

Presentation of an Assay Format for the Specific Assessment of Caspase 3

Martina Orth and Georg Fertig
 Roche Molecular Biochemicals, Penzberg, Germany
 Email: Georg.Fertig@Roche.com

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Introduction

Fourteen different caspases (cysteiny-l-aspartate-specific proteinases) have been reported to date. Caspases are classified and characterized in various ways. Detailed studies using a positional scanning substrate combinatorial library have demonstrated the optimal tetrapeptide recognition motifs for caspases. These preferred amino acid sequences are used for characterising caspase activity. For example, coumarin derivatives of DEVD and IETD are included in kits for the specific detection of activated caspase-3 and 8, respectively. In addition, aldehyde- and ketone derivatives of these tetrapeptides are used to inhibit specific caspases and confirm the origin of the signal generated. In this article, we show how combinatorial studies with different apoptotic cell lysates abrogate the specificity of such investigations. We also describe the development of a novel assay format for the specific determination of activated caspase 3.

Materials and Methods

Caspase 3 was detected with the specific assay. To detect phosphatidylserine exposure on apoptotic cells, annexin-V-fluorescein (Roche Molecular Biochemicals) was used in combination with propidium iodide. Briefly, 2 µg/ml annexin-V-fluorescein was prepared in HEPES-buffer (10 mM HEPES, 5 mM CaCl₂, 140 mM NaCl) containing 1 µg/ml propidium iodide. Cells of a vital culture were washed in PBS and stained with the annexin-V-fluorescein/PI solution for 10 min at RT. Subsequently, the stained cells were analyzed on a Coulter EPICS flow cytometer without further washing. Cells showing annexin-V binding without PI staining were counted as apoptotic.

Results

In order to investigate activation of caspase 8 prior to caspase 3, or vice versa, a specific assay for the respective protease is needed. Kits applying IETD-AFC for analysis of caspase 8 and DEVD-AFC for analysis of caspase 3 are commonly used. To test these assay format types, we used three different cellular models:

- I) Apoptosis was induced in U937 cells using camptothecin (CAM), where we expected caspase 3 activation without induction of caspase 8, because CAM works through the p53 pathway
- II) SKW 6.4 cells were treated with 1 µg/ml anti-CD95 for 3 h at 37°C to induce the FADD-mediated CD95-pathway, which is characterized by caspase 8 activation

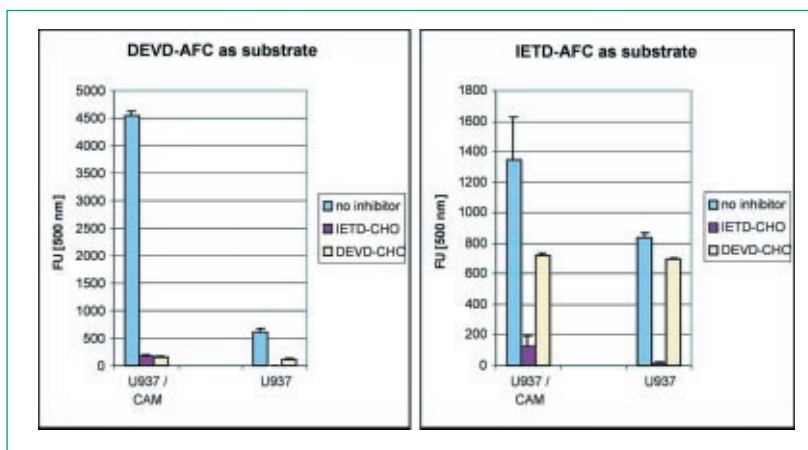


Figure 1a: Cleavage of different caspase substrates by lysates of U937 cells. The cells were treated with CAM to induce apoptosis or left without treatment as controls. The data show percentages of cleaved substrate related to non-inhibited lysates.

