ABSTRACT: Members of the oxytocinase subfamily of M1 aminopeptidases (ERAP1, ERAP2, and IRAP) play important roles in both the adaptive and innate human immune responses. Their enzymatic activity can contribute to the pathogenesis of several major human diseases ranging from viral and parasitic infections to autoimmunity and cancer. We have previously demonstrated that diaminobenzoic acid derivatives show promise as selective inhibitors for this group of aminopeptidases. In this study, we have thoroughly explored a series of 3,4-diaminobenzoic acid derivatives as inhibitors of this class of enzymes, achieving submicromolar inhibitors for ERAP2 (IC₅₀ = 237 nM) and IRAP (IC₅₀ = 105 nM). Cell-based analysis indicated that the lead compounds can be effective in downregulating macrophage activation induced by lipopolysaccharide and interferon-γ as well as cross-presentation by bone marrow-derived dendritic cells. Our results indicate that this class of inhibitors may be useful for the targeted downregulation of immune responses.

INTRODUCTION

The three members of the oxytocinase subfamily of M1 aminopeptidases, namely, endoplasmic reticulum aminopeptidases 1 and 2 (ERAP1 and ERAP2) and insulin regulated aminopeptidase (IRAP), henceforth referred to as antigen processing aminopeptidases (APAs), have been, during recent years, shown to have important biological functions, primarily in the regulation of human adaptive and innate immune responses. ERAP1 and ERAP2 act in intracellular antigen processing and are responsible not only for the correct generation of many antigenic epitopes but also for the destruction of others. They can regulate cellular immune responses to infected or diseased cells and contribute to immune evasion by pathogens and cancer cells as well as to autoimmunity. IRAP combines the specificity of ERAP1 and ERAP2 and functions in a specialized pathway of antigen processing and presentation called cross-presentation, present in dendritic cells (DCs), possibly regulating early stages of inflammatory immune responses. ERAP1 and ERAP2 are polymorphic, and several coding single nucleotide polymorphisms in these genes have been associated with predisposition to major human diseases, ranging from viral and parasitic infections to cancer and inflammatory diseases with autoimmune etiology (reviewed in refs 8 and 9). Recently, a secreted form of ERAP1 has also been implicated in innate immunity responses: macrophages activated by interferon-γ and lipopolysaccharides secrete ERAP1 in a TLR-dependent pathway, resulting in the enhancement of their phagocytic activity and inflammatory potential. Similarly, ERAP1 knockout mice show increased activation of NK and NKT cells, consistent with a role of ERAP1 in regulating both adaptive and innate immune responses. ERAP1 polymorphic variation was recently shown to quantitatively affect innate immune responses. Since natural polymorphic variation of ERAP1 and ERAP2 can generate a range of enzymatic activities in the population, it has been hypothesized that the activity of these enzymes is an important regulator of immune responses in humans. Inhibition of APAs is a promising approach for selectively regulating immune responses. ERAP1 knockout mice present a distinct repertoire of antigenic peptides on their cell sur-
Genetic downregulation of ERAP1 can lead to novel cellular immune responses, including nonclassical cellular responses, through the activation of cytotoxic T lymphocytes and natural killer cells. A pseudophosphinic peptide APA inhibitor has been demonstrated recently to enhance antigen presentation and elicit potent anti-cancer CTL responses toward a cryptic antigenic epitope that is normally destroyed by ERAP1. On the other hand, downregulation of ERAP1 has been recently shown to reduce CTL responses against a viral epitope associated with the pathogenesis of autoimmunity. As a result, the pharmacological regulation of APA activity has attracted significant scientific interest over the past few years as a promising area with therapeutic potential. Unfortunately, most characterized APA inhibitors suffer either from low potency or low selectivity, necessitating the exploration of other approaches. Selectively targeting only one of the members of this family of aminopeptidases may be highly desirable in order to fine-tune immune responses while minimizing potential side effects.

We recently demonstrated that using 3,4-diaminobenzoic acid (DABA) as a scaffold is a valid approach for generating inhibitors for this class of aminopeptidases with promising selectivity profiles. In this study, we explored the DABA derivatives more extensively, with the aim of improving their potency and selectivity toward ERAP1, ERAP2, and IRAP. By employing a rational, structure-based optimization procedure, we identified potent inhibitors for ERAP2 (237 nM) and IRAP (105 nM) as well as a highly selective ERAP2 inhibitor (755 nM) in comparison to ERAP1 (>100 μM).

To evaluate the biological potential of the discovered inhibitors, we utilized two specialized cellular assays relevant to innate and adaptive inflammatory immune responses. The low-micromolar ERAP1 inhibitor, 4a (2 μM), was able to inhibit macrophage activation by inflammatory mediators with submicromolar potency. The most potent IRAP inhibitor, 4u, was able to block the IRAP-dependent cross-presentation pathway in dendritic cells with low-nanomolar potency. These results suggest that these compounds may hold promise upon further development as targeted modulators of inflammatory immune responses.

## RESULTS AND DISCUSSION

### Inhibitor Design

Selection of the amino acid side chains used for inhibitor design was based on a previous study on the selectivity profile of the S1 subsite of ERAP1, ERAP2, and IRAP using a library of 82 fluorogenic substrates. Specifically, it was suggested that ERAP1 displays a general preference for substrates comprising long, aromatic or hydrophobic, R1 side chains (Figure 1). In contrast, the S1 subsite of ERAP2 exhibits higher selectivity for positively charged groups, especially Arg and homo-Arg, whereas IRAP combines the specificity of ERAP1 and ERAP2. All d-amino acid-based substrates were poorly processed by the three enzymes, suggesting that the tconfiguration is a prerequisite for binding. In our initial efforts, we utilized homo-Phe (hPhe), which is predicted to be ideally accommodated within the S1 specificity pocket of ERAP1 due to π-stacking interactions with a conserved aromatic residue (F433/450/544 for ERAP1/ERAP2/IRAP, respectively). In combination with the positively charged side chain of Lys for R2, 1,3-substituted derivative 4a displayed low-micromolar affinity for ERAP1 (IC50 = 2.0 ± 0.6 μM) and >10-fold selectivity for ERAP2. In this study, we explored the contribution of hydrophobic (Leu and nor-Leu), aromatic (Phe, Tyr, homo-Tyr(OBn), Ser-OBn), and positively charged Arg side chains at position R2, while keeping lysine in the R1 position (Figure 1).

Figure 1. (a) Crystallographic structure of ERAP1 in the closed conformation (PDB ID: 2YD0), illustrating the domain organization and the active site of the enzyme. The catalytic zinc is shown as a gray sphere, and protein residues are shown as sticks with carbon in cyan, nitrogen in blue, oxygen in red, and sulfur in yellow. (b) Schematic representation of the 1,4-disubstituted DABA inhibitors, where R1 and R2 are L-α-amino acid side chains targeting the S1 and Sn’ subsites of the aminopeptidases, respectively, and X = H, Me, or Bn. (c) Molecular model of the 1,4-disubstituted DABA scaffold (carbon atoms shown in orange) docked at the active site of ERAP1. The α-amino terminal docking residues are E183 and E320; the zinc-binding residues are H353, H357, and E376, whereas the catalytic E354 is expected to interact with the free aniline of the inhibitors. Catalytically important Y438 and conserved F433 that forms the bottom of S1 are also shown.

To evaluate the biological potential of the discovered inhibitors, we utilized two specialized cellular assays relevant to innate and adaptive inflammatory immune responses. The low-micromolar ERAP1 inhibitor, 4a (2 μM), was able to inhibit macrophage activation by inflammatory mediators with submicromolar potency. The most potent IRAP inhibitor, 4u, was able to block the IRAP-dependent cross-presentation pathway in dendritic cells with low-nanomolar potency. These
Chemistry. General synthetic routes for all new analogues are presented in Scheme 1. As described previously, 23 1,3-disubstituted DABA derivatives 4 were obtained through the coupling of N-Boc-protected amino acids, furnishing intermediates 2, followed by saponification of the corresponding methyl ester, formation of the second amide bond using O-methyl or O-benzyl protected amino acids, and global removal of the protecting groups. Following an analogous route, our approach was extended to furnish 1,4-disubstituted DABA derivatives 10 starting from 3-N-Boc protected DABA intermediate 7. In this case, the second amide bond formation at the less reactive 4-NH2 was achieved in higher yields by utilizing a combination of HATU as the coupling reagent and DBU as the base. As mentioned before, monosubstituted DABA derivatives 3, 6, and 9 were also prepared, as presented in Scheme 1, for direct comparison.

In Vitro Evaluation. The inhibitory potency of the synthesized compounds for the three enzymes was determined using an established fluorogenic assay. 23 Regarding monosubstituted DABA derivatives 3a–3g′ (Table 1), 6a–6f, and 9a–9d (Table 2), the in vitro evaluation did not reveal any low-micromolar (<10 μM) ERAP1 inhibition. In contrast, 4-amino substituted DABA derivatives 6e (R1=Nle) and 6f (R1=Arg) displayed IC50 values of 1.1 and 2.5 μM for ERAP2, respectively, whereas 6b (R1=Tyr(Obn)) exhibits IC50 = 1.7 μM for IRAP.

In the effort to optimize our previous hit 4a, we initially substituted R2=Lys for the shorter side chain of l-ornithine (Orn). Surprisingly, both the methyl ester and the carboxylic acid of 1,3-hPhe-DABA-Orn derivatives (4d and 4d′, respectively) were found to be inactive (IC50 > 100 μM) for all three aminopeptidases (Table 1), indicating a very important interaction associated with the longer Lys side chain. Substitution of hPhe by the shorter Phe side chain yielded compound 4e, which is inactive for ERAP1, although it is more potent than 4a for ERAP2 and IRAP, demonstrating the importance of the longer hPhe side chain, as proposed previously. 23 From the remaining 1,3-disubstituted DABA-Lys derivatives, 4e–4l, the in vitro evaluation revealed several moderate inhibitors (IC50 = 2–8 μM) for ERAP2 and IRAP only, with most compounds being inactive toward ERAP1 (Table 1).

