SCREEN FOR CALPAIN INHIBITORS USING A CELL-BASED, HIGH-THROUGHPUT ASSAY

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We developed a cell-based assay for endogenous calpain activity using a cell-permeant luminescent calpain substrate. Using the protocol described here, we were able to identify potent, reproducible inhibitors of calpain activity in a high-throughput screening application.

Introduction

The calpain family of proteases consists of several Ca^{2+}-dependent cysteine proteases. The two most widely studied calpains are μ- and m-calpain, with the μ and m referring to the molar concentrations of Ca^{2+} required for activation of the enzymes in activity assays in vitro. Both μ- and m-calpain consist of two subunits, an 80kDa subunit containing the active site of the protease as well as five potential Ca^{2+} binding domains, and a 28kDa subunit containing a hydrophobic domain and another five potential Ca^{2+} binding domains (1). The mechanisms of activation and regulation of calpain are complex and likely to differ in a cell-specific manner. One suggestion is that Ca^{2+} activates calpain by inducing a conformational change in the protein upon binding. A two-stage model for Ca^{2+}-dependent activation of calpain has been proposed (2,3). In this model, the 80 and 28kDa subunits dissociate following Ca^{2+} binding to both subunits, and this dissociation allows additional Ca^{2+} binding near the active site that in turn leads to opening of the active site cleft. While the mechanisms of calpain activation in vivo remain unclear, the protease does respond to changes in intracellular Ca^{2+} concentrations.

Many proteases recognize specific amino acids or amino-acid sequences on substrates, and cleavage by these proteases results in inactivation and degradation of the substrate. Unlike these other proteases, calpain cleaves substrates between domains, producing large fragments of the substrate that can retain biological activity. Often, this cleavage modulates the activity of the substrate, affecting cellular signaling pathways (1). An example of this process can be seen in the case of calpain-mediated cleavage of the ER-associated procaspase-12, which produces the active caspase-12 and activates the caspase-dependent apoptotic cascade (4). Another similar example is cleavage of the cdk5 regulatory protein p35, which results in formation of a p25/cdk5 complex and increased activation of cdk5 and phosphorylation of tau (5–8). Both of these pathways have been implicated in neuronal cell death following exposure to β-amyloid peptides (Aβ), a cell model of Alzheimer disease (6, 9–11).

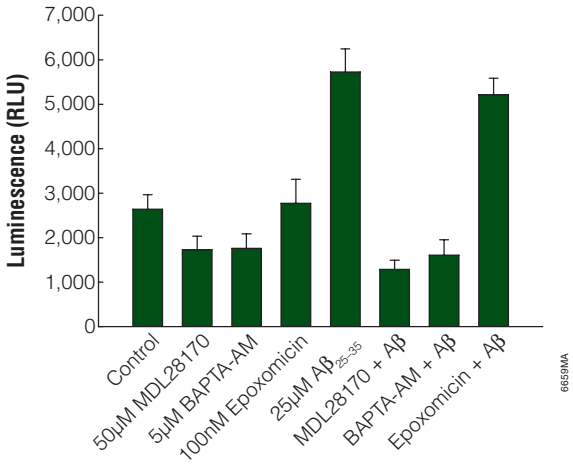

Figure 1. Validation of assay with known inhibitors. SH-SY5Y cells were plated in 384-well plates at a density of 10,000 cells per well in 30μl RPMI 1640 containing 1% serum. Twenty-four hours after plating, cells were exposed to 10μM all-trans-retinoic acid to induce differentiation. On day five after plating, cells were incubated with inhibitors (MDL-28170 or epoxomicin) or the cell-permeant Ca^{2+}-chelator BAPTA-AM for 30 minutes, then 20μM Suc-LLVY-aminoluciferin in Calpain-Glo™ buffer was added for a final concentration of 20μM. Finally, the cells were incubated with 25μM pre-aggregated $Aβ_{25–35}$ for 8 hours prior to lysis in 0.9% Triton® X-100 in PBS with 100μM MDL-28170. Twelve microliters of a preparation of Calpain-Glo™ luciferase detection reagent in Calpain-Glo™ buffer (double the concentration recommended in the Calpain-Glo™ protocol) was added to a final assay volume of 45μl for detection of free aminoluciferin. Ten minutes after adding detection reagent, luminescence was measured using an LJL Analyst® plate luminometer. Each bar represents the average of 16 wells, and error bars represent standard deviation from the mean.

Here we describe the development of a cell-based assay to identify small-molecule inhibitors of Aβ-induced calpain activation by modifying the protocol for the Calpain-Glo™ Protease Assay[a,b] (Cat.# G8501, G8502). The use of a cell-based assay for calpain activity allows identification of inhibitors that interact directly with the enzyme as well as compounds that may act upstream of calpain activation by Ca^{2+}, such as compounds that prevent the loss of cellular Ca^{2+} homeostasis induced by exposure to Aβ.