- III) Jurkat cells were exposed to 1 µg/ml anti-CD95 for 3 h at 37°C to induce apoptosis through the mitochondrial pathway. Additionally, it was assumed that inhibition of the specific pathway by the corresponding inhibitor would enhance the specificity of the assay

Figure 1 shows the effect of the two different caspase-substrates after preincubation with caspase-inhibitors

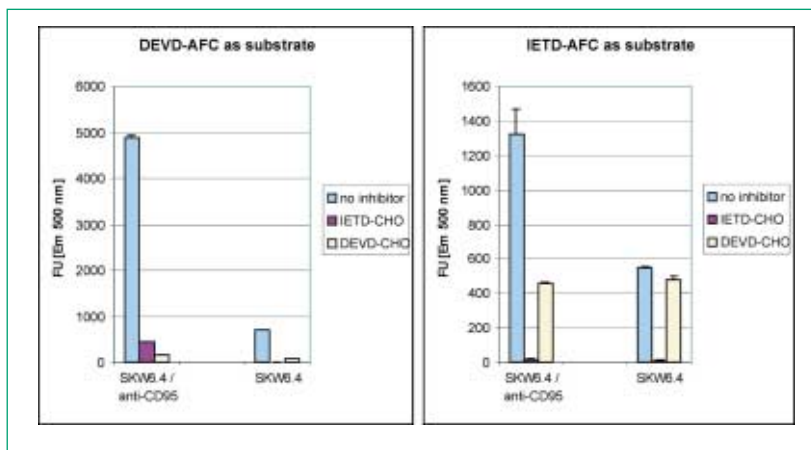


Figure 1b: Cleavage of different caspase substrates by lysates of SKW 6.4 cells. The cells were treated with anti-fas to induce apoptosis or left without treatment as controls. The data show relative fluorescence units (FU) of cleaved substrate related to non-inhibited lysates.

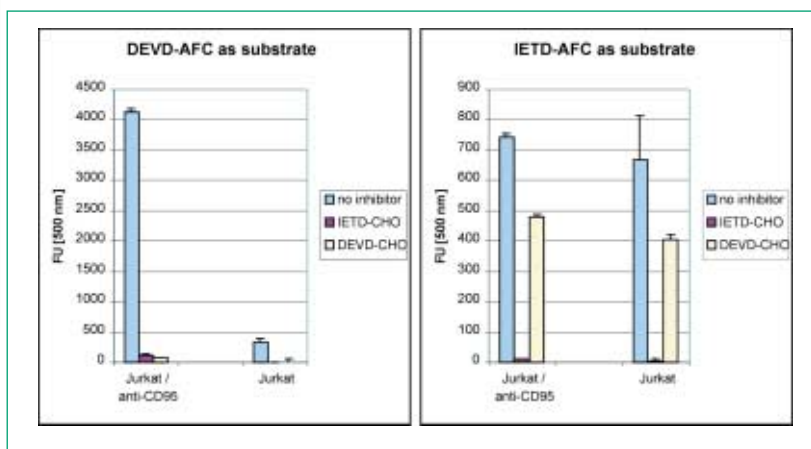


Figure 1c: Cleavage of different caspase substrates by lysates of Jurkat cells. The cells were treated with anti-fas to induce apoptosis or left without treatment as controls. The data show relative fluorescence units (FU) of cleaved substrate related to non-inhibited lysates.

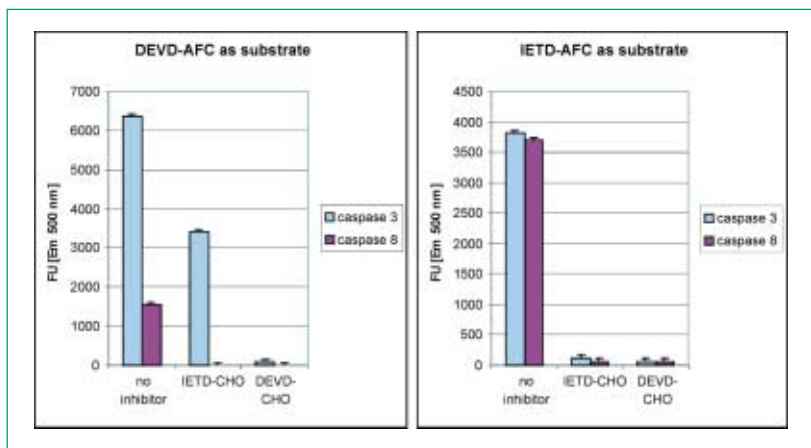


Figure 2: Cleavage of different caspase substrates by recombinant caspase 3 and 8. The data show relative fluorescence units (FU) of cleaved substrate with or w/o preincubation with aldehyde-inhibitors.