The next generation of 1,3-disubstituted DABA derivatives, 4m–4y, was designed by combining Tyr, hTyr, Nle, and Arg for R1 with Val, Tyr, and Trp for R2 (Table 1). Although Tyr in the R1 position yielded low-affinity inhibitors (4m–4p′) for all

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Scheme 1. Synthesis of the 1,3- and 1,4-Substituted 3,4-Diaminobenzoic Acid Derivatives

Reagents and conditions: (a) protected amino acid (1.1–1.5 equiv), HBTU (2.0–3.0 equiv), DIEA (3.0–4.0 equiv), DMF, 4–12 h, rt, 65–92%; (b) LiOH 1 M (20 equiv), dioxane/H2O (1:1), 3–6 h, rt, 87–95%. (c) LiOH 1 M (20 equiv), dioxane/H2O (1:1), 3–6 h, rt, or cat. Pd/C (10 wt %), H2, MeOH, 1–2 h, rt, 90–95%. (d) TFA/CH2Cl2 (1:2), 20–40 min, rt, 95–98%. (e) (Boc)2O (1.0 equiv), Gu-HCl (15 mol %), EtOH, 40 min, 45 °C, 78%. (f) Protected amino acid (2.0 equiv), HATU (3.0 equiv), DBU (4.0 equiv), DMF, 24–36 h, rt, 45–72%. R1: side chain of l-α-amino acid selected from Leu, nor-Leu, Ser(Obn), Phe, homo-Phe, Tyr(Obn), homo-Tyr(Obn), or Arg(Z)2. R2: side chain of l-α-amino acid selected from Val, Thr, Tyr(OtBu), Trp, Lys(Boc), ornithine(Boc), or Arg(Z)2. R1′, R2′: the corresponding deprotected l-amino acid side chains. X: H, OMe, or Obn.
three enzymes, hTyr in combination with Trp displayed low-micromolar inhibition for IRAP (4q and 4r). In particular, 4q proved to be the most selective IRAP inhibitor against both ERAP1 and ERAP2 (IC50 > 100 μM). The combination of R1= Nle (4s−4v) or Arg (4w−4y) with R2= Tyr or Trp provided the most potent inhibitors. Compound 4u is the most potent inhibitor of ERAP1 (IC50 = 0.92 μM) and IRAP (IC50 = 105 nM), followed by its corresponding carboxylic acid 4u′ (IC50 = 296 nM for IRAP). It is interesting to note that 4s and 4t are both significantly selective inhibitors of IRAP (IC50 = 2.4 μM) with respect to ERAP1 (IC50 > 100 μM). As predicted, Arg in the R1 position provided two more submicromolar inhibitors, 4x and 4y, for both ERAP2 and IRAP, with 4x (IC50 = 518 nM) displaying a ~20-fold selectivity with respect to ERAP1 (IC50 = 9.6 μM).

Concerning the in vitro evaluation of the 1,4-disubstituted DABA derivatives (Table 2), our results indicate a significant structural effect from the orientation of the two amino acid substituents on their inhibitory effects, especially regarding ERAP1. A striking example is inactive compound 10a, which is the regiosomer of the initial ERAP1 hit, 4a (IC50 = 2.0 μM). In contrast, the inhibitory effect of low-micromolar IRAP inhibitors 4b (IC50 = 1.3 μM) and 4c (IC50 = 2.8 μM) is marginally affected at their corresponding para-isomers 10d (IC50 = 2.4 μM) and 10c (IC50 = 1.3 μM), respectively.

By employing Tyr in the R1 position, we obtained two more potent inhibitors, 10h, with IC50 = 1.1 μM for ERAP1, and 10h′, with IC50 = 1.3 μM for IRAP. In particular, 10h displayed a 2-fold increase in selectivity for ERAP1 compared to ERAP2, with respect to that of the initial hit, 4a. Interestingly, compound 10j′ (R1= hTyr and R2= Trp), the topoisomer of ERAP1-inactive 4r, exhibits an IC50 = 0.8 μM for ERAP1 with a ~12-fold selectivity with respect to ERAP2. Similar to the 1,3-analagous, the use of Nle at R1 (10m−10o′) in combination with Tyr or Trp at R2 provided the most potent ERAP2 and IRAP inhibitors. Among them, 10n, with IC50 = 755 nM for ERAP2, is the most selective inhibitor against ERAP1 (>130-fold), and 10o′ is the most potent inhibitor of ERAP2 (IC50 = 237 nM). The corresponding benzyl ester of the latter, 10o, displayed a higher potency for IRAP (IC50 = 438 nM) with respect to ERAP2 (IC50 = 709 nM). Finally, 10q with R1= Arg, the para-isomer of potent ERAP2/IRAP inhibitor 4x, provided an equipotent inhibitor of the three enzymes (IC50 = 1.4−2.6 μM).

Overall, the in vitro evaluation of the designed DABA derivatives revealed several potent inhibitors for the three M1 aminopeptidases. ERAP1 appears to be the most challenging target in terms of inhibitor potency and selectivity, with 4u being the most potent inhibitor of ERAP1 (IC50 = 0.92 μM). The corresponding carboxylic acid 4u′ displayed a higher potency for IRAP (IC50 = 296 nM) with respect to ERAP2 (IC50 = 709 nM). Finally, 10q with R1= Arg, the para-isomer of potent ERAP2/IRAP inhibitor 4x, provided an equipotent inhibitor of the three enzymes (IC50 = 1.4−2.6 μM).
(IC$_{50}$ = 0.92 μM) and 10j’ (IC$_{50}$ = 0.80 μM) being the most effective inhibitors and 10h being the most selective with respect to ERAP2 (~20-fold). On the other hand, our optimization efforts resulted in significantly more potent inhibitors of ERAP2 (10f’, IC$_{50}$ = 237 nM) and IRAP (4u, IC$_{50}$ = 105 nM). In addition, we identified two remarkably selective inhibitors, 10n, displaying IC$_{50}$ = 755 nM for ERAP2 and IC$_{50}$ > 100 μM for ERAP1, and 4s, with IC$_{50}$ = 1.3 μM for IRAP and IC$_{50}$ > 100 μM for both ERAP1 and ERAP2. Conclusively, the inhibitory potency of the synthesized compounds revealed that Nle as the R$_{1}$ and Trp as the R$_{3}$ substituents provided the most potent inhibitors for the three aminopeptidases. It is possible that the linear side chain of Nle is easily accommodated within the S1 pocket and facilitates the proper orientation of the inhibitors in both topologies of the DABA derivatives. The indole ring of Trp is most probably mediating key aromatic interactions at the S-primed subsite in addition to potential hydrogen-bonding interactions. Similar observations were drawn from the crystallographic structure of ERAP1 in complex with a phosphinic transition state analogue tripeptide comprising a Trp residue, which was found to be stacked with catalytic Y438.

**Structure–Activity Relationships.** Recent crystallographic studies revealed that ERAP1 can adopt at least two distinct conformational states that involve large interdomain motions, a closed, active-form (Figure 1a) and an open, inactive conformation that exposes a large internal peptide-binding cavity to the solvent. Although ERAP2 has been crystallized in the closed conformation only, the absence of substrate access into the active site in the published structure suggests that ERAP2 should be able to adopt open conformations as well. These two states differ in the organization of key active site residues and the adjacent S1 specificity pocket, which is disordered in the open state, making inhibitor design a challenging task. Specifically, the conserved ERAP1 residue F433 that comprises the bottom of the S1 pocket as well as the catalytic Y438 are both disordered or displaced in the open-state crystal structure.

It is therefore plausible that inhibitors that target these aminopeptidases may form initial interactions inside the active site at different configurations than those expected by analyzing the closed crystallographic structures. This may be exemplified by a lack of a strong zinc binding group serving as anchor point. As a result, molecular docking calculations of the synthesized compounds reveal multiple putative binding poses inside the active sites of all three aminopeptidases. Still, the most potent inhibitors display a common binding motif among the top-ranked predicted conformations, where the carbonyl group of R$_{1}$ is bound to zinc so that the R$_{1}$ side chain can be accommodated within the S1 pocket (Figure 1c). In such conformation, the α-amino acid terminal amine displays salt bridge interactions with two conserved glutamates, E183/200/295 and E320/337/431 (residue numbering of ERAP1/ERAP2/IRAP, respectively), and the free aniline could provide an additional hydrogen bond with the catalytic E354/371/465 residue. It is therefore possible that only the best inhibitors bind in the expected conformation through an induced-fit mechanism that promotes correct folding of the APA active site.

The docked conformation of the most potent ERAP1 inhibitor, 10j’, displays the side chain of its hTyr stacked above F433 inside the S1 pocket and, at the same time, forms a hydrogen bond with the backbone NH of S869 (Figure 2a), a residue that belongs to the C-terminal domain IV. The indole ring of 10j’ could interact with the H873 residue of domain IV, whereas its terminal carboxylic acid might form salt bridges with K308 and K403. Considering the bound conformation of the most selective inhibitor of ERAP2 (>100-fold with respect to ERAP1), 10n is predicted to bind in a similar orientation as that for 10j’. However, the phenolic ring in 10n could be accommodated between the aromatic side chains of Y892 and W363 so that a hydrogen bond is formed between its hydroxylate group and R345 of ERAP2 (Figure 2b). The nonconserved W363 residue of ERAP2 corresponds to G346 of ERAP1 and therefore such an interaction might not be possible between ERAP1 and 10n. The most potent IRAP inhibitor, 4u, is predicted to interact mostly with conserved residues and thus displays low-micromolar affinity for ERAP1 and ERAP2 as well. The high binding affinity of 4u for IRAP (105 nM) can be attributed to a potential stacking interaction of the indole moiety with Y961 and the two hydrogen bonds with S546 (Figure 2c). The terminal benzyl group of 4u is predicted to provide minor contacts; therefore, the corresponding deprotected analogue, 4u’, displayed a marginally lower binding affinity for IRAP (296 nM).

