UC San Diego

UC San Diego Previously Published Works

Title

Novel selective inhibitors of aminopeptidases that generate antigenic peptides.

Permalink

<https://escholarship.org/uc/item/8bs4b9mj>

Journal

Bioorganic & medicinal chemistry letters, 23(17)

ISSN

0960-894X

Authors

Papakyriakou, Athanasios
Zervoudi, Efthalia
Theodorakis, Emmanuel A
et al.

Publication Date

2013-09-01

DOI

10.1016/j.bmcl.2013.07.024

Peer reviewed

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel selective inhibitors of aminopeptidases that generate antigenic peptides

Athanasios Papakyriakou^{a,b}, Efthalia Zervoudi^c, Emmanuel A. Theodorakis^a, Loredana Saveanu^d, Efstratios Stratikos^c, Dionisios Vourloumis^{b,*}

^a Department of Chemistry & Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA

^b Chemical Biology Laboratories, National Center for Scientific Research 'Demokritos', Agia Paraskevi Attikis, GR 15310, Greece

^c Protein Chemistry Laboratories, National Center for Scientific Research 'Demokritos', Agia Paraskevi Attikis, GR 15310, Greece

^d Institut National de la Santé et de la Recherche Médicale, Unité 1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine, 75015 Paris, France

ARTICLE INFO

Article history:

Received 3 June 2013

Revised 12 July 2013

Accepted 15 July 2013

Available online 23 July 2013

Keywords:

Endoplasmic reticulum aminopeptidase

ERAP1

ERAP2

IRAP

Selective inhibitor

Rational design

Molecular modeling

ABSTRACT

Endoplasmic reticulum aminopeptidases, ERAP1 and ERAP2, as well as Insulin regulated aminopeptidase (IRAP) play key roles in antigen processing, and have recently emerged as biologically important targets for manipulation of antigen presentation. Taking advantage of the available structural and substrate-selectivity data for these enzymes, we have rationally designed a new series of inhibitors that display low micromolar activity. The selectivity profile for these three highly homologous aminopeptidases provides a promising avenue for modulating intracellular antigen processing.

© 2013 Elsevier Ltd. All rights reserved.

Human aminopeptidases of the oxytocinase subfamily of M1 aminopeptidases have recently been shown to play important roles in the function of the human adaptive immune response.^{1–4} Endoplasmic reticulum aminopeptidases 1 and 2 (ERAP1/2), as well as Insulin regulated aminopeptidase (IRAP) can generate key antigenic peptides that help the human body fight pathogens and cancer but at the same time they can also destroy several antigenic peptides by over-trimming.^{5–7} Both functions can contribute to human disease by either promoting immune evasion or contributing to autoimmune responses.⁸ Genetic polymorphisms in ERAP1 and ERAP2 have been associated with the individual's predisposition to numerous human diseases (reviewed in Ref. 8) and it has been suggested that this link is due to changes in their specificity and activity.^{9–11}

Despite the important biological roles of ERAP1/2 on human health, very little is known on how to pharmacologically manipulate their function. Down-regulation of ERAP1 protein expression in experimental models has been shown to elicit novel cytotoxic responses in mice,⁷ to induce Natural Killer cell responses against malignant cells leading to tumor rejection,¹² and to elicit non-classical Major Histocompatibility Class Ib cytotoxic T-lymphocyte

responses *in vivo*.¹³ Some of these effects could be reproduced using the non-specific metalloprotease inhibitor Leucinethiol.^{7–13} These findings, along with the recent elucidation of the crystallographic structures of ERAP1,^{14,15} ERAP2,¹⁶ and the accumulation of a large amount of specificity data for these peptidases,¹⁷ have spurred interest towards the development of potent and selective inhibitors for these enzymes that could potentially control the generation of specific subsets of antigenic epitopes. Achievement of selectivity may be even more important than high potency in this case, since complete incapacitation of antigenic peptide generation may not be desired therapeutically as opposed to subtle modulation of a particular epitope's generation. This concept has been supported physiologically by demonstrating that relatively small changes in the enzymatic activity of ERAP1 due to a single nucleotide polymorphism can be either protective or predisposing to autoimmunity.^{8,10,18}

Since ERAP1, ERAP2 and IRAP are highly homologous, having sequencing identity at ~50%, the design of inhibitors that demonstrate any degree of selectivity between these enzymes is highly challenging. Towards our goal to achieve selectivity we utilized the acquired scientific experience from the matrix metalloproteinases (MMPs) family of zinc endopeptidases, which has been intensively pursued during the last three decades.^{18–20} Specifically, it has been demonstrated that employing strong zinc chelators,^{21–23}

* Corresponding author. Tel.: +30 210 6503624; fax: +30 210 6511766.

