Compare AMC, AFC, Rh110, and AnaRed™ Caspase-3 Substrate in One-step Homogenous Cell-based Assay

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Introduction

Apoptosis is involved in a variety of physiological and pathological events ranging from normal fetal development to diseases, such as cancer, organ failure and neurodegenerative diseases. Caspase-3 (CPP32/apopain) plays a key role in apoptosis pathway. It has been proven to cleave poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), topoisomerases and protein kinase C (PKC)δ.

In order to provide more flexibility and choice, we designed a series of one-step homogenous cell-based fluorimetric caspase-3 assays. These assays use fluorogenic caspase-3 substrates, AMC, AFC, Rh110, and AnaRed™ -linked DEVD. When these fluorophores are conjugated to the peptide, their fluorescence is weak; however, upon caspase-3 cleavage, the fluorophores are released and generate bright fluorescence with the emission wavelength ranging from 442 nm to 635 nm. These assays are ideal for measuring caspase-3 activity and high throughput screening apoptosis-inducers or inhibitors.

Materials and Methods

Experiment Aim: Measure EC_{50} of Camptothecin in inducing apoptosis in Jurkat cells.

Assay procedures:

1. **Plate cells**: Jurkat cells were counted and plated onto black-walled and clear-bottomed 96-well plates at the density of 2 x 10^4 cells/100 µL/well. The last two rows of 96-well plate contained growth medium only as non-cell control.
2. **Induce apoptosis**: Camptothecin was serially diluted in growth medium. 50 µL of diluted camptothecin was added into each well, including non-cell control wells. The final concentrations of camptothecin in cell culture from column 1 to 12 in a 96-well plate are 1.5, 0.75, 0.375, 0.188, 0.094, 0.047, 0.023, 0.012, 0.006, 0.003, 0.001, and 0 µM. The camptothecin-added Jurkat cells were further cultured for 6 hrs in a CO_2 incubator at 37°C.
3. **Prepare assay reagents**: AMC, AFC, Rh110, and AnaRed™ caspase-3 assay reagents were prepared according to the protocols in the following assay kits:

   Cat# 71118 EnzoLyte™ AMC Caspase 3 Assay Kit, Cat# 71114 EnzoLyte™ AFC Caspase 3 Assay Kit,

   Cat# 71141 EnzoLyte™ Rh110 Caspase 3 Assay Kit, Cat# 71122 EnzoLyte™ AR Caspase 3 Assay Kit,
4. **Detect caspase-3 activity**: The plate containing Jurkat cells (from Step # 2) was retrieved from the incubator. 50 µL of above caspase-3 assay reagents were added into each well, including non-cell control wells. The plate was gently shaken for 30-60 sec. The fluorescence signal was immediately monitored by FlexStation 384 II (Molecular Device, CA) at the excitation and emission wavelength for each fluorogenic caspase-3 substrate. Kinetic data were recorded every 3 min for a total of 30 minutes. The endpoint data were recorded at 30 min.

Note: *The assay buffer in the assay kit is a bi-functional buffer, serving both as lysis buffer and assay buffer. For most cells, the extra cell lysis and extraction steps are not necessary. For some hard to lyse cells, the extra cell extraction step may be still needed. If the caspase-3 activity is low, prolonged incubation time after adding caspase-3 assay reagents may be necessary to get adequate fluorescent signals.*

Table 1: Km and excitation/emission wavelength of caspase-3 substrates.

Caspase-3 Substrate	Km (uM)	Excitation/Emission (nm)
Z-DEVD-AMC	19	354 / 442
Ac-DEVD-AFC	39	380 / 500
(Z-DEVD)2-Rh110	26	496 / 520
Z-DEVD-AnaRed	2.4	595 / 635

Results

Fig 1a. Endpoint Data (T=30 min)