**Biological Evaluation of Inhibitors.** To assess the biological efficacy of this group of inhibitors we utilized two specialized cellular assays that relate to the function of the human innate and adaptive immune responses. It has been recently demonstrated that macrophages respond to the inflammatory modulator interferon-γ in combination with lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, by secreting ERAP1 in a
The aminopeptidase activity of the secreted ERAP1 induces the enhancement of phagocytosis by macrophages, which can be followed by the internalization of fluorescently labeled latex beads (Figure 3a). To evaluate the effect of the inhibitors on this process, we measured the total cell fluorescence in stimulated and unstimulated cells. Total fluorescence enhancement upon macrophage stimulation was in the range of 100−150%, which is a sufficient signal change to detect inhibitory effects (Figure 3b). Adding compounds 24 h premeasurement revealed a dose dependent reduction in phagocytosis with calculated ED_{50} values (Figure 3c,d) that correspond well to the in vitro IC_{50} values calculated for ERAP1 using recombinant protein, suggesting that the biological effect was indeed mediated through the inhibition of ERAP1.

Furthermore, we evaluated the effectiveness of the most potent IRAP inhibitor, 4u, on blocking cross-presentation by bone marrow-derived DCs (BMDCs). To assess the specificity of the inhibitor, we utilized BMDCs from both wild-type and IRAP knockout mice. We first confirmed that 4u is able to inhibit mouse IRAP using mouse recombinant enzyme and discovered that this compound is 2-fold more effective for mouse IRAP (IC_{50,mouse} = 52 nM, Supporting Information Figure S1) compared to that for human IRAP (IC_{50,human} = 105 nM). Mouse BMDCs were first exposed to yeast cells expressing surface ovalbumin and then added to CD8^+ T cells from the lymph nodes of transgenic mice expressing a T cell receptor specific for the peptide ovalbumin247−254. T cell activation by the cross-presented epitope was read out as IL-2 secretion (Figure 4a). The same experiment was performed in the presence of increasing doses of 4u (Figure 4b). In the presence of the inhibitor, cross-presentation was reduced in a dose-dependent manner with an ED_{50} of 6.5 nM. No effect was seen when we used BMDCs from IRAP knockout mice, indicating that the effect is specific for the IRAP-dependent cross-presentation pathway. The surprisingly high effectiveness of the inhibitor in this setting may be related to the compartmentalization of the cross-presentation process in which high local concentrations may be obtained due to continued inhibitor internalization into phagosomes and endosomes.

To date, only a series of phosphinic pseudopeptide transition state analogues has been reported as potent inhibitors of all three APAs,\textsuperscript{21} albeit with low selectivity among the three enzymes. Specifically, the most potent inhibitor displayed IC_{50} values of 33, 11, and 30 nM for ERAP1, ERAP2, and IRAP, respectively. On the basis of the observation that peptide inhibitors of IRAP can enhance cognitive functions, peptidomimetic analogues of angiotensin IV have been designed using a wide range of synthetic approaches.\textsuperscript{28−30} Among the most potent angiotensin IV mimetics reported, a series of macrocyclic compounds attained inhibition constants as low as 1.8 nM for IRAP.\textsuperscript{30} With regard to small molecule inhibitors of IRAP, a series of benzopyran compounds that had been identified through a virtual screening approach using a homology model of IRAP was led to the development of the first lead compound with K_i = 30 nM through a medicinal chemistry campaign.\textsuperscript{32} Recently, a series of arylsulfonamides was identified as being moderate inhibitors of IRAP, with the most potent exhibiting IC_{50} = 1.1 \mu M.\textsuperscript{33} These IRAP inhibitors, however, have either not been tested for their ability to inhibit ERAP1 and ERAP2 or have been tested and found not to be active.\textsuperscript{31} As a result, the 3,4-diaminobenzoic acid derivatives described here appear to constitute not only an alternative potent chemical tool for controlling APA activity but also one with very good selectivity properties.

**CONCLUSIONS**

In summary, we have investigated the ability of 3,4-diaminobenzoic acid derivatives to act as inhibitors of three critical aminopeptidases involved in the human innate and adaptive immune response, ERAP1, ERAP2, and IRAP. Our analysis revealed potent inhibitors for ERAP2 and IRAP as well as excellent selectivity profiles for all three enzymes. Furthermore, our analysis confirmed previously hypothesized difficulties in the rational design of inhibitors for this class of enzymes based on existing crystal structures, especially for ERAP1, possibly due to the conformational heterogeneity of ERAP1.
responses brought about by high ERAP2 expression, such as immune responses toward immune-evading cancer cells.35 solvents and reagents for organic synthesis were obtained from and subsequently to CD8+ T cells purified from mice were exposed to several ratios of ovalbumin-expressing yeast cells EXPERIMENTAL SECTION 34a This enzyme that greatly affects the folding of the S1 specificity pocket. Our best compounds were found to be active in specialized cellular assays related to their biological functions of the targeted enzymes in regulation of the adaptive and innate immune responses. We conclude that this class of compounds may find important applications in the regulation of inflammatory human immune responses for the treatment of a variety of major human diseases, varying from viral infections to cancer and autoimmunity. More specifically, ERAP1 inhibitors such as 4a may be useful for reducing inflammation by controlling macrophage activation11 and selective ERAP2 inhibitors like 10n may be useful for controlling autoimmune responses brought about by high ERAP2 expression, such as that seen in birdshot chorioretinopathy,34 or for enhancing immune responses toward immune-evading cancer cells.35 Finally, potent IRAP inhibitors such as 4u or 4u’ may find uses in controlling inflammatory autoimmune diseases or as cognitive enhancers.36,32

**EXPERIMENTAL SECTION**

**Materials and General Procedures.** Unless otherwise noted, all solvents and reagents for organic synthesis were obtained from commercial suppliers and used without further purification. Reactions requiring anhydrous conditions were carried out in flame-dried (vacuum <0.5 Torr) glassware, using anhydrous, freshly distilled solvents and under an Ar atmosphere. All reactions were stirred with Teflon-coated magnetic stir bars, and temperatures were measured externally. Yields refer to chromatographically and spectroscopically (1H NMR) homogeneous materials. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel plates (60 F254, E. Merck). Silica gel (60, particle size: 0.040–0.063 mm, E. Merck) was used for flash column chromatography. NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 298 K using residual undeuterated solvent for 1H NMR [δH = 7.26 (CDCl3), 2.50 (DMSO-d6), and 3.31 (MeOH-d4) ppm] and 13C undeuterated solvent for 13C NMR [δC = 77.16 (CDCl3), 39.52 (DMSO-d6), and 49.00 (MeOH-d4) ppm] as an internal reference. The following abbreviations were used to designate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Quantitation of the final compounds was achieved using an internal standard of 2,5-dimethylfuran (DMFu, 0.1 mM in MeOD).37 High-resolution mass spectra (HRMS) were measured on Agilent 6224 Accurate Mass TOF LC/MS at the Faculty of Chemistry and Chemical Technology, University of Ljubljana. Compounds were purified by reversed-phase HPLC on a C18 chromolith column (Merck) using a 0–50% (v/v) acetonitrile gradient in water containing 0.05% TFA while following the absorbance at 257 nm. The purity of all final compounds employed in the biological assays was greater than 95%, and the structures of the synthesized compounds were determined by 1H NMR, 13C NMR, and high-resolution mass spectroscopy (HRMS).

**General Methods for the Synthesis of the Inhibitors.** Amino Acid Coupling (Method A). A mixture of 1 (1.0 equiv), the desired N-Boc protected amino acid (1.1 equiv), HBTU (2.0 equiv), and DIEA (3.0 equiv) in anhydrous DMF (0.25 mM) was stirred under an Ar atmosphere at ambient temperature for 4 h. Similarly, a mixture of the carboxylic acid derivative of 2 or 7 (1.0 equiv), the desired O-Me or O-Bn protected amino acid (1.5 equiv), HBTU (3.0 equiv), and DIEA (4.0 equiv) in anhydrous DMF (0.25 mM) was stirred under an Ar atmosphere at ambient temperature for 8–12 h. For the preparation of the 1,4-substituted compounds, a mixture of 5 or 8 (1.0 equiv), the N-Boc protected amino acid (2.0 equiv), HATU (3.0 equiv), and DBU (4.0 equiv) in anhydrous DMF (0.25 mM) was stirred under an Ar atmosphere at ambient temperature for 24–36 h. The reaction mixture was then diluted with EtOAc and washed sequentially with aqueous HCl 1.0 N, sat. NaHCO3, and sat. NaCl. Consequently, the solvent was evaporated under reduced pressure to provide the crude product, which was purified using a gradient of EtOAc in hexanes to yield the desired products in yields of 45–92%.

**Saponification of Methyl Esters (Method B).** The desired compound was dissolved in a mixture of 1:1 dioxane/aqueous LiOH 1.0 M (20 equiv) under rigorous stirring. After 3–6 h (monitored by TLC) at ambient temperature, the mixture was diluted with aqueous sat. NaCl and the organic solvent was evaporated. The remaining aqueous solution (pH 12) was washed with EtOAc and then acidified with HCl 1.0 N until no further precipitation could be observed (pH 2). The aqueous phase was then extracted three times with EtOAc, and the combined organic extracts were dried with MgSO4, filtered, and washed with EtOAc. After evaporating the solvents and drying under high vacuum, the product was obtained at yields of 87–95% and purity >95% (NMR).

**General Procedure for the Deprotection of O-Bn and N-Cbz Groups (Method C).** The desired compound (0.1 mmol) was dissolved in MeOH (4 mL, 0.025 mM), and the solution was degassed under an Ar atmosphere. A catalytic amount of activated Pd/C 10% was added and degassed under Ar, followed by the introduction of H2 gas. The mixture was stirred under H2 at ambient temperature for 1–2 h (monitored by TLC) and then filtered through Celite and washed with MeOH. The resulting product was acquired in yields of 90–95%.

**General Procedure for the Deprotection of N-Boc and O-Tbu Groups (Method D).** The desired compound (0.1 mmol) was dissolved in a mixture of CH2Cl2/TFA (2:1 mL, 0.033 mM) and...
stirred at ambient temperature for 20–40 min (monitored by TLC). Consequently, the solvents were evaporated, and the product was treated with HCl 1.0 N (0.5 mL). The solvents were evaporated using toluene (2 × 2 mL) to azeotrop the remaining aqueous solution and dried under high vacuum overnight to yield the hydrochloric salt of the amine in yields of 95–99%.

