

Using Inhibitors to Improve Specificity in Cell-based Luminescent Caspase Assays

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1. Abstract

We have developed homogeneous, bioluminescent assays for several proteases, including caspases, calpain, DPPIV, and the proteasome. We are developing a new luminescent caspase-2 assay using the Z-VDVAD-aminoluciferin substrate. These luminescent assays are more sensitive, have a greater dynamic range, and are faster to perform than comparable fluorescent assays.

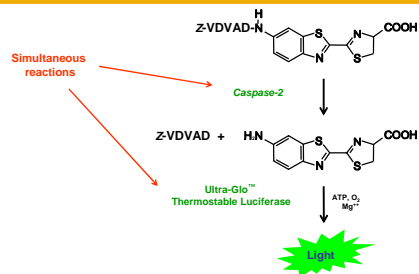
The improved sensitivity makes these assays amenable to HTS as cell-based assays. However, specificity of substrates is often a limitation for cell-based assays. Cross-reactivity between caspases has been notoriously problematic.

Our bioluminescent protease assays are coupled-enzyme assays that achieve a steady-state between the rate of protease cleavage and aminoluciferin consumption by luciferase. This steady-state feature simplifies the use of inhibitors in the reagent to remove non-specific activity.

Proteasomes exhibit non-specific activity on luminescent caspase substrates, including substrates for caspase-2, -8, and -9. The inhibition of proteasomes improves the specificity and quality of cell-based luminescent caspase assays.

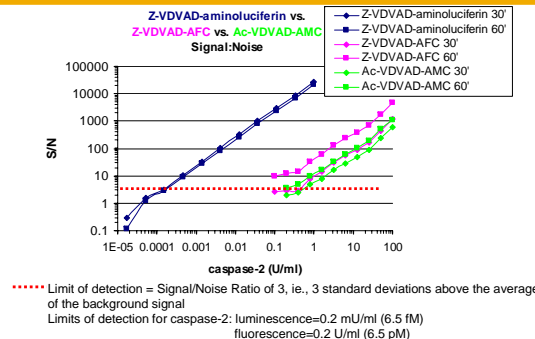
For a luminescent caspase-2 assay, we demonstrate that cross-reacting caspase-3 activity can be inhibited with low doses of a caspase-3 inhibitor while leaving caspase-2 activity intact. We used the Z-VDVAD-aminoluciferin substrate in combination with the caspase-3 inhibitor and proteasome inhibitor to monitor for caspase-2 activity in a heat shock apoptosis model.

2. Bioluminescent Assay Concept: Caspase-2 Example

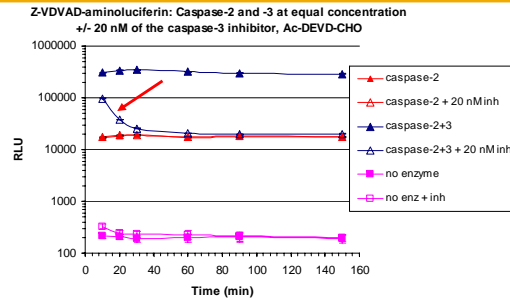


These are homogeneous assays with a coupled-enzyme format. The protease cleavage of the aminoluciferin-conjugated substrate and the consumption of the released aminoluciferin reach a steady-state producing a stable signal.

3. Bioluminescent Assays are More Sensitive than Fluorescent Assays Enabling HTS Cell-Based Assays

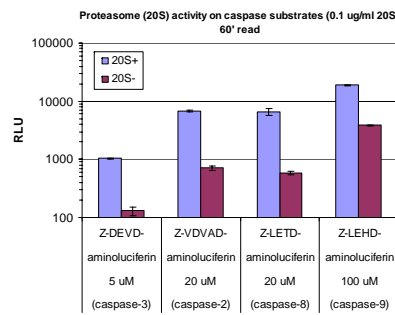


4. Coupled-enzyme Format of the Luminescent Assays Enables the Convenient Use of Inhibitors in the Reagent

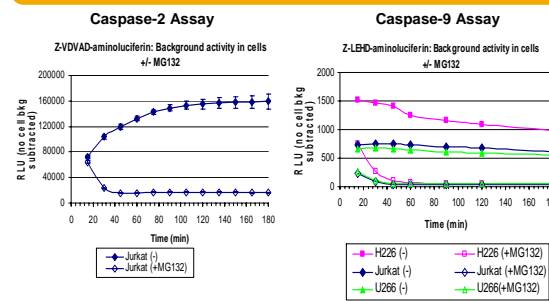


The luminescent substrates reach a steady-state in the coupled-enzyme format. An inhibitor in the reagent produces a new steady-state indicating equilibrium with the inhibitor.

5. 20S Proteasome Efficiently Cleaves Luminescent Caspase Substrates

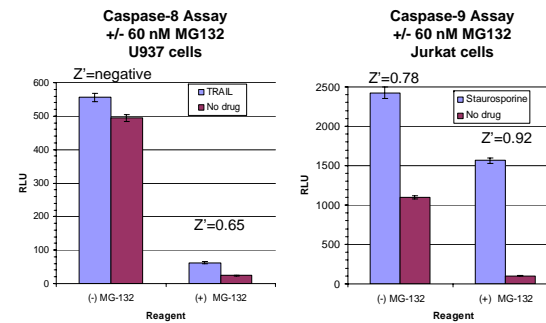


6. MG132, a Proteasome Inhibitor, Significantly Lowers Non-specific Background in Cell-based Luminescent Caspase Assays

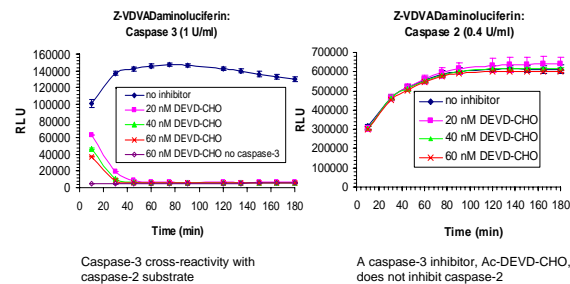


Untreated cells of various types have significant background caspase-like activity that can be inhibited with a proteasome inhibitor. The MG132 inhibitor (60 nM) does not inhibit caspase activity.

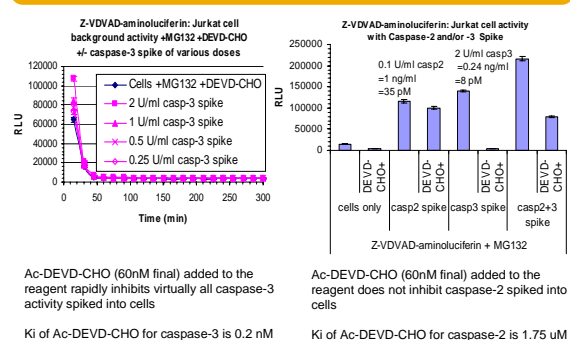
7. The Proteasome Inhibitor Improves Specificity and Z' Values in Luminescent Caspase-8 and -9 Assays



8. Activity has been Difficult to Monitor in Cells Because Caspase-2 Substrates have Significant Cross-reactivity with Caspase-3



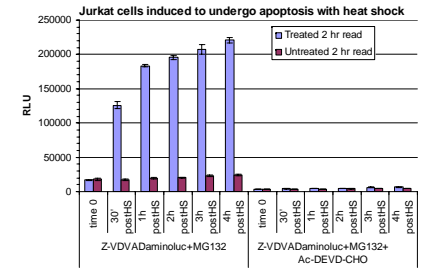
9. A Caspase-3 Inhibitor Can Selectively Inhibit Caspase-3 While Leaving Caspase-2 Intact in a Luminescent Cell-Based Caspase-2 Assay



Ki of Ac-DEV-CHO for caspase-3 is 0.2 nM

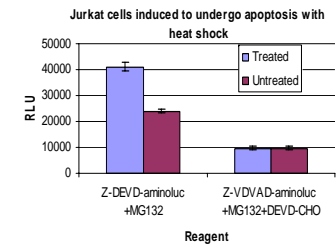
Ki of Ac-DEV-CHO for caspase-2 is 1.75 uM

10. Using the Luminescent Caspase-2 Assay + Inhibitors to Monitor Activity in Heat Shock Induction of Apoptosis



- Jurkat cells treated for 1 hr at 43°C with pre-warmed medium to induce apoptosis
- We detected significant activity 30' after the 1 hr heat shock treatment
- Using the proteasome inhibitor, MG132, and the caspase-3 inhibitor, Ac-DEV-CHO, we demonstrated that all the activity can be attributed to caspase-3/7 and not caspase-2
- We used an ATP assay, CellTiter-Glo®, to ensure equal cell numbers (data not shown)

11. Caspase-3 Activity is Detected Within 30 min of Initiating Heat Shock Treatment; There is No Detectable Caspase-2 Activity



- Jurkat cells were treated for 30' at 43°C with pre-warmed medium to induce apoptosis
- Cells were then assayed immediately for caspase activity

There is controversial information regarding whether caspase-2 is the initiator caspase during heat shock induction of apoptosis.^{1,2}

Our results do not indicate that caspase-2 is acting as an initiator caspase during heat shock.

We demonstrate that caspase-3 can be detected very early after heat shock with our bioluminescent assay.

Milleron and Bratton (2006)² propose that an unknown protease initiates the apoptotic pathway during heat shock activation.

12. Summary

- We have developed a new bioluminescent caspase-2 assay. The limit of detection for this assay is in the femtomolar range of caspase-2.
- Adding a proteasome inhibitor to our bioluminescent caspase-2, -8 and -9 assays (Caspase-Glo® 8 Assay and Caspase-Glo® 9 Assay), improves the specificity and quality of these cell assays.
- Adding a caspase-3/7 inhibitor at a low concentration in the caspase-2 assay eliminated caspase-3/7 cross-reactivity with this substrate. The steady-state format for this bioluminescent assay simplifies the addition of inhibitors in the reagent.
- We used the new luminescent caspase-2 assay to address the question of whether caspase-2 is the initiator caspase in heat shock-induced apoptosis. We do not detect caspase-2 activity during heat shock, but caspase-3 is activated extremely early in this pathway. Our results are consistent with Milleron and Bratton.²

References

1. Tu, S. et al. (2006) *In situ* trapping of activated caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nature Cell Biol.* 8: 72-77.
2. Milleron R. S. and Bratton S. B. (2006) Heat shock induces apoptosis independently of any known initiator caspase-activating complex. *J. Biol. Chem.* 281: 16991-17000.

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