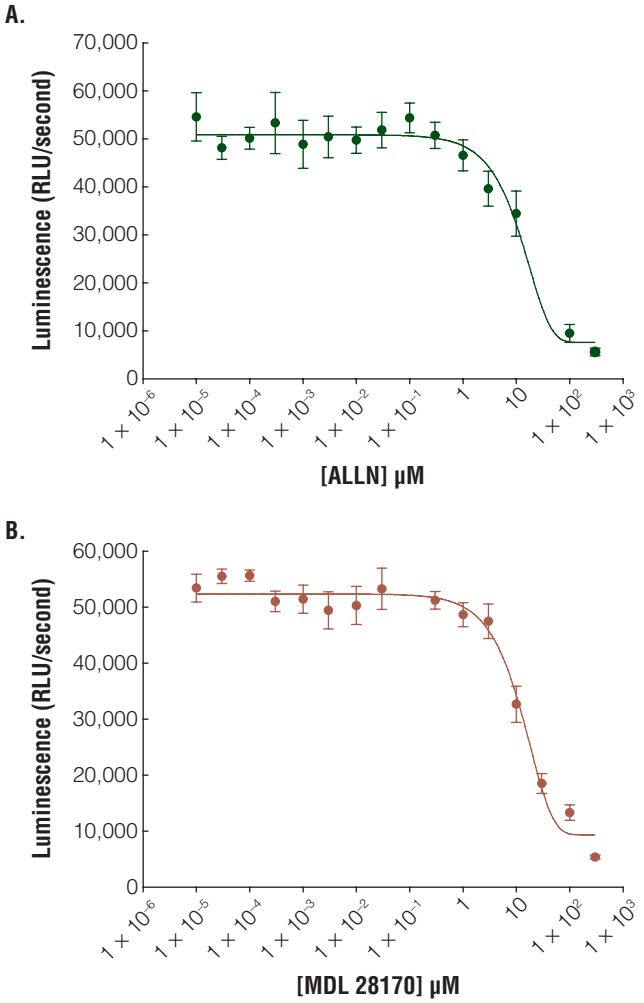

Figure 2. EC_{50} determination of known calpain inhibitors. Cells were plated as described for Figure 1. Serial dilutions of inhibitors ALLN (**Panel A**) and MDL-28170 (**Panel B**) were prepared in neat DMSO and diluted in serum-free RPMI-1640 so that the final concentration of DMSO in the assay was 0.06%. Five microliters of inhibitor was added to retinoic acid-differentiated SH-SY5Y cells, after which the cells were incubated for 30 minutes. Suc-LLVY-aminoluciferin substrate was diluted in Calpain-Glo™ buffer and added to cells for a final concentration of 20µM in the assay. Following a 1-hour incubation of the cells with the substrate in the presence or absence of inhibitor, the cells were lysed, and free aminoluciferin was detected as described for Figure 1. Each data point represents the average of eight wells, and error bars represent standard deviation from the mean.

Assay Protocol

Current methods for measuring calpain activity in cells use a cell-permeant fluorescent calpain substrate, Suc-LLVY-AMC. However, this method may not be conducive to high-throughput screening (HTS) for two reasons: the magnitude of the signal generated by cleavage of the substrate may not be sufficient for miniaturization in a 384-well format, and there is potential for compound interference with the fluorescent signal, either through inherent fluorescence of the test

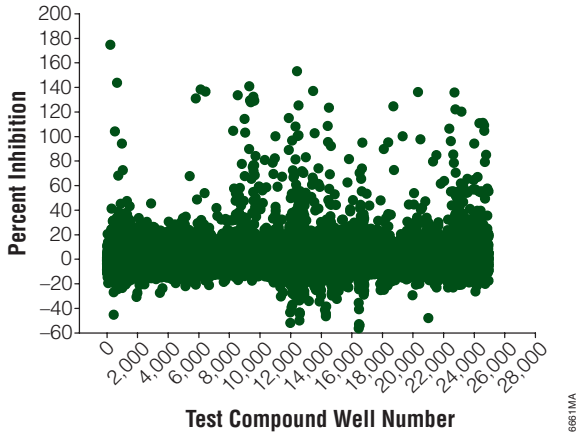

Figure 3. Representative data from primary screen. Cells were plated as described for Figure 1 using a Multidrop® 384 cell dispenser (Thermo Labsystems) and exposed to 1µM compound for 30 minutes prior to adding 20µM substrate (final concentration). Following a 30-minute incubation with substrate, 25µM $A\beta_{25-35}$ was added (final concentration), and the cells were incubated for eight hours prior to lysis. Luminescence was detected as described for Figure 1. Liquid handling was performed with a Beckman Biomek® FX robotics system. Data points represent results from single-well tests of approximately 25,000 individual compounds. Percent inhibition was calculated relative to Aβ-treated cells as a maximum and 50µM MDL-28170-treated cells as a minimum. (Note that compounds exhibiting "negative" inhibition actually indicate increased cleavage above that observed in cells treated with $A\beta_{25-35}$ alone. Compounds exhibiting greater than 100% inhibition, inhibit cleavage of the substrate more strongly than does 50µM MDL-28170.)

compound or due to quenching of the signal. However, an aminoluciferin-conjugated substrate generates a stable signal in a 384-well assay format and reduces the possibility of signal interference by the test compound.