**Methyl 4-Amino-3-((tert-butoxycarbonyl)amino)benzoate (5).** To a stirred solution of 1 (500 mg, 3.0 mmol) and guHCl (45 mg, 0.45 mmol) in 5 mL of EtOH was added (Boc)2O (650 mg, 3.0 mmol), and the mixture was heated at 45°C for 40 min. Subsequently, ethanol was evaporated under reduced pressure, and the reaction mixture was redissolved in EtOAc and washed sequentially with aqueous HCl 1.0 N, sat. NaHCO3, and sat. NaCl. The organic layer was evaporated under reduced pressure to provide the crude product, which was purified using a gradient of 35–65% EtOAc in hexanes to yield the N-Boc protected amine 5 (665 mg, 2.5 mmol, 83%).

**1H NMR (500.13 MHz, CDCl3):** δ 7.82 (s, 1H), 7.56 (dd, J = 8.4, 1.7 Hz, 1H), 6.76 (s, 1H), 6.58 (d, J = 8.4 Hz, 1H), 4.39 (s, 2H), 3.74 (s, 3H), 2.80 (m, 2H). 13C NMR (125.76 MHz, CDCl3): δ 171.9, 169.4, 160.1, 145.8, 138.6, 131.8, 131.8, 130.5, 130.5, 129.5, 129.5, 128.9, 128.9, 128.6, 127.9, 127.9, 119.3, 116.7, 116.7, 71.1, 56.4, 52.6, 38.0. HRMS (ESI): m/z calcd for C25H27N3O4 +H+ [M + H+] 434.2074; found, 434.2079.

**Methyl 4-Amino-3-(2-amino-4-(benzylxoy)phenyl)butanamido)benzoate (3c).**

**1H NMR (500.13 MHz, MeOH-d4):** δ 7.95 (s, 1H), 7.90 (dd, J = 8.4, 1.8 Hz, 1H), 7.42–7.37 (m, 2H), 7.37–7.32 (m, 2H), 7.32–7.25 (m, 2H), 7.20 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 5.02 (s, 2H), 4.33–4.26 (m, 1H), 3.87 (s, 3H), 2.84–2.74 (m, 2H), 2.42–2.33 (m, 1H), 2.32–2.24 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 169.9, 167.3, 158.9, 138.8, 133.5, 130.4, 130.4, 130.1, 130.1, 130.1, 129.4, 128.9, 128.9, 128.5, 128.5, 127.9, 127.9, 127.3, 122.1, 116.2, 116.2, 71.1, 55.0, 52.8, 34.5, 31.3. HRMS (ESI): m/z calcd for C24H23N3O4 +H+ [M + H+] 344.1605; found, 344.1607.

**Methyl 4-Amino-3-(2-amino-4-(4-hydroxyphenyl)pentanamido)benzoate (3e).**

**1H NMR (500.13 MHz, MeOH-d4):** δ 7.93 (s, 1H), 7.86 (d, J = 8.4 Hz, 1H), 7.21 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 8.3 Hz, 2H), 6.73 (d, J = 8.3 Hz, 2H), 4.30–4.22 (m, 1H), 3.88 (s, 3H), 2.80–2.68 (m, 2H), 2.41–2.28 (m, 1H), 2.28–2.20 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 169.9, 167.6, 157.1, 140.4, 131.9, 130.3, 130.3, 130.2, 129.1, 126.7, 125.8, 121.0, 116.5, 116.5, 54.9, 52.7, 34.8, 31.3. HRMS (ESI): m/z calcd for C25H25N3O4 +H+ [M + H+] 344.1605; found, 344.1607.
(S)-4-Amino-3-(2-amino-4-methylpentanamido)benzoic Acid (3e).  

\[
\text{H NMR (500.13 MHz, MeOH-} d_4\text{): } \delta 7.92 (d, J = 8.3 \text{ Hz, } 1H), 7.69 (s, 1H), 7.55 (d, J = 8.3 \text{ Hz, } 1H), 7.47–7.34 (m, 5H), 4.64–4.55 (m, 1H), 4.53–4.46 (m, 1H), 3.51–3.43 (m, 1H), 3.29–3.21 (m, 1H), 3.00–2.93 (m, 2H), 2.12–1.88 (m, 2H), 1.83–1.70 (m, 2H), 1.68–1.50 (m, 2H).  
\]

13C NMR (125.76 MHz, MeOH-\(d_4\)): \(\delta\) 175.0, 173.0, 169.8, 168.3, 153.4, 150.8, 143.0, 130.3, 130.3, 129.1, 128.1, 127.1, 127.0, 125.0, 56.4, 54.2, 40.5, 38.4, 31.8, 28.1, 24.2. HRMS (ESI): \(m/z\) calcd for \(C_{23}H_{20}NO_5 + H^+ [M + H]^+\), 428.2292; found, 428.2299.

(S)-6-Amino-2-(4-amino-3-(4-hydroxyphenanilamido)benzamido)hexanoic Acid (4e).

\[
\text{H NMR (500.13 MHz, MeOH-} d_4\text{): } \delta 7.92 (d, J = 8.3 \text{ Hz, } 1H), 7.82 (s, 1H), 7.59 (d, J = 8.3 \text{ Hz, } 1H), 7.25 (d, J = 8.1 \text{ Hz, } 2H), 6.83 (d, J = 8.1 \text{ Hz, } 2H), 4.62–4.55 (m, 1H), 4.49–4.38 (m, 1H), 3.45–3.37 (m, 1H), 3.19–3.10 (m, 1H), 3.02–2.91 (m, 2H), 2.09–1.90 (m, 2H), 1.85–1.70 (m, 2H), 1.67–1.48 (m, 2H).  
\]

13C NMR (125.76 MHz, MeOH-\(d_4\)): \(\delta\) 175.1, 170.0, 168.3, 158.3, 141.3, 131.8, 129.6, 129.5, 128.1, 127.0, 125.9, 125.3, 117.0, 117.0, 56.6, 54.3, 40.5, 37.6, 31.7, 28.1, 24.2. HRMS (ESI): \(m/z\) calcd for \(C_{23}H_{20}NO_5 + H^+ [M + H]^+\), 444.2241; found, 444.2251.
(S)-Methyl 6-Amino-2-(4-amino-3-((S)-2-amino-4-(4-hydroxyphenyl)butanamido) benzamido)hexanoate (4g).

\[ \text{(S)-Methyl 6-Amino-2-(4-amino-3-((S)-2-amino-4-(4-benzoyloxy)phenyl)butanamido) benzamido)hexanoate (4i).} \]

\[ \text{(S)-Methyl 6-Amino-2-(4-amino-3-((S)-2-amino-4-(4-benzoyloxy)propanamido) benzamido)hexanoate (4h).} \]

\[ \text{(S)-Methyl 6-Amino-2-(4-amino-3-((S)-2-amino-3-(4-hydroxyphenyl)butanamido) benzamido)hexanoate (4i).} \]

\[ \text{(S)-Methyl 6-Amino-2-(4-amino-3-((S)-2-amino-3-(4-benzoyloxy)propanamido) benzamido)hexanoate (4j).} \]

\[ \text{(S)-6-Amino-2-(4-amino-3-((S)-2-amino-4-methylpentanamido)benzamido)hexanoic Acid (4j).} \]
1H NMR (500.13 MHz, MeOH-d4): δ 7.78 (d, J = 8.2 Hz, 1H), 7.69 (s, 1H), 7.41–7.29 (m, 6H), 7.21 (d, J = 8.3 Hz, 2H), 6.83 (d, J = 8.3 Hz, 2H), 6.31 (d, J = 8.4 Hz, 2H), 6.10 (d, J = 8.4 Hz, 2H), 4.87 (s, 1H), 3.72 (s, 3H), 3.34–3.15 (m, 2H), 3.06–2.90 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 173.7, 169.9, 168.4, 158.5, 157.4, 132.9, 131.8, 131.8, 131.2, 129.0, 128.9, 128.0, 127.2, 125.9, 123.1, 117.1, 117.1, 116.9, 116.3, 116.3, 56.6, 56.2, 52.8, 37.8, 37.4. HRMS (ESI): m/z calcd for C21H26N4O5 +H+ [M + H+], 415.1976; found, 415.1982.

(S)-Methyl 2-(4-Amino-3-((S)-2-amino-3-(4-hydroxyphenyl)propanoamido)benzamido)-3-methylbutanoic Acid (4m).

1H NMR (500.13 MHz, MeOH-d4): δ 7.80 (d, J = 8.4 Hz, 1H), 7.69 (s, 1H), 7.26 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.3 Hz, 2H), 6.31 (d, J = 8.4 Hz, 2H), 6.10 (d, J = 8.4 Hz, 2H), 4.87 (s, 1H), 3.72 (s, 3H), 3.34–3.15 (m, 2H), 3.06–2.90 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 169.6, 169.2, 165.1, 158.5, 158.2, 131.7, 131.7, 128.3, 127.5, 125.9, 120.6, 120.5, 120.4, 117.1, 117.1, 68.6, 60.3, 56.7, 52.8, 37.4, 20.1. HRMS (ESI): m/z calcd for C22H25N3O5 +H+ [M + H+], 431.1925; found, 431.1931.

(S)-Benzyl 2-(4-Amino-3-((S)-2-amino-3-(4-hydroxyphenyl)propanamido)benzamido)-3(1H-indol-3-yl)propanoate (4p).

1H NMR (500.13 MHz, MeOH-d4): δ 7.55 (d, J = 7.9 Hz, 1H), 7.49 (s, 1H), 7.46–7.41 (m, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.30–7.25 (m, 3H), 7.19–7.14 (m, 2H), 7.14–7.05 (m, 3H), 7.03–6.97 (m, 2H), 6.78–6.71 (m, 3H), 5.07 (s, 2H), 4.93–4.87 (m, 1H), 4.38–3.76 (m, 1H), 3.43–3.37 (m, 1H), 3.35–3.33 (m, 1H), 3.05–2.97 (m, 1H), 2.95–2.86 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 175.3, 173.8, 169.8, 157.5, 148.0, 138.1, 137.1, 131.6, 131.6, 129.5, 129.2, 129.2, 128.9, 128.8, 128.3, 127.7, 124.5, 123.1, 122.5, 122.2, 122.0, 119.2, 117.2, 116.7, 116.6, 116.6, 116.2, 112.5, 110.6, 68.2, 57.8, 55.6, 41.5, 28.6. HRMS (ESI): m/z calcd for C34H33N5O5 +H+ [M + H+], 592.2554; found, 592.2564.

