Eliminating caspase-7 and cathepsin B cross-reactivity on fluorogenic caspase-3 substrates†

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11 FRET-based fluorogenic substrates were constructed using the pentapeptide template Asp-Glu-X2-Asp-X1, and evaluated with caspase-3, caspase-7 and cathepsin B. The sequence Asp-Glu-Pro-Asp-Ser was able to selectively quantify caspase-3 activity in vitro without notable caspase-7 and cathepsin B cross-reactivity, while exhibiting low µM KM values and good catalytic efficiencies (7.0–16.9 µM⁻¹ min⁻¹).

Caspases (cysteine-aspartate proteases) are a family of endopeptidases that play a key role in the controlled initiation, execution, and regulation of apoptosis, and are essential in the development and homeostasis of mammals. Their deregulation can lead to a number of human pathologies including autoimmune diseases, neurodegenerative disorders and cancer. Caspases are produced as proenzymes and undergo post-translational activation by the ‘caspase cascade’ following apoptotic stimuli and initiation of the extrinsic, intrinsic, or granzyme B apoptotic pathways. Inactivation of the apoptotic intrinsic pathway is often regarded as a ‘hallmark of cancer’ as it leads to the uncontrolled proliferation of cells. The intrinsic pathway responds to intercellular stress triggers, including oncogene activation and DNA damage, and therefore is often targeted in the treatment of cancer. In addition, since caspas es are usually directly involved in the early stages of apoptosis, they are attractive targets for molecular imaging, especially executioner caspase-3, which is down regulated in a variety of cancers.

Current methods of caspase-3 detection include the use of antibodies and fluorescent inhibitors. In addition, FRET-based fluorogenic substrates, consisting of either small molecule fluorophores or fluorescent fusion proteins, have been developed, typically applying the substrate sequence Asp-Glu-Val-Asp (DEVDD). Commercially available substrates, such as Ac-Asp-Glu-Val-Asp-AFC (AFC = 7-amino-4-trifluoromethyl-coumarin) and MCA-Asp-Glu-Val-Asp-Ala-Pro-Lys-DNP (MCA = 7-methoxycoumarin-4-ylacetyl, [DNP = dinitrophenyl]), have been utilised to analyse caspase-3 activity in cell lysates; however, they are unable to measure active caspase in intact cells for real-time analysis due to their inability cross the cell membrane and poor optical properties. Asp-Glu-Val-Asp-NucView, which displays fluorescence upon cleavage and subsequent DNA binding, can be used to measure caspase-3 activity in cell-based assays. All the above substrates are, however, based on the recognition sequence Asp-Glu-Val-Asp and therefore cross-react especially with caspase-7 and cathepsin B. Low expression of proapoptotic caspase-7 is also associated with many cancers, and cathepsin B is often overexpressed in cancerous cells, highlighting the necessity to develop substrates and probes that can detect caspase-3 activation without cross-reactivity with these other enzymes. Recently, caspase-3 selectivity over other caspase isoforms was achieved by incorporation of unnatural amino acids into a pentapeptide recognition sequences to enable imaging of caspase-3 activity in live cells. Similarly, substitution of glutamic acid in Asp-Glu-Val-Asp with pentafluorophenylalanine gave 4.5-fold selectivity over caspase-7 in vitro along with good selectivity over isoforms 6, 8, 9 and 10.

In this study, internally quenched fluorogenic substrates to selectively detect caspase-3 over caspase-7 and cathepsin B were designed and synthesised, and evaluated against human recombinant caspase-3, caspase-7 and cathepsin B. A focused 11-member library of internally quenched substrates was designed incorporating a pentapeptide recognition sequence (X4-X3-X2-X1-X1'). Residue X4 is where the most variability is found within substrates of the caspase family, with preference absolute for aspartic acid for caspase-3 and -7. Caspases are not as selective for the X3 position, although glutamic acid is by far the most favoured residue. This gives rise to the possibility of improving specificity towards caspase-3 by altering the X3 position. Similarly, substitution of valine at the X2 position...
of Asp-Glu-Val-Asp with proline is known to abolish binding to cathepsin B. The X position was incorporated into the recognition sequence to explore if selectivity could be tuned by looking at the residue next to the cleavage/recognition site. At the X position, natural caspase-3 substrates typically show a high preference for small amino acids. Therefore, based on these known substrate requirements for caspase-3 and -7 and cathepsin B, a substrate library was designed, incorporating a FRET pair constructed using carboxyfluorescein as the donor \( \lambda_{\text{Em, max}} = 492/517 \text{ nm} \) and methyl red \( \lambda_{\text{max}} = 480 \text{ nm} \) as the acceptor/quencher (see Fig. 1). In the substrates, the X position contained either valine or proline, and X1' glycine, alanine or serine. In addition, a 6-aminohexanoic acid (Ahx) spacer was positioned between the X and the methyl red moiety in combination with the proline residue. In the preliminary screen, no significant preference was observed for glycine, alanine or serine at the X position. Substrate 9 showed a 3- and 5-fold higher increase in fluorescence than Ac-Asp-Glu-Val-Asp-AFC (AFC) and MCA-Asp-Glu-Val-Asp-Ala-Pro-Lys-DNP (MCA), respectively (ESI† Fig. S2). In the initial screens, caspase-7 showed significantly lower cleavage of substrates containing proline at X2 position, confirming that selectivity can be achieved by replacing valine in that position (ESI† Fig. S3 and S4).