on lysates of apoptosis-induced/non-induced U937 cells (1a), SKW 6.4 (1b) and Jurkat (1c). Although IETD-CHO should not diminish the DEVD signal, and DEVD-CHO should not erase the IETD-signal, both inhibitors block the cleavage of caspase substrates non-specifically in all cellular models. Interestingly, the IETD-AFC substrate showed a high turnover even in uninduced cells that could only be partially - but not totally - blocked by DEVD-CHO inhibitor.

For elimination of a possible non-specific protease cleavage of the IETD-AFC substrate, we supplemented the incubation buffer with the following inhibitors: leupeptin, aprotinin and pepstatin. The resulting data showed no alteration compared to Figures 1 a-c (data not shown). For further clarification, recombinant caspases were used to support the non-specificity of the aldehyde inhibitors.

Figure 2 shows the effect on the two different caspase-substrates after preincubation with inhibitors on recombinant caspase 3 and 8. The turnover of DEVD-AFC by caspase 8 is lower compared to that with caspase 3. By contrast, the turnover of IETD-AFC by caspase 8 is fully comparable with caspase 3 turnover. Caspase 8 is blocked by both IETD-CHO and DEVD-CHO, regardless of the substrate (DEVD- or IETD-AFC) used. Caspase 3 is totally blocked by DEVD-CHO and IETD-CHO when IETD-AFC is used as substrate. Interestingly, caspase 3 is not totally blocked by IETD-CHO when DEVD-AFC is used as a substrate.

The following conclusions were drawn from this experiment:

- DEVD-AFC is cleaved by caspase 3 as well as by caspase 8
- IETD-AFC is cleaved by caspase 3 as well as by caspase 8
- DEVD-AFC is a better substrate for caspase 3 than IETD-AFC (supports prior assumption)
- IETD-AFC is a better substrate for caspase 8 than DEVD-AFC (supports prior assumption)
- DEVD-CHO is more efficient at blocking caspase 3 than IETD-CHO when DEVD-AFC is used as a substrate
- IETD-CHO is fully comparable with DEVD-CHO in blocking caspase 3 when IETD-AFC is used as a substrate
- Caspase 3 is only partially blocked by IETD-CHO
- Caspase 8 is blocked by both DEVD-CHO and IETD-CHO

Importantly, conclusions (a) and (b) clearly show that caspase 8 contributes to the caspase 3 turnover of any substrate, and vice versa. Inhibitor studies and the choice of substrate do not allow the origin (caspase 3

or 8) of the signal within a cellular lysate to be specified. Another approach must be used to specify the cellular pathway. We therefore captured the specific caspase from the lysate by means of a monoclonal antibody against CPP32 coated to a MTP. All non-specific proteases were removed (by washing) after capture. The specific caspase 3 activity could be monitored by the addition of DEVD- or IETD-AFC.

Figure 3 shows the analysis of captured recombinant caspases, or captured caspase 3 from cellular lysates in the novel assay format. The data show the following features:

- 1) Both substrates could be used, although DEVD-AFC shows a better turnover
- 2) Both IETD-CHO and DEVD-CHO are capable of blocking the turnover of both substrates, although DEVD-CHO is more effective.
- 3) Non-specific cleavage of IETD-AFC substrate, which was apparent in Figure 1, is eliminated

To verify whether the data for the specific caspase 3 assay correspond to another apoptosis-specific assay (annexin-V-staining): we conducted a dose-response experiment (Figure 4), and performed a kinetic study (Figure 5) of U937 cells treated with CAM.