(S)-2-(4-Amino-3-((S)-2-amino-3-(4-hydroxyphenyl)propanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (4p).

1H NMR (500.13 MHz, MeOH-d4): δ 7.71–7.50 (m, 3H), 7.39–7.29 (m, 2H), 7.20 (d, J = 8.4 Hz, 2H), 7.15 (s, 1H), 7.11–7.02 (m, 1H), 7.02–6.63 (m, 1H), 6.82 (d, J = 8.4 Hz, 2H), 4.95–4.90 (m, 1H), 4.42–4.30 (m, 1H), 3.55–3.40 (m, 1H), 3.40–3.32 (m, 1H), 3.25–3.15 (m, 1H), 3.14–3.04 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 175.1, 169.9, 168.2, 158.4, 138.1, 133.9, 131.7, 131.7, 129.5, 128.8, 127.9, 127.0, 125.8, 124.5, 123.6, 122.4, 122.1, 119.9, 119.3, 117.1, 117.1, 112.4, 111.1, 56.5, 55.5, 37.8, 28.3. HRMS (ESI): m/z calcd for C42H41N5O5 +H+ [M + H+], 696.3180; found, 696.3180.

(S)-Benzyl 2-(4-Amino-3-((S)-2-amino-4-(benzoxyl)phenyl)butanamido) benzamido)-3-(1H-indol-3-yl)propanoate (4q).

1H NMR (500.13 MHz, MeOH-d4): δ 7.75–7.65 (m, 2H), 7.55–7.48 (m, 1H), 7.41–7.11 (m, 1H), 7.07–6.86 (m, 5H), 5.06 (br.d, 2H), 4.99 (br.d, 2H), 4.95–4.89 (m, 1H), 4.38–4.22 (m, 1H), 3.49–3.35 (m, 1H), 3.36–3.30 (m, 1H), 2.87–2.67 (m, 2H), 2.41–2.31 (m, 1H), 2.29–2.19 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 173.4, 170.1, 168.4, 158.9, 138.7, 138.0, 137.0, 134.9, 133.5, 132.8, 130.4, 130.4, 129.4, 129.4, 129.2, 129.1, 128.9, 128.8, 128.8, 128.7, 128.5, 128.5, 128.1, 127.0, 124.6, 123.2, 122.5, 119.9, 119.1, 116.2, 116.2, 112.4, 110.7, 71.0, 68.0, 55.9, 54.9, 34.6, 31.4, 28.3. HRMS (ESI): m/z calcd for C45H41N4O6 +H+ [M + H+], 702.2085; found, 702.2080.
(S)-2-(4-Amino-3-(3-(S)-2-amino-4-(4-hydroxyphenyl)butanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (4r).

1H NMR (500.13 MHz, MeOH-d4): δ 7.65 (s, 1H), 7.59–7.39 (m, 3H), 7.29 (br.d, 1H), 7.14–6.91 (m, 5H), 6.74 (br.d, 2H), 4.71–4.49 (m, 1H), 4.30–4.04 (m, 1H), 3.46–3.38 (m, 1H), 3.30–3.28 (m, 1H), 2.76–2.64 (m, 2H), 2.52–2.22 (m, 1H), 2.23–2.13 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 175.3, 169.8, 168.9, 157.1, 141.2, 138.0, 132.0, 130.3, 130.3, 128.4, 128.2, 127.6, 127.3, 125.5, 124.4, 122.4, 120.0, 119.8, 119.0, 118.9, 118.2, 118.2, 7.83 (m, 2H), 7.51 (d, J = 7.8 Hz, 1H), 7.48 (d, J = 7.8 Hz, 1H), 7.40–7.32 (m, 2H), 7.40–7.25 (m, 3H), 7.22–7.16 (m, 2H), 7.11–7.06 (m, 1H), 7.04 (s, 1H), 7.01–6.95 (m, 1H), 5.09 (s, 2H), 4.24–4.16 (m, 1H), 3.48–3.38 (m, 1H), 3.38–3.32 (m, 2H), 2.12–2.04 (m, 1H), 2.03–1.93 (m, 1H), 1.56–1.39 (m, 4H), 1.02–0.94 (m, 3H). 13C NMR (125.76 MHz, MeOH-d4): δ 173.4, 170.4, 168.3, 157.0, 133.3, 129.5, 129.5, 129.2, 129.9, 129.1, 128.7, 128.0, 127.0, 124.5, 123.5, 123.4, 122.5, 119.9, 119.1, 112.4, 110.8, 68.0, 55.9, 55.1, 32.3, 28.3, 28.1, 14.0. HRMS (ESI): m/z calcd for C30H29N4O4 + H+ [M + H]+, 452.2762; found, 452.2759.

(5)-Benzyl 2-(4-Amino-3-(3-(S)-2-aminohexanamido)benzamido)-3-methylbutanoate (4s).

1H NMR (500.13 MHz, MeOH-d4): δ 7.78 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.54 (d, J = 7.8 Hz, 1H), 7.40–7.30 (m, 2H), 7.30–7.25 (m, 3H), 7.22–7.16 (m, 2H), 7.11–7.06 (m, 1H), 7.04 (s, 1H), 7.01–6.95 (m, 1H), 5.09 (s, 2H), 4.24–4.16 (m, 1H), 3.48–3.38 (m, 1H), 3.38–3.32 (m, 2H), 2.12–2.04 (m, 1H), 2.03–1.93 (m, 1H), 1.56–1.39 (m, 4H), 1.02–0.94 (m, 3H). 13C NMR (125.76 MHz, MeOH-d4): δ 173.4, 170.4, 168.3, 157.0, 133.3, 129.5, 129.5, 129.2, 129.9, 129.1, 128.7, 128.0, 127.0, 124.5, 123.5, 123.4, 122.5, 119.9, 119.1, 112.4, 110.8, 68.0, 55.9, 55.1, 32.3, 28.3, 28.1, 14.0. HRMS (ESI): m/z calcd for C30H29N4O3 + H+ [M + H]+, 365.2182; found, 365.2177.

(5)-Methyl 2-(4-Amino-3-(3-(S)-2-aminohexanamido)benzamido)-3-(4-hydroxyphenyl)propanoate (4t).

1H NMR (500.13 MHz, MeOH-d4): δ 7.80 (s, 1H), 7.70–7.64 (m, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.41–7.36 (m, 1H), 7.32 (d, J = 7.8 Hz, 1H), 7.15 (s, 1H), 7.09–7.03 (m, 1H), 7.00–6.93 (m, 1H), 4.93–4.90 (m, 1H), 4.26–4.18 (m, 1H), 3.53–3.44 (m, 1H), 3.37–3.32 (m, 1H), 2.14–2.04 (m, 1H), 2.02–1.94 (m, 1H), 1.55–1.40 (m, 4H), 1.02–0.92 (m, 3H). 13C NMR (125.76 MHz, MeOH-d4): δ 175.1, 170.5, 168.2, 138.0, 134.3, 133.2, 129.9, 128.8, 127.9, 126.9, 124.5, 124.0, 122.4, 119.8, 119.2, 112.3, 111.2, 55.5, 55.1, 32.2, 28.2, 28.1, 14.4. HRMS (ESI): m/z calcd for C30H29N4O3 + H+ [M + H]+, 452.2292; found, 452.2302.
(S)-2-(4-Amino-3-((S)-2-amino-5-guanidinopentanamido)-benzamido)-3-methylbutanoic Acid (4w).

1H NMR (500.13 MHz, MeOH-d4): δ 7.91 (s, 1H), 7.83 (d, J = 8.3 Hz, 1H), 7.44–7.37 (m, 1H), 4.50–4.45 (m, 1H), 4.33–4.27 (m, 1H), 3.36–3.32 (m, 2H), 2.35–2.05 (m, 3H), 1.89–1.80 (m, 2H), 1.08–1.01 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 174.9, 169.9, 169.1, 169.0, 158.7, 158.3, 128.1, 127.2, 122.5, 122.3, 60.0, 54.6, 41.8, 31.7, 29.7, 25.6, 19.7, 19.6. HRMS (ESI): m/z calc'd for C13H10N3O4 + H+ [M + H+], 240.2354; found, 240.2354.

(S)-Methyl 2-(4-Amino-3-((S)-2-amino-5-guanidinopentanamido)benzamido)-3-(4-hydroxyphenyl)propanoate (4x).

1H NMR (500.13 MHz, MeOH-d4): δ 7.90 (s, 1H), 7.76 (brd, 1H), 7.60–7.47 (m, 1H), 7.08 (brd, 2H), 6.70 (brd, 2H), 4.82–4.74 (m, 1H), 4.39–4.29 (m, 1H), 3.72 (s, 3H), 3.40–3.27 (m, 2H), 3.26–3.14 (m, 1H), 3.13–2.97 (m, 1H), 2.25–2.06 (m, 2H), 1.92–1.76 (m, 2H). 13C NMR (125.76 MHz, MeOH-d4): δ 173.6, 170.0, 167.9, 158.6, 157.3, 151.2, 135.8, 131.2, 131.2, 129.0, 127.9, 126.8, 126.6, 125.3, 116.3, 116.3, 56.3, 54.6, 52.8, 41.8, 37.3, 29.6, 25.6. HRMS (ESI): m/z calc'd for C20H17N3O4 + H+ [M + H+], 409.2459; found, 408.2456.

(S)-2-(4-Amino-3-((S)-2-amino-5-guanidinopentanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (4y).