E-mail address: vourloumis@chem.demokritos.gr (D. Vourloumis).

such as the hydroxamic acid zinc-binding group, usually raises undesirable off-target activity against other metalloproteins, as exemplified by the first generation of MMP inhibitors.^{24,25} Additionally, it has been proposed that since the metal site is the most conserved feature across all MMPs, inhibition based on non-zinc chelating inhibitors would minimize or even eliminate the interaction with the catalytic zinc, leading to improved selectivity.^{26–28} The latter was proven to be very successful for targeting enzymes with deep pockets, such as the S1' subsite of MMP-13.²⁹

It has become clear that a careful consideration of inhibitor backbones for targeting the substrate pockets of individual metalloenzymes, can lead to the development of highly selective inhibitors, irrespective of the zinc-binding group used. On these grounds, we based our design strategy on a relatively weak zinc chelator, aminobenzamide, borrowed from class I histone deacetylase inhibitors.³⁰ The amino-functionality at position 1 (numbering as of Fig. 1A) could either coordinate to the active-site zinc ion, or probably interact with a catalytically important glutamic acid residue (Glu-354/371/465 for ERAP1/ERAP2/IRAP), which is engaged in most zinc metalloproteases to polarize the water molecule for nucleophilic attack on the carbonyl group of the scissile bond.³¹ The 2-benzamide functionality incorporates the coordinating carbonyl group and the α -amino acid-based moiety, as required for the enzymatic function in all natural substrates. Thus, the strong electrostatic interaction with the two conserved glutamic acid residues that harbor the α -amino terminus of natural substrates is preserved (Fig. 1). The P1 side-chain could be selected according to the target member and is expected to significantly contribute in achieving selectivity for one aminopeptidase over

another, based on their individual spatial and electrostatic requirements. Expansion towards the primed subsites could be achieved through functionalization of the carboxylic acid at position 4, properly directing the inhibitor side-chain(s) Pn' to target the corresponding substrate pockets.

The selectivity profile of the S1 subsite of ERAP1, ERAP2 and IRAP was recently investigated using a library of 82 fluorogenic substrates.¹⁷ These studies suggested that ERAP1 displays a general preference for substrates comprising long, aromatic or hydrophobic P1 side-chains. In contrast, the S1 subsite of ERAP2 exhibits higher selectivity for positively charged groups (e.g., L-Arg), whereas IRAP combines the specificity of ERAP1 and ERAP2.¹⁷ For these reasons we selected L-homo-phenylalanine (hPhe) as one of the preferred occupants for the S1 subsite. This decision was further supported by our molecular modeling calculations, which indicated that the long side-chain of hPhe would be ideally accommodated in the S1 pocket of ERAP1, providing π -stacking interactions with a conserved aromatic residue (Phe-433/Phe-450/Phe-544 for ERAP1/ERAP2/IRAP). Higher flexibility was allowed for the Pn' position by employing a compilation of polar, non-polar and aromatic natural amino acids to attain the desired selectivity.