The experiments shown in Figures 4 and 5 present convincing data for the reliable quantification of caspase 3 activity, the data corresponding to those of a cellular method.

Discussion

In this study, we examined the suitability of two different coumarin-based peptide substrates, and corresponding aldehyde-peptides for analyzing specific caspase activities. By positional scanning of coumarin-conjugated tetrapeptide substrates, DEVD and IETD were found to be preferred substrates for caspase 3 and 8, respectively. From a detailed scrutiny of these studies, one may conclude that the tetrapeptide-derived substrates can serve as optimized substrates, but only without absolute specificity. Nevertheless, it was assumed that these substrates, used in combination with the corresponding inhibitors DEVD-CHO and IETD-CHO, could serve as tools for studying certain specific pathways of caspase 3 and 8. The experimental data shown in Figure 1 underline the incorrectness of this assumption.

In all three cellular models the usage of different substrates, in combination with or without inhibitors, showed that the preferred substrates could not be used to conclude or exclude the presence of any specific caspase activity. The DEVD-AFC substrate was completely blocked by both IETD-CHO and DEVD-CHO.

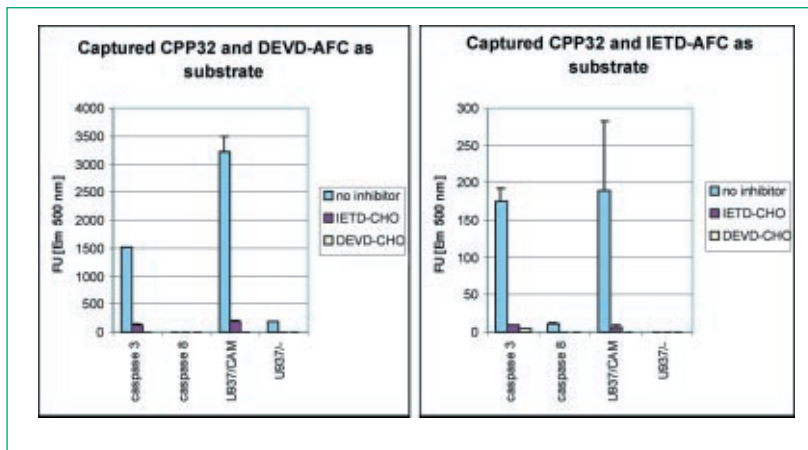


Figure 3: Cleavage of different caspase substrates by recombinant caspase 3, 8, or in a cellular model after capturing of caspase 3 to a microtiterplate. The data show relative fluorescence units (FU) of cleaved substrate with or w/o preincubation with aldehyde-inhibitors.

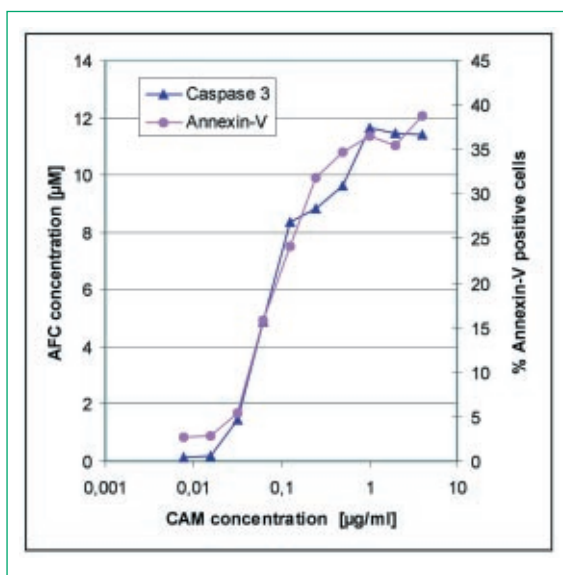


Figure 4: Dose-Response-Curve of U937 cells treated with different concentrations of camptothecin for 4h at 37°C. A sample of the cell culture was removed for annexin-V-staining prior to lysis for the caspase 3 specific assay.