1H NMR (500.13 MHz, MeOH-d4): δ 7.83–7.55 (m, 2H), 7.39–7.21 (m, 2H), 7.20–6.88 (m, 4H), 4.32–4.24 (m, 1H), 3.93–3.81 (m, 1H), 3.69–3.49 (m, 2H), 3.34–3.40 (m, 2H), 2.17–2.00 (m, 2H), 1.88–1.76 (m, 2H). 13C NMR (125.76 MHz, MeOH-d4): δ 174.1, 170.5, 169.7, 168.6, 158.6, 138.0, 128.0, 127.2, 125.9, 124.5, 122.5, 121.6, 120.1, 119.8, 119.6, 119.1, 112.4, 110.9, 55.6, 54.5, 41.8, 29.7, 28.3, 25.6. HRMS (ESI): m/z calc'd for C20H17N3O4 + H+ [M + H+], 495.2463; found, 495.2470.

(S)-Methyl 3-Amino-4-(2-amino-4-phenylbutanamido)benzoate (6a).

1H NMR (500.13 MHz, MeOH-d4): δ 8.17–8.06 (m, 2H), 7.67–7.58 (m, 1H), 7.35–7.27 (m, 4H), 7.25–7.18 (m, 1H), 4.45–4.36 (m, 1H), 3.94 (s, 3H), 2.90–2.80 (m, 2H), 2.48–2.37 (m, 1H), 2.36–2.26 (m, 1H). 13C NMR (125.76 MHz, MeOH-d4): δ 170.1, 166.4, 141.2, 135.6, 131.2, 131.1, 130.7, 129.7, 129.6, 129.4, 127.6, 127.1, 126.6, 126.5, 55.2, 53.1, 34.5, 32.3. HRMS (ESI): m/z calc'd for C16H14N3O3 + H+ [M + H+], 328.1652; found, 328.1661.
(S)-Methyl 6-Amino-2-(3,4-diaminobenzamido)hexanoate (9a).

1H NMR (500.13 MHz, MeOH-d$_4$): δ 7.81 (s, 1H), 7.69 (dd, J = 8.3 Hz, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.20–7.11 (m, 4H), 7.11–6.88 (m, 4H), 5.09 (s, 2H), 4.92–4.85 (m, 1H), 3.47–3.34 (m, 2H). $^{13}$C NMR (125.76 MHz, MeOH-d$_4$): δ 173.6, 168.8, 152.0, 143.4, 138.1, 137.1, 132.1, 129.5, 129.2, 128.6, 126.9, 125.4, 125.4, 124.5, 119.9, 119.2, 118.3, 117.6, 112.4, 110.6, 68.0, 55.8, 28.3. HRMS (ESI): $m/z$ calc for C$_{29}$H$_{34}$N$_4$O$_4$ +H$^+$ [M + H$^+$], 503.2653; found, 503.2649.

(S)-Methyl 2,3,4-Diaminobenzamido)-3-(4-hydroxyphenyl)-propanoate (9b).

1H NMR (500.13 MHz, MeOH-d$_4$): δ 7.63 (s, 1H), 7.58–7.46 (m, 2H), 7.38–7.23 (m, 4H), 7.20–7.11 (m, 2H), 7.11–6.88 (m, 4H), 5.09 (s, 2H), 4.92–4.85 (m, 1H), 3.47–3.34 (m, 2H). $^{13}$C NMR (125.76 MHz, MeOH-d$_4$): δ 173.6, 168.8, 152.0, 143.4, 138.1, 137.1, 132.1, 129.5, 129.2, 128.6, 126.9, 125.4, 125.4, 124.5, 122.5, 119.9, 119.2, 118.3, 117.6, 112.4, 110.6, 68.0, 55.8, 28.3. HRMS (ESI): $m/z$ calc for C$_{29}$H$_{34}$N$_4$O$_4$ +H$^+$ [M + H$^+$], 503.2619; found, 503.2649.

(S)-2,3,4-Diaminobenzamido)-3-(1H-indol-3-yl)propanoic Acid (9c).

1H NMR (500.13 MHz, MeOH-d$_4$): δ 7.63 (s, 1H), 7.58–7.46 (m, 2H), 7.38–7.23 (m, 4H), 7.20–7.11 (m, 2H), 7.11–6.88 (m, 4H), 5.09 (s, 2H), 4.92–4.85 (m, 1H), 3.47–3.34 (m, 2H). $^{13}$C NMR (125.76 MHz, MeOH-d$_4$): δ 173.6, 168.8, 152.0, 143.4, 138.1, 137.1, 132.1, 129.5, 129.2, 128.6, 126.9, 125.4, 125.4, 124.5, 122.5, 119.9, 119.2, 118.3, 117.6, 112.4, 110.6, 68.0, 55.8, 28.3. HRMS (ESI): $m/z$ calc for C$_{29}$H$_{34}$N$_4$O$_4$ +H$^+$ [M + H$^+$], 503.2619; found, 503.2649.

H NMR (500.13 MHz, MeOH-d$_4$): δ 8.13 (s, 1H), 8.08 (dd, J = 8.4, 1.8 Hz, 1H), 7.71 (d, J = 8.4 Hz, 1H), 4.40–4.33 (m, 1H), 3.94 (s, 3H), 3.29–3.24 (m, 2H), 2.25–2.16 (m, 1H), 2.13–2.04 (m, 1H), 1.87–1.78 (m, 2H). $^{13}$C NMR (125.76 MHz, MeOH-d$_4$): δ 167.9, 166.7, 158.3, 145.0, 131.6, 130.3, 126.8, 121.1, 118.6, 53.5, 52.8, 41.8, 29.3, 25.4. HRMS (ESI): $m/z$ calc for C$_7$H$_7$N$_2$O$_3$ +H$^+$ [M + H$^+$], 152.0937; found, 152.0935.

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DOI: 10.1021/jm001867s
J. Med. Chem. 2015, 58, 1524–1543
1H NMR (500.13 MHz, CDCl₃): δ 7.89 (s, 1H), 7.84 (d, J = 8.3 Hz, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.31–7.28 (m, 4H), 7.24–7.15 (m, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 4.82–4.76 (m, 1H), 4.43–4.32 (m, 1H), 3.72 (s, 3H), 3.24–3.15 (m, 1H), 2.98–2.91 (m, 2H), 2.24–2.20 (m, 1H). 13C NMR (125.76 MHz, CDCl₃): δ 173.6, 170.1, 167.6, 157.4, 141.2, 134.6, 134.5, 131.2, 129.7, 129.4, 129.3, 128.9, 127.5, 127.1, 126.8, 125.2, 116.3, 116.3, 56.3, 55.1, 52.8, 37.3, 34.4, 32.3. HRMS (ESI): m/z calcd for C₂₇H₂₈N₄O₇ [M + H]+ [M + H]+, 493.2289; found, 493.2289.

(S)-Methyl 2-(3-Amino-4-(S)-2-amino-4-phenylbutanamido)benzamido)-3-(4-hydroxyphenyl)-propanoate (10c).

1H NMR (500.13 MHz, CDCl₃): δ 7.75 (s, 1H), 7.66 (d, J = 8.4, 1.7 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.46 (d, J = 8.4 Hz, 1H), 7.36–7.15 (m, 1H), 7.12–6.96 (m, 3H), 5.10 (s, 2H), 4.98–4.92 (m, 1H), 4.40–4.32 (m, 1H), 3.50–3.40 (m, 1H), 3.38–3.32 (m, 1H), 2.90–2.77 (m, 2H), 2.45–2.35 (m, 1H), 2.14–2.11 (m, 1H). 13C NMR (125.76 MHz, CDCl₃): δ 173.4, 169.9, 168.2, 141.2, 138.1, 137.0, 134.5, 132.8, 130.3, 129.7, 129.7, 129.5, 129.4, 129.4, 129.2, 128.7, 127.6, 126.9, 126.7, 126.7, 124.5, 123.6, 122.5, 119.9, 119.1, 112.4, 110.7, 68.1, 55.9, 55.1, 34.5, 32.2, 28.3. HRMS (ESI): m/z calcd for C₁₅H₁₄N₃O₄ + H⁺ [M + H]+, 390.2759; found, 390.2759.

(S)-Methyl 2-(3-Amino-4-(S)-2-amino-4-phenylbutanamido)benzamido)-5-guanidinopentanoate (10e).

1H NMR (500.13 MHz, CDCl₃): δ 7.07 (d, J = 7.9 Hz, 1H), 7.41 (d, J = 7.9 Hz, 1H), 7.23 (d, J = 7.9 Hz, 1H), 7.19 (d, J = 7.9 Hz, 1H), 7.07 (br, 2H), 6.82 (d, J = 8.3 Hz, 2H), 6.69 (br, 2H), 4.82–4.76 (m, 1H), 4.41–4.35 (m, 1H), 3.73 (s, 3H), 3.28–3.09 (m, 3H), 3.06–2.99 (m, 1H). 13C NMR (125.76 MHz, CDCl₃): δ 173.6, 169.8, 167.8, 158.4, 157.4, 133.4, 131.8, 131.8, 131.2, 128.8, 129.8, 128.7, 127.0, 127.0, 123.7, 121.7, 116.8, 116.3, 71.0, 56.5, 56.2, 52.8, 37.7, 37.4. HRMS (ESI): m/z calcd for C₂₇H₂₈N₄O₇ + H⁺ [M + H]+, 493.2082; found, 493.2082.

(S)-Benzy l 2-(3-Amino-4-((S)-2-amino-3-(4-(benzyloxy)phenyl)propanamido)benzamido)-3-(1H-indol-3-yl)propanoate (10g).

1H NMR (500.13 MHz, CDCl₃): δ 7.799 (brs, 1H), 7.74 (brd, 1H), 7.64 (brd, 1H), 7.17 (brd, 1H), 7.07 (brd, 2H), 6.82 (d, J = 8.3 Hz, 2H), 6.69 (brd, 2H), 4.82–4.76 (m, 1H), 4.41–4.35 (m, 1H), 3.73 (s, 3H), 3.28–3.09 (m, 3H), 3.06–2.99 (m, 1H). 13C NMR (125.76 MHz, CDCl₃): δ 173.6, 169.8, 167.8, 158.4, 157.4, 133.4, 131.8, 131.8, 131.2, 128.9, 128.8, 127.0, 127.7, 127.4, 127.0, 123.7, 121.7, 116.8, 116.3, 71.0, 56.5, 56.2, 52.8, 37.7, 37.4. HRMS (ESI): m/z calcd for C₃₀H₂₆N₅O₅ + H⁺ [M + H]+, 493.2082; found, 493.2082.