We were able to measure cleavage of the substrate by endogenous calpain in the cellular system by first incubating the cells with the Suc-LLVY-aminoluciferin substrate provided in the Calpain-Glo™ Assay, and then lysing the cells in the presence of a known, direct calpain inhibitor (MDL-28170) to halt the reaction for detection. We next added the luciferase detection reagent and recorded luminescence. We have used this assay protocol to detect calpain activation by $A\beta_{25-35}$ peptides in retinoic acid-differentiated SH-SY5Y cells.

Results

Because the Suc-LLVY-aminoluciferin substrate is also a substrate for the 20S proteasome, we first demonstrated the specificity of the substrate for cleavage by calpain under our assay conditions. Both the known calpain inhibitor MDL-28170 and the cell-permeant Ca^{2+} chelator BAPTA-AM inhibited the signal, while the 20S proteasome inhibitor epoxomicin failed to inhibit the signal (Figure 1). Additionally, EC_{50} values were obtained for the known calpain inhibitors MDL-28170 and ALLN (Figure 2).

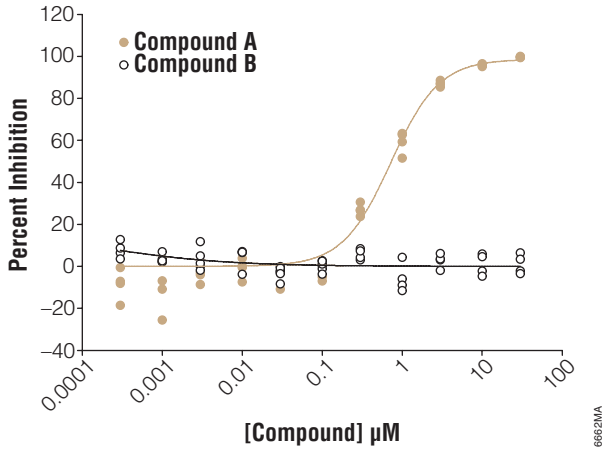

Figure 4. Activity of two compounds identified as calpain inhibitors in the cell-based assay tested in a purified calpain II assay. Purified calpain II activity was measured according to the protocol supplied with the Calpain-Glo™ Assay. Serial dilutions of the compounds were prepared in neat DMSO, then diluted in assay buffer for a final concentration in the assay of 0.03%. Purified calpain II enzyme (100nM) was incubated with the compound for 15 minutes; then the Calpain-Glo™ assay reagent with 2.5mM Ca^{2+} was added to the reaction. The reaction mixture was incubated at room temperature for 15 minutes, and luminescence was detected using an LJL Analyst® plate luminometer. Data points represent 4 wells for each concentration of inhibitor, and percent inhibition was calculated relative to enzyme-plus-Ca^{2+} control as a maximum and enzyme-minus-Ca^{2+} control as a minimum. (Note that data points showing "negative" inhibition indicate cleavage that is greater than the positive control; whereas data points with inhibition greater than 100% indicate substrate cleavage less than that of the minus-Ca^{2+} control.)

Approximately 115,000 compounds were screened in the assay with Z′-factors averaging 0.7 ± 0.1 (Figure 3). Three hundred thirty six compounds exhibiting ≥60% of Aβ-induced calpain activation were selected for retesting, representing a 0.28% hit rate. Eighty percent of the compounds identified in the primary screen reproducibly inhibited the assay signal in a dose-dependent manner upon retesting. Compounds identified in the primary cell-based screen were also tested in a purified calpain assay. As expected, some compounds inhibited purified calpain II activity, while others did not (Figure 4).

Summary

Using the Calpain-Glo™ aminoluciferin-conjugated calpain substrate allowed the efficient screening of a 115,000 compound library in a cell-based format. This HTS assay identified potent, reproducible inhibitors that either directly or indirectly inhibit Aβ-induced calpain activation.

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Protocol

Calpain-Glo™ Protease Assay Technical Bulletin #TB344
(www.promega.com/tbs/tb344/tb344.html)

Ordering Information

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml	G8501
	50ml	G8502

For Laboratory Use.

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.