The targeted analogues were synthesized according to the procedure presented in Scheme 1. Thus, Boc-protected L-homo-phenylalanine **2**, selected as the amino acid of choice for optimizing the S1 lipophilic interactions as presented before, was coupled with di-aniline **1** under standard coupling conditions (HBTU, DIPEA) furnishing the corresponding amide in 82% yield. The high regioselectivity of the coupling transformation is

Figure 1. Inhibitor design strategy. (A) Schematic representation of the designed inhibitors (black) showing the interacting residues of the enzyme in blue. P1 and Pn' represent amino acid side-chains that are accommodated at the S1 and S1' subsites of the aminopeptidases, respectively. (B) Molecular model of the designed scaffold with carbon atoms shown in orange. The active-site residues of ERAP1 are shown with cyan-stick carbons and Zn²⁺ with a green sphere; all oxygen and nitrogen atoms are red and blue, respectively. The α -amino terminal docking residues are Glu-183 and Glu-320. The zinc-coordinated residues His-353, His-357 and Glu-376, and the catalytically important Tyr-438 are shown transparent. (C) Surface representation of the active site of ERAP1 indicating the S1 and the putative S1'–S3' subsites.

attributed to the increased nucleophilic nature of the meta-NH₂ compared to the para-one, resulting in the exclusive formation of **3**. Saponification of the methyl ester was followed by a second amide formation with a series of protected L-amino acids producing intermediates **5–11** (Scheme 1). In particular for the lysine- and valine-containing intermediates, their methyl and benzyl esters were cleaved in order to evaluate the potential effect of the carboxylic acid versus the ester in the binding potential of the designed inhibitors. Finally, acidic cleavage of the P1 α -amino protecting groups furnished compounds **12–20** (Scheme 1) that were further evaluated for their ability to inhibit the enzymes. In addition, acidic cleavage of the α -amino Boc-group of intermediates **3** and **4** yielded the non-substituted derivatives **21** and **22**, respectively, which were also evaluated as reference compounds.

ERAP1, ERAP2 and IRAP were expressed in insect cells (Hi5 cells) as recombinant proteins after infection with recombinant baculoviruses carrying the desired gene and then purified to homogeneity by affinity chromatography (see Supplementary data for details). The inhibitor potency of all compounds towards the three enzymes was determined using an established fluorogenic assay (Supplementary data, Fig. S1). Our initial screen showed that these

inhibitors exhibit low micromolar affinity and a high degree of selectivity between the three enzymes (Table 1). In particular, **16** proved to be the most potent ERAP1 inhibitor with >10-fold selectivity with respect to ERAP2 (Fig. 2A), whereas **20** is the most potent and selective inhibitor for IRAP. On the other hand, compounds with a short P1' side-chain (**12–15**) proved to be inactive for all three aminopeptidases, with the exception of **13** that carries a C-terminus benzylic group and exhibits comparable affinity for ERAP2 and IRAP. Finally, the non-substituted derivatives **21** and **22** were not able to inhibit ERAP1 or ERAP2 at easily achievable concentrations, albeit **21** is a modest inhibitor of IRAP. These results verify the value of the L-amino acid incorporation for both potency and selectivity, although they indicate that some selectivity for IRAP can be achieved even in the absence of the additional amino acid.

To investigate the mechanism of inhibition of ERAP1 by the most potent compound we performed a standard Michaelis–Menten (MM) analysis. Since the K_M value for L-Leucine-7-amido-4-methyl coumarin (L-AMC) is very high to allow reliable calculation of MM kinetics, we instead used the chromogenic substrate L-Leucine-4-nitroanilide (L-pNA) as described before.¹⁴ Analysis indicated that compound **16**, only affected the K_M parameter of the enzymatic reaction and not the V_{max} or k_{cat} parameters (Fig. 2B), consistent with a competitive inhibition mechanism. A major concern for this type of inhibitors was whether they can be hydrolytically cleaved by the enzymes. In order to investigate their stability we incubated the most potent ERAP1-inhibitor **16** with active enzyme and analyzed the reaction products on reversed-phase HPLC. This analysis suggested that **16** was resistant towards hydrolysis by ERAP1 (see Supplementary data, Fig. S2).