(S)-Benzy l 2-(3-Amino-4-((S)-2-amino-3-(4-(benzyloxy)phenyl)propanamido)benzamido)-3-(1H-indol-3-yl)propanoate (10h).
122.5, 119.9, 119.1, 116.6, 116.6, 115.5, 112.5, 110.7, 70.9, 68.1, 56.5, 55.9, 37.6, 28.3. HRMS (ESI): m/z calc for C<sub>35</sub>H<sub>30</sub>N<sub>5</sub>O<sub>5</sub> + H<sup>+</sup> [M + H<sup>+</sup>], 582.3024; found, 582.3020.

(S)-2-[(3-Amino-4-((S)-2-amino-3-(4-hydroxyphenyl)propanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (10h)].

1H NMR (500.13 MHz, MeOH-d<sub>4</sub>): δ 7.89 (s, 1H), 7.84 (brs, 1H), 7.58–7.50 (m, 2H), 7.33 (d, J = 8.3 Hz, 1H), 7.14–6.96 (m, 4H), 6.76–6.69 (m, 3H), 4.93–4.91 (m, 1H), 4.40–4.31 (m, 1H), 3.83–3.32 (m, 2H), 2.81–2.70 (m, 2H), 2.43–2.32 (m, 1H), 2.31–2.20 (m, 1H). 13C NMR (125.76 MHz, MeOH-d<sub>4</sub>): δ 175.0, 170.3, 167.6, 157.1, 138.1, 134.7, 131.9, 130.4, 129.5, 127.1, 125.5, 124.5, 122.5, 122.4, 119.9, 119.2, 119.1, 116.4, 112.4, 112.4, 55.7, 55.1, 34.7, 31.4, 28.2. HRMS (ESI): m/z calc for C<sub>35</sub>H<sub>29</sub>N<sub>5</sub>O<sub>5</sub> + H<sup>+</sup> [M + H<sup>+</sup>], 516.2241; found, 516.2249.

(S)-Benzyl 2-((3-Amino-4-((S)-2-amino-4-methylpentanamido)benzamido)-3-(1H-indol-3-yl)propanoate (10k).

1H NMR (500.13 MHz, MeOH-d<sub>4</sub>): δ 7.94 (s, 1H), 7.77 (brs, 1H), 7.54 (d, J = 7.7 Hz, 1H), 7.50 (d, J = 8.3 Hz, 1H), 7.36–7.25 (m, 4H), 7.22–7.16 (m, 2H), 7.11–6.96 (m, 3H), 5.13–5.06 (s, 2H), 4.98–4.92 (m, 1H), 4.30–4.21 (m, 1H), 3.39–3.33 (m, 1H), 1.96–1.76 (m, 3H), 1.11–1.04 (brs, 6H). 13C NMR (125.76 MHz, MeOH-d<sub>4</sub>): δ 173.3, 171.7, 168.1, 138.1, 137.1, 134.8, 133.2, 129.8, 129.5, 129.4, 129.3, 129.2, 128.8, 128.2, 127.3, 127.0, 124.5, 124.0, 122.5, 119.9, 119.2, 112.4, 110.8, 68.1, 55.9, 53.7, 41.4, 28.3, 25.5, 23.4, 21.9. HRMS (ESI): m/z calc for C<sub>35</sub>H<sub>28</sub>N<sub>5</sub>O<sub>5</sub> + H<sup>+</sup> [M + H<sup>+</sup>], 542.2762; found, 542.2761.

(S)-Methyl 6-Amino-2-((3-amino-4-((S)-2-amino-4-(4-hydroxyphenyl)propanamido)benzamido)hexanoate (10l).

1H NMR (500.13 MHz, MeOH-d<sub>4</sub>): δ 7.77 (s, 1H), 7.72 (brs, 1H), 7.52 (d, J = 7.7 Hz, 1H), 7.52–7.02 (m, 2H), 7.11–6.96 (m, 3H), 5.13–5.06 (s, 2H), 4.98–4.92 (m, 1H), 4.30–4.21 (m, 1H), 3.39–3.33 (m, 1H), 2.38–2.19 (m, 1H). 13C NMR (125.76 MHz, MeOH-d<sub>4</sub>): δ 173.3, 170.7, 168.1, 138.1, 137.1, 134.8, 133.2, 129.8, 129.5, 129.4, 129.3, 129.2, 128.8, 128.2, 127.3, 127.0, 124.5, 124.0, 122.5, 119.9, 119.2, 112.4, 110.8, 68.1, 55.9, 53.7, 41.4, 28.3, 25.5, 23.4, 21.9. HRMS (ESI): m/z calc for C<sub>35</sub>H<sub>28</sub>N<sub>5</sub>O<sub>5</sub> + H<sup>+</sup> [M + H<sup>+</sup>], 542.2762; found, 542.2761.

(S)-2-((3-Amino-4-((S)-2-amino-4-(4-hydroxyphenyl)butanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (10j).

1H NMR (500.13 MHz, MeOH-d<sub>4</sub>): δ 7.99–7.93 (m, 2H), 7.60 (d, J = 8.4 Hz, 1H), 4.65–4.57 (m, 1H), 4.32–4.23 (m, 1H), 3.76 (s, 3H), 3.00–2.90 (m, 2H), 2.11–1.81 (m, 5H), 1.79–1.68 (m, 2H), 1.67–1.46 (m, 2H), 1.13–1.04 (m, 6H). 13C NMR (125.76 MHz, MeOH-d<sub>4</sub>): δ 173.9, 170.8, 168.2, 134.6, 133.9, 128.4, 128.3, 127.1, 124.6, 54.4, 53.6, 52.9, 41.4, 40.5, 31.7, 28.1, 25.5, 24.2, 23.4, 21.8. HRMS (ESI): m/z calc for C<sub>35</sub>H<sub>29</sub>N<sub>5</sub>O<sub>5</sub> + H<sup>+</sup> [M + H<sup>+</sup>], 408.2605; found, 408.2604.
(S)-Benzyl 2-((3-Amino-4-((S)-2-aminoheptanamido) benzamido)-3-methylbutanoate (10m).

$^1$H NMR (500.13 MHz, MeOH-$d_4$): δ 7.99–7.92 (m, 2H), 7.60 (d, $J = 7.9$ Hz, 1H), 4.51–4.46 (m, 1H), 4.29–4.22 (m, 1H), 2.34–2.24 (m, 1H), 2.15–2.06 (m, 1H), 2.05–1.95 (m, 1H), 1.58–1.35 (m, 4H), 1.08–1.03 (m, 3H), 1.02–0.96 (m, 3H). $^{13}$C NMR (125.76 MHz, MeOH-$d_4$): δ 174.7, 170.5, 168.2, 135.0, 134.3, 130.7, 129.0, 127.3, 127.0, 125.1, 60.1, 55.2, 52.2, 31.6, 28.1, 23.4, 19.7, 18.9, 14.1. HRMS (ESI): m/z calcd for C$_{23}$H$_{30}$N$_4$O$_5$ [M + H$^+$], 443.2289; found, 452.2297.

(S)-Methyl 2-((3-Amino-4-((S)-2-aminoheptanamido)benzamido)-3-(4-hydroxyphenyl)propanoate (10n).

$^1$H NMR (500.13 MHz, MeOH-$d_4$): δ 8.07–7.94 (m, 2H), 7.62 (d, $J = 8.3$ Hz, 1H), 4.63–4.57 (m, 1H), 4.32–4.22 (m, 1H), 3.75 (s, 3H), 3.00–2.90 (m, 2H), 2.17–1.90 (m, 4H), 1.91–1.66 (m, 2H), 1.65–1.32 (m, 6H), 1.06–0.83 (m, 3H). $^{13}$C NMR (125.76 MHz, MeOH-$d_4$): δ 173.9, 170.4, 168.1, 134.4, 134.3, 128.9, 127.5, 127.0, 125.0, 55.2, 54.4, 52.9, 40.5, 32.2, 31.6, 28.1, 28.0, 24.2, 23.4, 14.1. HRMS (ESI): m/z calcd for C$_{25}$H$_{32}$N$_4$O$_5$ [M + H$^+$], 408.2605; found, 408.2608.

(S)-Methyl 2-((3-Amino-4-((S)-2-amino-5-guanidinopentanamido)benzamido)-3-(4-hydroxyphenyl)propanoate (10q).

$^1$H NMR (500.13 MHz, MeOH-$d_4$): δ 7.95–7.82 (m, 2H), 7.67 (d, $J = 8.4$ Hz, 1H), 7.09 (d, $J = 8.0$ Hz, 2H), 6.71 (d, $J = 8.0$ Hz, 2H), 4.82–4.74 (m, 1H), 4.40–4.28 (m, 1H), 3.73 (s, 3H), 3.38–3.32 (m, 2H), 3.23–3.15 (m, 1H), 3.09–2.99 (m, 1H), 2.30–2.17 (m, 1H), 2.16–2.05 (m, 1H), 1.95–1.76 (m, 2H). $^{13}$C NMR (125.76 MHz, MeOH-$d_4$): δ 173.6, 169.9, 167.6, 158.6, 157.4, 134.7, 134.6, 131.2, 131.2, 129.4, 128.9, 127.1, 126.4, 125.4, 116.3, 116.3, 56.3, 54.7, 52.8, 41.8, 37.3, 29.6, 25.7. HRMS (ESI): m/z calcd for C$_{26}$H$_{32}$N$_4$O$_5$ [M + H$^+$], 486.2459; found, 484.2458.
(S)-2-(3-Amino-4-((S)-2-amino-5-guanidinopentanamido)benzamido)-3-(1H-indol-3-yl)propanoic Acid (10r).

