Supporting information

A peptide-based fluorescent probe images ERAAP activity in cells and in high throughput assays

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Experimental section

Table of contents

<table>
<thead>
<tr>
<th>I.</th>
<th>Materials and instrumentations</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.</td>
<td>Synthesis of Ep</td>
</tr>
<tr>
<td>III.</td>
<td>Kinetics assay</td>
</tr>
<tr>
<td>IV.</td>
<td>Calculation of fluorescence quantum yield</td>
</tr>
<tr>
<td>V.</td>
<td>Computational docking</td>
</tr>
<tr>
<td>VI.</td>
<td>Fluorescence live cell imaging</td>
</tr>
<tr>
<td>VII.</td>
<td>High throughput screening using Ep-based assay</td>
</tr>
<tr>
<td>VIII.</td>
<td>Steady-state time dependent inhibition</td>
</tr>
<tr>
<td>IX.</td>
<td>Supporting figures</td>
</tr>
</tbody>
</table>

Page

1-2
2
2-3
3
3-4
4
4-5
5
5-9

I. Materials and instrumentations

The precursor peptide for Ep, K(Dnp)KSIINFECL, was purchased from GenScript Inc. The thiol reactive BODIPY dye, BODIPY\textsuperscript{TM} FL Iodoacetamide, was purchased from ThermoFisher. Phosphate buffered saline (PBS, 1x, pH 7.4), pipette tips, and centrifugation tubes were purchased from VWR. All other chemicals were purchased from common commercial chemical suppliers like Sigma Aldrich and VWR, and used without further purifications. High-resolution mass spectra (HRMS) were obtained on an AB SCIEX TOF/TOF 5800 system and are reported as m/z (relative intensity). Accurate masses are reported for the molecular ion (M\textsuperscript{+}) or a suitable fragment ion. Fluorescence assays were performed using a Tecan Microplate reader. The wild type fibroblast cell line and the ERAAP knockout fibroblast cell line were generated from B6 wild
type (WT) and from an ERAAP knockout (ERAAP-KO) mouse. A LSM 710 was used for laser scanning confocal microscopy of fibroblast cells.

II. Synthesis of Ep

The peptide K(Dnp)KSIINFECL (3.0 mg) was dissolved in 600 µL DMSO and then mixed with 1380 µL of D.I. H₂O and 220 µL 10x PBS (pH 7.4). To this solution, 60 µL of TCEP solution (10 mg/mL, pH 7.0) was added and the resulting mixture was stirred in the dark for 20 min. 210 µL of 10 mM BODIPY™ FL iodoacetamide, in DMSO, was added to the reaction solution and the reaction was stirred in the dark overnight. The crude mixture was diluted with D.I. H₂O and further purified by semi-preparative HPLC, and lyophilized to obtain an orange powder. HPLC Method: mobile phase A: H₂O with 0.1% TFA; mobile phase B: Acetonitrile with 0.1% TFA. 0-2 min: 0% B, 2-23 min: 0%-100% B. Product retention time: 13.2 min. HRMS (ESI): calculated for C₇₄H₁₀₈BF₂N₁₈O₂₀S¹⁺ [M+H]+, 1649.7764; found, 1649.7777

III. ERAAP kinetic assays

All steady-state kinetic assays were carried out at room temperature in a 96-well plate. A concentration of 1 µg/mL of ERAAP were used for the kinetic assay measurements. After addition of substrates (Ep) in 1x PBS containing ERAAP, the fluorescence intensity of the reaction solution (110 µL) at 510 nm was measured as a function of time with intervals of 30 s using a microplate reader for 5 min. These “fluorescence intensity vs time” plots were then used to obtain the slope (Slope_initial) at the initial point of each reaction by determining the first derivative of each curve using OriginPro 9.0 software. The initial reaction velocity (v) was then calculated by Slope_initial / ε(fl), where ε(fl) = [the fluorescent intensity (510 nm) difference of 110 µL solutions of Ep with excess amount of ERAAP and without ERAAP for overtime incubation] / [concentration of Ep]. The plots of initial velocity (v) against Ep concentrations were fitted by nonlinear regression using the Michaelis-Menten equation. The apparent kinetic parameters were calculated using the Michaelis-Menten equation v = V_max × [E] / (K_m + [E]), where V_max
denotes the maximal reaction velocity, [E] represents the concentration of ERAAP, and $K_m$ is the Michaelis constant. $K_m$ and $V_{\text{max}}$ were obtained from Lineweaver-Burk plot.