In an effort to gain further insight into the structure–activity relationships of the designed inhibitors, we performed molecular modeling calculations using the crystallographic structures of ERAP1, ERAP2 and a homology model of IRAP (Supplementary data). The large conformational space of such flexible molecules (16 active torsions for compound **16**) represents a challenging task for most of the widely used molecular docking methods. However, we were able to predict meaningful structures within the highest ranked docked poses, in terms of their zinc-binding geometry within the active site and their interactions with key-catalytic residues. More specifically, the complex between **16** and ERAP1

Table 1
Results of the in vitro evaluation for **12–22**

ID	R	ERAP1	ERAP2	IRAP
12	L-Ala-OMe	NI	NI	38 ± 3
13	L-Val-OBn	95.5 ± 3.3	11.5 ± 0.6	3.9 ± 0.1
14	L-Val-OH	NI	NI	>100 ^a
15	L-Thr-OMe	NI	NI	46 ± 2
16	L-Lys-OMe	2.0 ± 0.6	24.9 ± 1.2	10.3 ± 0.6
17	L-Lys-OH	2.6 ± 0.2	8.9 ± 0.5	6.0 ± 0.2
18	L-Arg-OMe	NI	NI	9.6 ± 0.5
19	L-Tyr-OMe	7.7 ± 0.4	>100 ^a	2.8 ± 0.2
20	L-Trp-OBn	NI	23.9 ± 0.8	1.3 ± 0.1
21	OMe	NI	>100 ^a	16.3 ± 0.8
22	OH	NI	>100 ^a	>100 ^a

NI = No inhibition observed at 50 μ M.

^a Limited inhibition (up to 20%) was evident in the 50–100 μ M range indicating an IC₅₀ value >100 μ M.

Figure 2. (A) Inhibitory potency of **16** for the three enzymes, calculated by titrating increasing amounts of the compound while following the kinetics of hydrolysis of small fluorogenic substrates (L-AMC for ERAP1 and IRAP, and R-AMC for ERAP2). (B) Effect of **16** on the enzymatic parameters of ERAP1 calculated by Michaelis–Menten analysis using the chromogenic substrate L-pNA.

Figure 3. Molecular models of the two most potent inhibitors bound into the aminopeptidases' active site, illustrating the key-interacting residues for (A) ERAP1 complex with **16** and (B) IRAP complex with **20**. Colors are as described in Figure 1.

(Fig. 3A) displays the accommodation of hPhe side-chain inside the S1 pocket so that the phenyl group is stacked over Phe-433. The lysine moiety of **16** is extended towards a putative S2' subsite of ERAP1, with its side-chain amine group interacting electrostatically with two aspartic acid residues (Asp-435 and Asp-439, Fig. 3A). Interestingly, both of these residues are not conserved in the other two M1 aminopeptidases (Glu-452 and Asn-454 in ERAP2, Ser-546 and Phe-550 in IRAP), which might account for the selectivity of **16** for ERAP1 over ERAP2 and IRAP. The C-terminus methyl ester is probably hydrogen bonded with the hydroxylic group of Ser-869 (Tyr-892 in ERAP2 and Tyr-961 in IRAP). The corresponding hydrolyzed carboxylic acid **17** displays comparable affinity for ERAP1 with respect to **16**, albeit with lower selectivity for ERAP2 and IRAP. Surprisingly, the longer side-chain of arginine in **18** displayed no affinity for either ERAP1 or ERAP2, which indicates that the planar guanidinium group might not be suitable for the electrostatic interactions predicted for the more flexible side-chain of lysine in **16**. Another potentially good candidate for exploring the primed subsites of ERAP1 is the phenolic group of tyrosine, though **19** displayed high potency for IRAP, as well.

With regard to the more potent inhibitor of IRAP, compound **20** is predicted to bind within the active site in a similar configuration as described for **16** (Fig. 3B). The P1' tryptophan side-chain of **20** engaged in aromatic interactions with the catalytically active Tyr-549 residue (conserved amongst M1 aminopeptidases), Phe-550 (Asp-439 in ERAP1 and Asn-454 in ERAP2) and Tyr-961 (Ser-869 in ERAP1 and conserved in ERAP2). The carboxylate terminus benzyl group of **20** is found in the assumed S2' subsite, interacting with Phe-926 (Gln-834 in ERAP1 and Ala-857 in ERAP2) and Tyr-961, as well. Similar interactions of the terminal O-benzyl group in **13** might account for its increased affinity for IRAP with respect to the non-active carboxylic acid **14**.