\[
\text{H NMR (500.13 MHz, MeOH-d}_4\): } \delta 7.80 \text{ (s, 1H), 7.74 (d, } J = 8.1 \\
\text{Hz, 1H), 7.63–7.56 (m, 2H), 7.32 (d, } J = 8.1 \text{ Hz, 1H), 7.14 (s, 1H, 7.10–7.05 (m, 1H), 7.01–6.94 (m, 1H), 4.97–4.89 (m, 1H), 4.37– \\
4.26 (m, 1H), 3.37–3.32 (m, 2H), 3.25–3.20 (m, 2H), 2.28–2.03 (m, 2H), 1.91–1.79 (m, 2H).} \\
\text{1C NMR (125.76 MHz, MeOH-d}_4\): } \delta 175.0, 169.9, 167.8, 158.7, 138.1, 133.8, 128.6, 126.9, 124.6, 124.4, \\
122.5, 122.4, 119.9, 119.2, 119.1, 112.4, 111.2, 64.9, 54.7, 41.8, \\
29.7, 28.3, 25.7. \\
\text{HRMS (ESI): } m/z \text{ calc for } C_{29}H_{28}N_{10}O_{11} + H^+ [M + H^+] = 549.2463; \text{ found, 549.2468.}
\]

(S)-Methyl 6-Amino-2-(3-amino-4-((S)-2-amino-5-guanidinopentanamido)benzamido)hexanoate (10s).

\[
\text{H NMR (500.13 MHz, MeOH-d}_4\): } \delta 8.02–7.94 (m, 2H), 7.70 (br.d, 1H), 4.65–4.58 (m, 1H), 4.37–4.30 (m, 1H), 3.76 (s, 3H), 3.37–3.32 (m, 2H), 2.99–2.91 (m, 2H), 2.14–1.90 (m, 4H), 1.89–1.69 (m, 4H), 1.65–1.48 (m, 2H). \\
\text{1C NMR (125.76 MHz, MeOH-d}_4\): } \delta 173.9, 169.9, 168.1, 158.8, 134.5, 134.1, 128.6, 127.1, 126.7, 124.9, 54.7, 54.4, \\
52.9, 41.9, 40.6, 31.7, 29.7, 28.1, 25.7, 24.1. \\
\text{HRMS (ESI): } m/z \text{ calc for } C_{29}H_{28}N_{10}O_{11} + H^+ [M + H^+] = 549.2776; \text{ found, 549.2778.}
\]

Protein Expression and Purification. The expression and purification of recombinant human endoplasmic reticulum aminopeptidase 1 (ERAP1), endoplasmic reticulum aminopeptidase 2 (ERAP2), and insulin-regulated aminopeptidase (IRAP) have been described before. Recombinant baculovirus containing each ERAP1 variant was produced in sf9 cells according to the manufacturer’s instructions (Bac-to-Bac baculovirus expression system, Invitrogen). Recombinant proteins were expressed in Hi5 cells after infection with the appropriate recombinant baculovirus and purified as previously described. Proteins were aliquoted in and stored in a buffer containing 10 mM Heps, pH 7, 100 mM NaCl, and 10% glycerol at −80 °C, until needed.

Enzymatic Assays. The enzymatic activity of the enzymes was calculated by following the time-dependent increase in fluorescence at 460 nm (excitation was at 380 nm) of the fluorogenic substrates l-leucine-7-amido-4-methyl coumarin (L-AMC; Sigma) for ERAP1 and IRAP and l-arginyl-7-amido-4-methyl coumarin (R-AMC; Sigma) for ERAP2. All measurements were performed on a TECAN infinite M200 microplate fluorescence reader. For evaluation of the effect of the compound on activity, 30 nM ERAP1, 6 nM ERAP2, or 6 nM IRAP was added to each well along with 50 μM substrate and varied concentrations of compound. In all cases, the enzyme concentration used was significantly less than the calculated IC50 values for the inhibitors in order to avoid ligand depletion artifacts. The reaction was followed for 5–10 min, and activity was calculated by measuring the slope of the time course. For calculation of the in vitro IC50 values, experimental data were fit to the following equation using the GraphPad Prism software package

\[
Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\text{Log IC}_{50} - X)\times \text{Hill Slope}})
\]

where \(Y\) is the enzymatic activity and \(X\) is the inhibitor concentration.

Phagocytosis Assay. Murine macrophage cell line RAW264.7 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. For assays, cells were transfected to and cultured in a 96-well black culture plate (2 × 10^5 cells/well) for 24 h at 37 °C, 5% CO2. The next day, they were washed twice with cold phosphate-buffered saline (PBS) and activated with IFN-γ (100 IU/mL) and LPS (1 μg/mL) in the presence or absence of various inhibitor concentrations. Phagocytosis was assessed by measuring the amount of uptake of latex beads coated with FITC-labeled rabbit IgG into cells using a phagocytosis assay kit (FITC) (Cayman Chemical, Ann Arbor, MI) according to the instruction manual. In brief, cells with or without various stimulants were treated with the beads and cultured in RPMI 1640 without FBS (1% FBS) for 24 h at 37 °C, 5% CO2. Twenty-four hours after stimulation, cells were washed twice with cold phosphate-buffered saline (PBS). The uptake of the beads into cells was calculated by measuring fluorescence intensity in a TECAN infinite M200 microplate fluorescence reader using an excitation of 485 nm and an emission of 535 nm. The cell supernatant from treated cells was used for measuring aminopeptidase activity. The enzymatic activity of ERAP1 was determined with l-leucine-7-amido-4-methyl coumarin (L-AMC; Sigma). The reaction mixture containing 100 μM Leu-AMC and 50 μL of culture medium in 50 mM HEPES pH 7.0, 100 mM NaCl. The amount of 7-amino-4-methylcoumarin released was measured by a TECAN infinite M200 microplate fluorescence reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. For calculation of the ED50 values, experimental data were fit to the equation described in the Enzymatic Assay section using the GraphPad Prism software.

Mice. Previously described IRAP knockout mice on an Sv129 background obtained from S. Keller were backcrossed up to 10 times to C57BL/6 mice obtained from Janvier (St. Quentin-Fallavier, France). Control mice were mixed-background mice bred in our facility. RAG1-deficient OT-I T cells receptor transgenic mice were obtained from Taconic (Germantown, NY) and bred in our animal facility. Animal experimentation was approved by the Comité d’étique pour l’expérimentation animale Paris Descartes (no. P2.LS.156.10).

In Vitro Cross-Presentation Assays. Murine BMDCs were generated in vitro by culturing progenitor cells extruded from large bones for 7 days in complete medium (IMDM medium completed with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 mM β-mercaptoethanol) supplemented with JS88 supernatant containing 20 μg/mL GM-CSF. On day 6, BMDCs were seeded at 50 000 cells/well into a 96-well flat bottom culture plate with increasing concentrations of inhibitor 4u. On day 7, cells were washed twice with PBS and incubated with serial dilutions of yeast cells expressing ovalbumin on their cell surface in complete medium (prepared as described in ref 39), in the presence of the same concentration of 4u as that used before. After 6 h, the cells were washed twice with PBS, and CDB+ T cells purified from lymph nodes of OT-1 mice were added to the culture for 20 h at a ratio T/BMDCs of 1:5:1, again in the presence of inhibitor 4u. To assess T cell activation, the IL-2 concentration in supernatants was measured by sandwich ELISA using Nunc Maxisorp plates, streptavidin/horse radish peroxidase (Thermo Scientific), and OptEIA TMB substrate (BD Biosciences). As negative controls, an aliquot of BMDCs were fixed with 0.04% glutaraldehyde (as described in ref 39) prior to the addition of antigen, and the signal obtained was set as the background and subtracted from all OD values. Results represent the means of duplicate wells.

Computational Methods. The crystallographic structures of ERAP1 (PDB ID: 2YD0), ERAP2 (PDB ID: 3S6E), and IRAP (PDB ID: 4IP6) were used without any further refinement. Polar hydrogen atoms were added and Gasteiger charges were applied using Autodock Tools 1.5.6.42 The initial conformations of the inhibitors were generated from SMILES representations using the program Omega 2.4, and then Gasteiger charges were applied. The search space was defined by a grid box centered next to the catalytic zinc and comprised 81 × 81 × 81 grid points of 0.375 Å spacing. For each
complex, 100 docking rounds were calculated with AutoDock 4.2 using the Lamarckian genetic algorithm with the default parameters of AutoDock 3.4. The maximum number of energy evaluations was set to $10 \times 10^6$, and the docked conformations were clustered using a tolerance of 2.0 Å. Visual examination of the complexes and rendering of the figures were performed with VMD 1.9. Calculations were carried out using Intel Xeon workstations running Linux 2.6.32 kernels.

**ASSOCIATED CONTENT**

**Supporting Information**
1H and 13C NMR spectra of biologically active compounds 4a, 4u, 4x, and 10n; titration curve of 4u with recombinant mouse IRAP. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

*Corresponding Authors*
E.A.T. co-supervised the project along with D.V. and E.S., who contributed to the preparation of the manuscript and have conceived the experiments and analyzed the results. All authors A.P. performed the computational design, synthesis, and characterization of the compounds; E.Z. prepared the 1H and 13C NMR spectra of biologically active compounds. Biochim. Biophys. Acta

**AUTHOR CONTRIBUTIONS**
A.P. performed the computational design, synthesis, and characterization of the compounds; E.Z. prepared the recombinant enzymes and performed the in vitro and phagocytosis assays measurements and HPLC analysis; S.T. assisted in the synthesis of the compounds; F.-X.M. and P.v.E. designed and performed the cross-presentation assay; G.S and D.C.M. helped to establish the macrophage activation assay; E.A.T. co-supervised the project along with D.V. and E.S., who conceived the experiments and analyzed the results. All authors contributed to the preparation of the manuscript and have approved its final version.

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**
This research was financed by the European Union (European Social Fund) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework: Research Funding Program of the General Secretariat for Research & Technology (grant nos. LS7-2199 and ERC-14). F.-X.M. was supported by a grant from INSERM (poste d’accueil). Work in the laboratory of P.v.E. was supported by the Fondation pour la Recherche Médicale (grant no. DEQ20130326539).

**ABBREVIATIONS USED**
DABA, 3,4-diaminobenzoic acid; hPhe, l-homophenylalanine; hTyr, l-homotyrosine; Nle, l-norleucine; TFA, trifluoroacetic acid; HBTU, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)-uronium hexafluorophosphate; HATU, 1-[(bis(dimethylamino)-methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIEA, N,N-diisopropylethylamine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, N,N-dimethylformamide

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