To evaluate the effectiveness of the substrates, as well as to further investigate selectivity, the \( K_M \) and \( k_{\text{cat}} \) values of 1–11 were determined for caspase-3 and caspase-7 (Table 2). All the substrates exhibited good affinity for caspase-3 \( (K_M < 5 \text{ M}) \) compared to AFC and MCA \( (K_M > 13.5 \text{ M}) \), respectively). Substrates bearing a valine at X2 position typically showed lower \( K_M \) values compared to the corresponding peptides bearing a X2 proline, with the exception of substrate 10 (sequence Asp-Glu-Pro-Asp-Ahx, \( K_M = 1.8 \text{ M} \)) which showed the same binding as 11 (Asp-Glu-Val-Asp-Ahx, \( K_M = 1.7 \text{ M} \)). When the substrates were ranked by their catalytic efficiency \( k_{\text{cat}}/K_M \), a more of a division between them was observed. Peptides 2 and 8, both bearing proline at the X2 position and alanine at the X1', were poor substrates for caspase-3 with \( k_{\text{cat}}/K_M \) values of 1.8 and 3.2 \( \text{M}^{-1} \text{min}^{-1} \), respectively. Within the series, 6 (Asp-Glu-Pro-Asp-Ahx) and 9 (Asp-Glu-Val-Asp-Ala-Ahx) had the highest catalytic efficiency (22.2 and 24.2 \( \text{M}^{-1} \text{min}^{-1} \), respectively) proving that the enzyme.

Table 1 11-membered FRET-based substrate library FAM-Ahx-Asp-Glu-X2'-Asp-X1'-spacer-Lys(MR)-NH2 (see Fig. 1 for structures)*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>X2</th>
<th>X1'</th>
<th>Spacer</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>11</td>
<td>Val</td>
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</tbody>
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* For the solid-phase synthesis and characterisation of the fluorogenic substrates, see ESI. Ahx = 6-aminohexanoic acid.
can efficiently cleave substrates with proline at the X₃ position. Based on the catalytic efficiency, the X₄ position overall had a slight preference for glycine and serine over alanine, especially with substrates incorporating proline at X₅.

With caspase-7, substrates MCA and AFC had a $K_M$ values of 15.1 and 10.8 $\mu$M. At the X₄ position, caspase-7 had a clear preference for glycine with 1 and 6 having $k_{cat}/K_M$ values of 8.6 and 8.4 $\mu$M⁻¹ min⁻¹, respectively. Substrates 2 (Asp-Glu-Pro-Asp-Ala) and 3 (Asp-Glu-Pro-Asp-Ser) showed negligible affinity to the enzyme ($K_M$ could not be determined). Remarkably, 10, which was efficiently cleaved by caspase-3 ($k_{cat}/K_M$ 16.9 $\mu$M⁻¹ min⁻¹), was a poor substrate for caspase-7 ($k_{cat}/K_M$ 0.5 $\mu$M⁻¹ min⁻¹) further demonstrating that the sequence ASP-Glu-Pro-Asp-Ser-(Ahx) provides good specificity for caspase-3. Fig. 2 shows a direct comparison of fluorescence increase of probes 3, 10 and 11 after incubation with caspase-3 and caspase-7.

To further examine the specificity of the substrates, reactivity with cathepsin B was evaluated (ESI, Fig. S5). The sequences incorporating proline at the X₅ position (peptides 5, 6, 8, and 10) proved to be poor substrates for cathepsin B and were cleaved with a much lower efficiency than their valine counterparts, with 5 (Asp-Glu-Pro-Asp-Ahx) showing no increase in fluorescence. Substrates 6 and 10 showed significantly reduced cleavage by cathepsin B. To establish the relative affinity of cathepsin B and caspase-3 for the same substrate, the cleavage rates by the two enzymes were directly compared, with 5, 6, and 10 showing the highest selectivity towards caspase-3 even with a 1.7-fold higher cathepsin B concentration (Fig. 3).

**Conclusions**

11 FRET-based fluorogenic substrates, having a pentapeptide sequence with two variable positions, were designed and synthesised with the aim of identifying a caspase-3 selective peptide. Replacement of the valine with a proline in the traditional, non-selective Asp-Glu-Val-Asp recognition sequence yielded substrates with good selectivity over caspase-7 and cathepsin B. In particular peptide sequence Asp-Glu-Pro-Asp-Ser (substrates 3 and 10), was able to selectively quantify caspase-3 activity in vitro without notable caspase-7 and cathepsin B cross-reactivity. Furthermore, the binding affinities of these new substrates for caspase-3 were significantly increased (>3-fold) compared to the two widely used substrates MCA and AFC, compared to the two commercially available fluorogenic caspase-3 substrates, while also exhibiting good catalytic efficiency. The substrates based on Asp-Glu-Pro-Asp-Ser, have the potential to solve experimental issues caused by the lack of enzyme selectivity of
commonly used substrates, providing a more accurate analysis of caspase-3 activity in cancer and beyond. Application of these selective substrates to probes enabling caspase-3 imaging in cell-based assays is currently underway.

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Notes and references