In conclusion, we have rationally designed and synthesized a novel family of inhibitors for zinc aminopeptidases that display promising selectivity profiles for three, highly homologous members of antigen-processing enzymes. Their facile synthesis and flexibility in incorporating a plethora of P1 and Pn' moieties may constitute, after further optimization, a promising avenue to modulating intracellular antigen processing. Additional examples and substitution patterns along with co-crystallization efforts and cell-based inhibition assays are currently underway.

Author contributions

A.P. performed the molecular modeling calculation and the synthesis of the inhibitors. E.Z. prepared the recombinant enzymes, performed fluorogenic assay measurements and HPLC analysis. L.S. established the expression system for IRAP. E.S. and D.V. conceived the experiments and analyzed data. E.A.T. co-supervised the project. All authors contributed to the preparation of the manuscript and have approved its final version.

Acknowledgments

This work was supported by the Action 'Supporting Postdoctoral Researchers' grant LS7_2199 of the Operational Program 'Education and Lifelong Learning', and by the 'ARISTEIA I' action grant 986, co-financed by the European Social Fund (ESF) and the General Secretariat for Research and Technology of Greece. L.S. acknowledges financial support from the French 'Agence Nationale de Recherche', ANR for the IRAP-DC (NT09_522096) project'. We thank Damijana Urankar and Janez Košmrlj (University of Ljubljana) for the HRMS analysis.

Supplementary data

Supplementary data (experimental procedures for the synthesis and characterization of compounds **3–22** along with the enzyme production and purification; description of the enzymatic activity assay with characteristic titration curves for **12**, **19** and **20**; HPLC analysis of digestion products by ERAP1 with the chromatogram for **16** and computational modeling details) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.07.024>.