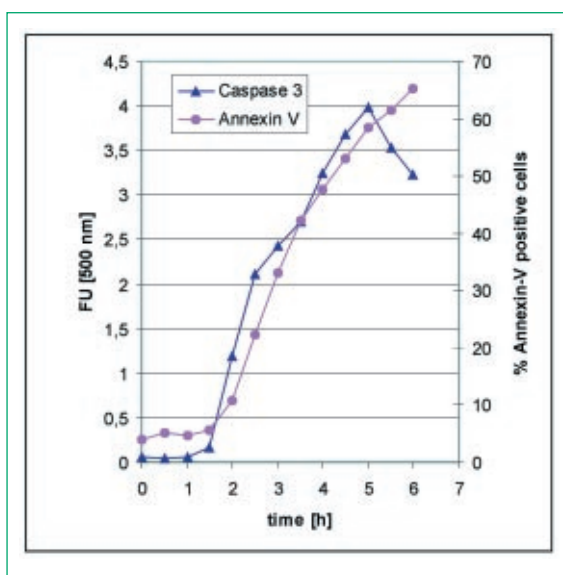


Figure 5: Kinetic study of U937 cells exposed to 2 µg/ml CAM at 37°C. A sample of the cell culture was removed for annexin-V-staining prior to lysis for the caspase 3 specific assay.

Furthermore, the IETD-AFC substrate seemed to be cleaved to a considerable extent by proteases other than caspases, because its turnover reached very high levels in the non-induced cellular lysates. Assuming that the portion of DEVD-CHO-blocked signal represents the caspase 8 activity, there is almost no caspase 8 activation left following subtraction of the uninduced signal. Interestingly, only IETD-CHO - i.e. none of the other applied protease inhibitors - was able to totally block the IETD-AFC signal.

Figure 1c shows that the IETD-AFC turnover of uninduced Jurkat cells almost reached the level of induced cells. This suggests that there is a naturally-occurring active protease with high specificity for IETD as a substrate, which cannot be blocked by leupeptin, aprotinin or pepstatin inhibitors. Furthermore, these inhibitors even elevated the signal in the non-induced lysates compared to the induced lysates. This leaves room for further speculation e.g., that there is a protease that inhibits activated caspase 8 in normal cells only to a certain degree. After excessive activation of caspase 8 by e.g. DISC assembly, this inhibitory protease is not able to totally block the caspase 8 activity any more.

Further supporting experiments for the non-specificity of the substrates were performed with recombinant caspases and are shown in Figure 2. Both substrates are cleaved by both caspases and the cleavage could not be assigned to one specific caspase. Using this approach, caspase 8 contributes to the signal of caspase 3 and vice versa. The inhibitors could not specifically block one of the caspases, and therefore this assay principle is not suitable for detecting the activation of a specific caspase related to a certain pathway.

In order to obtain data on specific caspase 3 activity, we looked for an antibody that is capable of capturing caspase 3 specifically while not blocking the enzymatic activity. After screening several different moAbs, we found one clone (#19) that was suitable for capturing human CPP32 while remaining an active enzyme. Although the antibody captures inactive procaspase 3 as well as activated caspase 3, it allowed us to detect the activation of CPP32 by the addition of DEVD-AFC. The advantage of this technique is that every non-captured protease existing in the lysate is removed (by washing) following the sole capture of caspase 3.

Figure 3 shows that the signals generated can only be related to caspase 3 because no signal is generated by recombinant caspase 8. Once again, it could be demonstrated that both inhibitors, IETD-CHO and DEVD-CHO, are capable of blocking the activity of caspase 3 to a certain degree.

The final experiments were performed to verify the suitability of the new caspase assay for detecting apoptosis (Figures 4 and 5). The comparison with an existing, established method, annexin-V-staining and

PI exclusion of apoptotic cells, showed a very good correlation between the microtiter plate-based assay and the cellular FACS analysis.



Product	Cat. No.	Pack Size
Annexin-V-FLUOS	1 828 681	1 kit (250 tests)
Caspase 3 ActivityAssay	2 012 952	1 kit