Abstract
The cleavage of proteins by intracellular or extracellular proteases results in protein and peptide products that are hallmark markers in many pathologies, such as Alzheimer’s and cardiovascular diseases. Therefore, the identification of specific modulators for these proteases is of significant importance in the current drug discovery efforts. Here, we describe the application of the AlphaLISA® technology for monitoring protease cleavage activity of whole protein substrates. Using two protease models, J-secretase (BACE-1) and Caspase-3, we show that physiological protein substrates can be cleaved into products that can be directly detected and quantified. Interestingly, both proteases have a higher affinity for these physiological protein substrates compared to short peptides encompassing the enzyme cleavage sites. Known inhibitors of these two enzymes generated IC50 values in agreement with the literature. Protease assays with whole-protein substrates offer the advantage of using physiological enzymes in substrates that could uncover novel regulators. In addition, assays developed using full-length substrates are applicable to cell-based measurements, which open new possibilities for better understanding complex protease regulation.

Materials and Methods

BACE-1

Cleavage (reagents are diluted in reaction buffer (RB1): 10 mM Sodium acetate buffer, 1% Triton X-100, 0.5% FBS) promote cleavage of the BACE-1 (Ub-MOD 203-450) incubate 60 minutes at 29°C. Detection (Reagents are diluted in 100 mM Hepes buffer, 0.1% Casin, 0.5% Triton X-100, 1 mg/mL Dextran T-500, 0.05% Proclin-300, pH 8.0): 20 μL of biotinylated anti-BACE-1 (10 nM final) anti-BACE-1 acceptor beads (PerkinElmer) (10 μM final) incubate 20 minutes at 29°C. Substrate samples were read with a 384-well OptiPlates using the Envision® Multilabel Plate Reader (PerkinElmer).

Cleavage (reagents are diluted in RB2: 25mM HEPES, 0.1% Casin, 1mg/mL Dextran, 0.05% Triton X-100, 0.05% Proclin-300 pH 7.4): 20 μL of Fetzr (ROB+30) polymers, PARP protein (BD systems 4687-250) 20 μL of C-terminal sequence-specific probe, 3-caspase-3 enzyme (SB/3074) substrate (10 μM final) incubate 60 minutes at 37°C. Detection (Bead mixtures are diluted in RB2): 20 μL of biotinylated anti-PARP cleavage site (Newa Biologics) (10 nM final) anti-PARP linker conjugate to streptavidin beads (PerkinElmer) (10 μM final) incubate 60 minutes at 29°C. 20 μL of Streptavidin Donor Beads (PerkinElmer) (60 μM final) incubate for 30 minutes at 29°C. Samples were read with a 384-well Optiplates using the Envision Multilabel Plate Reader (PerkinElmer).

BACE-1 Assay

Excitation 680 nm
Emission 615 nm

Caspase-3 Assay

Excitation 680 nm
Emission 615 nm

Protein substrate

Kcat = 30 nM
Substrate PARP-1 triturations were performed using three different Caspase-3 concentration. The enzyme reaction was initiated for 60 minutes followed by the addition of the 3-caspase-3 enzyme (SB/3074) substrate (10 μM final) incubate for 60 minutes at 37°C. The reaction was converted to quantities of cleaved peptide by using a standard curve produced in parallel under the same assay conditions.

Inhibition of BACE-1 Activity

Inhibitor

<table>
<thead>
<tr>
<th>IC50 (nM)</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
</tr>
</tbody>
</table>

Inhibition of Caspase-3 Activity

Inhibitor

<table>
<thead>
<tr>
<th>IC50 (nM)</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
</tr>
</tbody>
</table>

Summary

We developed high throughput assays for monitoring protease cleavage activity on whole protein substrates using AlphaLISA® technology. BACE-1 and Caspase-3 have higher affinity (lower Kd) for their target substrates compared to the tested peptides harbouring the cleavage site. The observed Kd values are lower than those reported in the literature for fluorogenic peptides (BACE-1: 9 μM, Caspase-3: 9.7 μM).

Inhibitors of these enzymes resulted in IC50 values in agreement with the literature.

The Kd values obtained were superior to 0.8, indicating suitability and robustness of the platform for HTS applications.

The tested antibodies against the cleavage site non-epitope allows for signal generation assays.

The AlphaLISA® technology platform is well-suited for compound characterisation in monitoring protease activity and is physiologically relevant targets.

Chris Abenes, Shihadeh Anani, Simonne Harvey-Lavoie, Alexandre Marcil, Philippe Roby, Lucille Beaudet, Sophie Dahan, Gregory Cosentino

Plates were loaded with 62.5 ng/mL of BACE-1 and 0.2 ng/mL of PARP-1 in the presence or the absence of inhibitor (0.5 μM) pre-incubated 30 minutes at 23°C. The enzymatic reaction was incubated at 37°C for 60 minutes. The Z’-factor values obtained were superior to 0.8, indicating the suitability of the platform for HTS application. This is a representative result of three independent experiments.

Plates were loaded with 62.5 ng/mL of CASP3 and 0.2 μg/mL of PARP-1 in the presence or the absence of inhibitor (0.5 μM) pre-incubated 30 minutes at 23°C. The enzymatic reaction was incubated at 37°C for 60 minutes. The Z’-factor values obtained were compared to 0.8, indicating the suitability of the platform for HTS application. This is a representative result of three independent experiments.

PL-MAK 295-425 4-J5-5A