome of cervical carcinoma suggesting the manipulating ERAP1 activity may enhance anti-viral responses [30]. Immune responses towards Toxoplasma gondii also require proper antigenic peptide precursor trimming by ERAP1 [22]. Finally, immune responses towards HIV can be greatly affected by ERAP1 and ERAP2 and can even promote natural resistance to HIV-1 infection [99, 100]. Interestingly, a ERAP2 SNP that leads to nonsense-mediated decay and lower protein expression has been found to be protective versus HIV infection and be preserved through balancing selection [40] suggesting that inhibiting ERAP2 may be a tractable approach to enhancing anti-HIV immune responses.

### 1.11. Other Biological Roles of ERAP1

Besides its major role in antigen presentation, ERAP1 is also involved in innate immunity. ERAP1 has been proposed to modulate inflammatory events by generating the soluble forms of cytokines receptors although this function has not been investigated further [101]. Goto *et al.,* demonstrated that ERAP1 can be secreted from macrophages upon activation with LPS and IFN-γ, resulting in enhancement of their phagocytic activity [102]. Additionally, ERAP1 knock-out mice exhibited increased activation levels of NK and NKT cells both in liver and spleen and presented enhanced potential to release pro-inflammatory cytokines and phagocytic capabilities in response to innate stimuli [103]. The potential roles of ERAP1 in regulating innate immune responses may be complementary to its effects in adaptive immunity and as a result pharmacological interventions aiming at the role of ERAP1 in adaptive immune responses may have complementary or synergistic effects in the innate immune system. Such synergisms have not been explored yet however.

Although the role of ERAP1 in antigen processing has dominated the literature during the past decade, several studies have proposed that it is also involved in other biological processes. ERAP1 may contribute to blood pressure regulation by hydrolyzing bioactive peptides to their inactive derivatives. Co-localization of ERAP1 with kallidin in the human kidney and the ability to trim angiotensin II, suggest that ERAP1 can affect the production of bradykinin and angiotensin IV [104]. Indeed, ERp44, an ER protein that contributes to disulfide bond formation in nascent proteins, can form mixed disulfide bonds with ERAP1 and control its secretion in a redox-dependent manner, presumably as a mechanism for controlling blood pressure [105]. During differentiation of mouse epithelial cells, ERAP1 was expressed after VEGF stimulation suggesting a role in post-natal angiogenesis [106]. Furthermore, ERAP1 silencing reduces VEGF-dependent transition in ECs due to lack of activation of 6S kinase [107]. Finally, it has been proposed that ERAP1 is involved in activation of endothelial integrins during cell migration [108].

## 2. FUTURE DIRECTIONS AND CHALLENGES

Almost 15 years of basic research on the roles of ERAP1 and ERAP2 in the functioning of the human immune system has led to the accumulation of experimental evidence ranging from genetic and population studies to *in vitro* functional studies and *in vivo* models that corroborate that these enzymes can now be perceived as tractable pharmacological targets for particular clinical applications. Furthermore, different preliminary medicinal approaches have generated potent first-generation inhibitors with good bioactivity suggesting that regulating ERAP1 and ERAP2 activity in a clinical setting is feasible. While medicinal chemistry approaches will keep improving available inhibitors, it appears that the field has now matured sufficiently to warrant pre-clinical development. Targeting cancer immunotherapy may be the obvious first choice, in particular due to the rising interest in increasing the immunogenicity of tumors, although modulating ERAP activity in inflammatory autoimmunity or for boosting immune responses against pathogens are also promising. Discovery of small-molecule activators for these enzymes would undeniably be a powerful tool for pharmacological approaches since inhibition may not always be appropriate. Limited selectivity especially amongst homologous aminopeptidases still remains a challenge and future studies may have to take into account the conformational plasticity of these enzymes [109]. The main challenge to be addressed by future studies however is the complexity that inhibition may have on the cellular immunopeptidome. Since ERAP1 and possibly ERAP2 should probably be considered as

optimizers rather than just generators for the peptide repertoire, their inhibition may influence T-cell responses in hard-to-predict manners that would need to be analyzed first on a case-to-case basis. Still, generating tools to affect how cells are recognized by the immune system can be a powerful approach that can have applications that have not even been imagined yet.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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