**IV. Calculation of fluorescence quantum yield**

Rhodamine 6G ($\phi_n = 95\%$ in water solution)$^{[1]}$ was used as a standard to determine the fluorescence quantum yields of Ep before and after enzymatic reaction with ERAAP. Both samples and reference dye were prepared under identical conditions and the absorption and emission spectra were acquired under same experimental conditions. The areas for all fluorescence spectra were calculated by integration of peak using OriginLab Pro 9.0. The plot of integrated area versus absorbance at excitation wavelength was drawn for the corresponding reference data as well as the data for the test sample. A linear regression was then employed to determine the slope of the line (gradient) for the reference and the sample respectively. The fluorescence quantum yield of each sample was obtained by using the following equation:

$$\Phi_x = \Phi_{st} \left( \frac{\text{Grad}_x}{\text{Grad}_{st}} \right) \left( \frac{\eta_x^2}{\eta_{st}^2} \right)$$

Where the subscripts ‘st’ and ‘x’ represent standard and test samples, respectively; $\Phi$ is the fluorescence quantum yield; Grad stands for the gradient from the plot of integrated fluorescence intensity versus absorbance, and $\eta$ is the refractive index of the solvent.

**V. Computational docking**

The computational docking studies were performed using Molecular Graphics software at the Computation Facility (MGCF) at the College of Chemistry, UC Berkeley. The conformational structures of tetrapeptide (first 4 amino acid residues from N-terminal) and tripeptide (last 3 amino acid residues from N-terminal) were built and optimized using Meatro software. The conformational structures (receptor grid) of ligand-free catalytic site and regulatory site were obtained by removing the ligand and water molecules (distance to pocket molecules >3 Å) from the solved structure of the Bestatin-ERAP1 complex (PDB 2DY0) using Meatro software. The ligand (tetrapeptide or tripeptide) was eventually docked into the receptor grid (catalytic site or
regulatory site) using Meatro software and compared with the structure of the solved Bestatin-ERAP1 complex (PDB 2DY0).

VI. **Fluorescence live cell imaging**

The wildtype (WT) and ERAAP knockout (ERAAP-KO) fibroblast cell lines are generated from B6 wildtype mouse and B6 ERAAP-/- mouse, respectively. WT and ERAAP-KO cells were incubated with 10 μM or 20 μM of Ep for 2 or 3 hours at 37 °C. Cells were co-incubated with nucleus stains 10-15 min before the completion of incubation. Cells were then washed with PBS 3 times and the live cell fluorescence images were obtained using laser scanning confocal microscopy (GFP channel).

VII. **High throughput screening using the Ep-based assay**

Each test compound was dissolved in DMSO to give ~200 μM stock solution. The assay was initiated by mixing of 100 μL of 10 μg/mL of recombinant ERAAP in 1x PBS (pH 7.4) with 1.0 μL of the test compound stock solution. The mixture was incubated at room temperature for 30 min. 10 μL of 30 μM Ep solution in 1x PBS was then added and mixed homogenously (final Ep concentration is 2.7 μM). The mixture was further incubated at room temperature for 1 hour and then the fluorescence intensity (510 nm) of the solution was measured by a plate reader with the excitation wavelength of 490 nm. The positive control was performed via replacing the compound stock with pure DMSO. The negative control was performed via replacing the compound stock with pure DMSO and replacing ERAAP solution with pure buffer solution (1X PBS).

The inhibition rate (%) was calculated by the equation below:

\[
\text{Inhibition rate} \% = 1 - \frac{(FL_{\text{compound}} - FL_{\text{Ep only}})}{(FL_{\text{control}} - FL_{\text{Ep only}})}
\]

Where “FL” represents the fluorescent intensity value at 510 nm. \(FL_{\text{Ep only}}\) is the fluorescence intensity value of negative control.