References and notes

- Serwold, T.; Gonzalez, F.; Kim, J.; Jacob, R.; Shastri, N. *Nature* **2002**, *419*, 480.
- Saveanu, L.; Carroll, O.; Lindo, V.; Del Val, M.; Lopez, D.; Lepelletier, Y.; Greer, F.; Schomburg, L.; Fruci, D.; van Niedermann, G.; Endert, P. M. *Nat. Immunol.* **2005**, *6*, 689.
- Saveanu, L.; Carroll, O.; Weimershaus, M.; Guernonprez, P.; Firat, E.; Lindo, V.; Greer, F.; Davoust, J.; Kratzer, R.; Keller, S. R.; van Niedermann, G.; Endert, P. *Science* **2009**, *325*, 213.
- Evnouchidou, I.; Papakyriakou, A.; Stratikos, E. *Curr. Pharm. Des.* **2009**, *15*, 3656.
- York, I. A.; Chang, S. C.; Saric, T.; Keys, J. A.; Favreau, J. M.; Goldberg, A. L.; Rock, K. L. *Nat. Immunol.* **2002**, *3*, 1177.
- York, I. A.; Brehm, M. A.; Zendzian, S.; Towne, C. F.; Rock, K. L. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9202.
- Hammer, G. E.; Gonzalez, F.; James, E.; Nolla, H.; Shastri, N. *Nat. Immunol.* **2007**, *8*, 101.
- Haroon, N.; Inman, R. D. *Nat. Rev. Rheumatol.* **2010**, *6*, 461.
- Haroon, N.; Tsui, F. W.; Uchanska-Ziegler, B.; Ziegler, A.; Inman, R. D. *Ann. Rheum. Dis.* **2012**, *71*, 589.
- Evnouchidou, I.; Kamal, R. P.; Seregin, S. S.; Goto, Y.; Tsujimoto, M.; Hattori, A.; Voulgari, P. V.; Drosos, A. A.; Amalfitano, A.; York, I. A.; Stratikos, E. *J. Immunol.* **2011**, *186*, 1909.
- Evnouchidou, I.; Birtley, J.; Seregin, S.; Papakyriakou, A.; Zervoudi, E.; Samiotaki, M.; Panayotou, G.; Giastas, P.; Petrakis, O.; Georgiadis, D.; Amalfitano, A.; Saridakis, E.; Mavridis, I. M.; Stratikos, E. *J. Immunol.* **2012**, *189*, 2383.
- Lo Monaco, E.; Forloni, M.; Giorda, E.; Petrini, S.; Tremante, E.; Pende, D.; Locatelli, F.; Giacomini, P.; Fruci, D. *Cancer Res.* **2011**, *71*, 1597.
- Nagarajan, N. A.; Gonzalez, F.; Shastri, N. *Nat. Immunol.* **2012**, *13*, 579.
- Nguyen, T. T.; Chang, S. C.; Evnouchidou, I.; York, I. A.; Zikos, C.; Rock, K. L.; Goldberg, A. L.; Stratikos, E.; Stern, L. J. *Nat. Struct. Mol. Biol.* **2011**, *18*, 604.
- Kochan, G.; Krojer, T.; Harvey, D.; Fischer, R.; Chen, L.; von Vollmar, M.; Delft, F.; Kavanagh, K. L.; Brown, M. A.; Bowness, P.; Wordsworth, P.; Kessler, B. M.; Oppermann, U. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 7745.
- Birtley, J. R.; Saridakis, E.; Stratikos, E.; Mavridis, I. M. *Biochemistry* **2012**, *51*, 286.
- Zervoudi, E.; Papakyriakou, A.; Georgiadou, D.; Evnouchidou, I.; Gajda, A.; Poreba, M.; Salvesen, G. S.; Drag, M.; Hattori, A.; Swevers, L.; Vourloumis, D.; Stratikos, E. *Biochem. J.* **2011**, *435*, 411.
- Evans, D. M.; Spencer, C. C.; Pointon, J. J., et al. *Nat. Genet.* **2011**, *43*, 761.
- (a) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735; (b) Nuti, E.; Tuccinardi, T.; Rossello, A. *Curr. Pharm. Des.* **2007**, *13*, 2087.
- Georgiadis, D.; Yiotakis, A. *Bioorg. Med. Chem.* **2008**, *16*, 8781.
- Jacobsen, J. A.; Major Jourden, J. L.; Miller, M. T.; Cohen, S. M. *Biochim. Biophys. Acta* **2010**, *1803*, 72.
- Puerta, D. T.; Cohen, S. M. *Inorg. Chem.* **2003**, *42*, 3423.
- Puerta, D. T.; Lewis, J. A.; Cohen, S. M. *J. Am. Chem. Soc.* **2004**, *126*, 8388.
- Brown, S.; Meroueh, S. O.; Fridman, R.; Mobashery, S. *Curr. Top. Med. Chem.* **2004**, *4*, 1227.
- For certain examples MMP inhibition modulated by the zinc binding groups see: (a) Puerta, D. T.; Mongan, J.; Tran, B. L.; McCammon, J. A.; Cohen, S. M. *J. Am. Chem. Soc.* **2005**, *127*, 14148; (b) Agrawal, A.; Romero-Perez, D.; Jacobsen, J. A.; Villarreal, F. J.; Cohen, S. M. *ChemMedChem* **2008**, *3*, 812.
- Morales, R.; Perrier, S.; Florent, J.-M.; Beltra, J.; Dufour, S.; De Mendez, I.; Manceau, P.; Tertre, A.; Moreau, F.; Compere, D.; Dublanchet, A.-C.; O'Gara, M. J. *Mol. Biol.* **2004**, *341*, 1063.
- Pochetti, G.; Montanari, R.; Gege, C.; Chevrier, C.; Taveras, A. G.; Mazza, F. J. *Med. Chem.* **2009**, *52*, 1040.
- For promising examples of mechanism based inhibitors see: Brown, S.; Bernardo, M. M.; Li, Z.-H.; Kotra, L. P.; Tanaka, Y.; Fridman, R.; Mobashery, S. *J. Am. Chem. Soc.* **2000**, *122*, 6799.
- Li, J. J.; Nahra, J.; Johnson, A. R.; Bunker, A.; O'Brien, P.; Yue, W.-S.; Ortwine, D. F.; Man, C.-F.; Baragi, V.; Kilgore, K.; Dyer, R. D.; Han, H.-K. *J. Med. Chem.* **2008**, *51*, 835.
- Wang, D. F.; Helquist, P.; Wiech, N. L.; Wiest, O. *J. Med. Chem.* **2005**, *48*, 6936.
- Addlagatta, A.; Gay, L.; Matthews, B. W. *Biochemistry* **2008**, *47*, 5303.