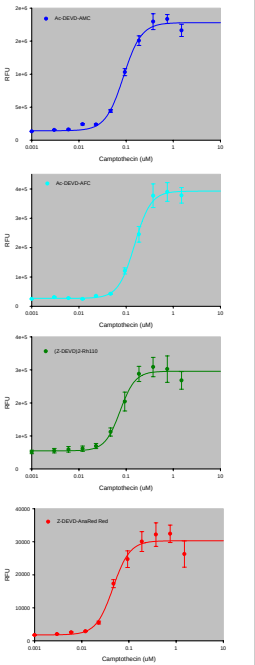

Fig 1b. Kinetic Data

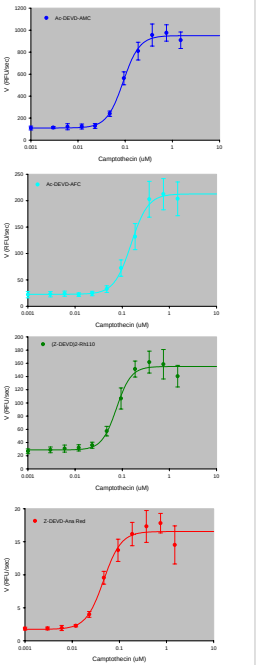

Table 2: EC_{50} of camptothecin in inducing caspase-3 in Jurkat cells.

Caspase-3 Substrate	EC50 of Camptothecin (uM)
Z-DEVD-AMC	0.088
Ac-DEVD-AFC	0.152
(Z-DEVD)2-Rh110	0.075
Z-DEVD-AnaRed	0.046

Figure 1. The dose response curve of camptothecin in inducing caspase-3 activity in Jurkat cells. The caspase-3 activity was measured with Ac-DEVD-AMC, Ac-DEVD-AFC, (Z-DEVD)2-Rh110, and Z-DEVD-AnaRed™.

a. The endpoint data at T=30 min. The fluorescence reading (relative fluorescence unit, RFU) from each well was subtracted with the reading from its corresponding non-cell control. Non-cell control contains the fluorescence generated by test compound, camptothecin, and caspase-3 substrate.

b. The kinetic data. The initial velocity (ΔRFU/sec) of caspase-3 hydrolyzing its substrate was plotted against the concentration of camptothecin. All caspase-3 substrates have very low self-hydrolysis rate. Therefore the initial velocity from sample wells was not corrected with the self-hydrolysis rate of the substrates.

The EC50 of camptothecin tested by each substrate was listed in Table 2.

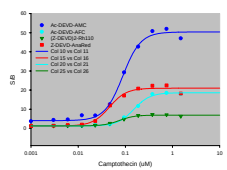

Figure 2. The signal/background (S/B) of caspase-3 substrates in measuring caspase-3 activity in Jurkat cells.

Ac-DEVD-AMC gave the highest S/B (maximal=50), while the (Z-DEVD)2-Rh110 gave the lowest S/B (maximal=7). But the S/B of (Z-DEVD)2-Rh110 was adequate to provide sensitive assay.

The S/B was calculated from the endpoint data of T=30 min. First, the fluorescence reading (relative fluorescence unit, RFU) from each well was subtracted with the reading from its corresponding non-cell control. Then, S/B was calculated according to the following formula:

$S/B = RFU_{camptothecin\ 1.5\text{-}0\ \mu M} / RFU_{camptothecin\ 0\ \mu M}$.

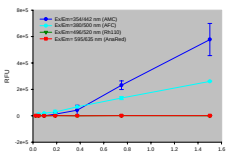

Figure 3. The autofluorescence of camptothecin at the Ex/Em wavelength of each caspase-3 substrate.

Camptothecin has strong autofluorescence at the Ex/Em wavelength of AMC and AFC, but negligible at the spectral channel of Rh110 and AnaRed™. If autofluorescence of test compound is a concern in your assay, the Rh110 or AnaRed™ based caspase-3 assay are better choices than AMC and AFC-based assay.

Summary

We developed a series of fluorimetric homogenous cell-based caspase-3 assays, which provides the following advantages.

1. Four fluorophores, AMC, AFC, Rh110, and AnaRed™, were conjugated to caspase-3 specific substrate, DEVD. The Ex/Em wavelength of these substrates ranges from 442 nm to 635 nm, which provides researchers more choices for multiplex fluorescence assays. All substrates provided sensitive caspase-3 assay with comparable Km and good S/B.
2. The red (AnaRed™) and green (Rh110) fluorogenic caspase-3 substrates potentially avoid the autofluorescence interference from test compounds. If the autofluorescence is not a concern, AMC and AFC substrates are good choices.
3. A bi-functional assay buffer is used in all four assays, which can lyze cells and at the same time support the optimal caspase-3 activity. The extra step for preparing cell lysate is not necessary. All assays are in simple mix-and-read format, ideal for high throughput screening.