To calculate the Z’-factor, the negative control (2.7 μM Ep only) and positive control (2.7 μM Ep + 10 μg / mL ERAAP) were performed using this Ep-based fluorescent assay. The Z’-factor was then calculated according to the method developed by Zhang, J. et al in 1999 on J. Biomol. Screen. [2]
VIII. Steady-state time dependent inhibition (Figure S6)

10 μg/mL of ERAAP was incubated with 200 μM of compound A, B or C for various times (10, 60, 120, 180, 270 min). To this solution, 10 μL of 30 μM Ep solution was added and mixed homogenously. The fluorescent intensity (510 nm) of the reaction solution was then immediately measured by a microplate reader for 3 min with a time interval of every 30 seconds. The “fluorescence intensity vs time” plots were then used to obtain the slope \(S_{\text{compound}}\) at the initial point of each reaction by determining the first derivative of each curve using OriginPro 9.0 software. The inhibition efficiency (%) was calculated by the equation below:

\[
\text{Inhibition Efficiency} (\%) = 1 - \frac{S_{\text{compound}} - S_{\text{Ep only}}}{S_{\text{control}} - S_{\text{Ep only}}} 
\]

Where “S” represents the slope of fluorescence intensity vs time plot; subscript “control” means no compound was incubated with ERAAP, subscript “Ep” denotes the condition of the solution only containing Ep.

IX. Supporting figures

![Figure S1. The HRMS of Ep](image)

Figure S1. The HRMS of Ep
Figure S2. The comparison of fluorescence images of the solutions of Ep (5 µM), ERAAP (5 µg/mL), and the mixture of Ep (5 µM) with ERAAP after 10 min incubation. The quantum yield (φ) of Ep in 1x PBS (sample 1 in the image) was determined as 0.01 and its quantum yield after enzymatic reaction with ERAAP was determined as 0.22. Rhodamine 6G (φ = 0.95 in water[1]) was used as the reference standard for quantum yield calculations.

Figure S3. a: The steady-state kinetics of enzymatic reaction between ERAAP and Ep. The initial velocity (v) was measured using 1 µg/mL of Ep in 1x PBS at room temperature and the plot of v against Ep concentrations was fitted using the Michaelis-Menten equation.; b: Lineweaver–Burk plot generated from a, linear fitting was performed to determine kinetic parameters.
**Figure S4.** The fluorescence images of wildtype (WT) and ERAAP knockout (ERAAP-KO) fibroblast cells stained with 4 µM carboxyfluorescein succinimidyl ester (CFSE) for 2 h, which was used as a positive control group for the fluorescence live cell imaging of ERAAP using Ep. The results indicated that both WT and ERAAP-KO cell lines were able to take up CFSE similarly without any significant difference in fluorescence intensity observed.

**Figure S5.** Z’ factor of the Ep-based fluorescence assay for ERAAP activity. The negative control (2.7 µM Ep only) and positive control (2.7 µM Ep + 10 µg / mL ERAAP) were performed using this Ep-based fluorescent assay. The Z’-factor was then calculated according to the method developed by Zhang, J. et al in 1999 on J. Biomol. Screen. [1]
Figure S6. Steady-state time dependent inhibition curves of compounds A, B and C. Both compounds A and C showed enhanced inhibition efficiency upon increased compound-ERAAP pre-incubation time but not B. Compared with C, compound A showed constant higher inhibition rate and can generate almost 100% of inhibition after 4.5 h pre-incubation. These reveal the irreversible inhibitory property of A and C because the covalent binding relies on both substrate concentration and reaction time. It also demonstrated that A has best inhibition properties towards ERAAP among all compounds we have screened.

Figure S8. Time dependence of 5 μM Ep fluorescence (510 nm) in presence of 0.2 μg/mL ERAAP in 1x PBS at room temperature. 0.2 μg/mL of ERAAP triggered a rapid fluorescence enhancement of the Ep solution (5 μM) within 10 min and then a plateau was reached.

X. References: