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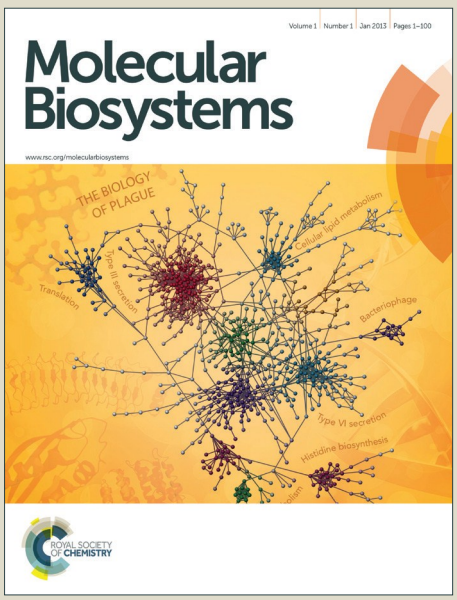

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Eliminating Caspase-7 and Cathepsin B Cross-Reactivity on Fluorogenic Caspase-3 Substrates†

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11 FRET-based fluorogenic substrates were constructed using the pentapeptide template Asp-Glu-X_2-Asp-X_1', 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 K_M values and good catalytic efficiencies (7.0–16.9 $\mu M^{-1} min^{-1}$).

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.[1] Their deregulation can lead to a number of human pathologies including autoimmune diseases,[2] neurodegenerative disorders[3] and cancer.[4] Caspases are produced as procaspases and undergo post-translational activation by the 'caspase cascade' following apoptotic stimuli and initiation of the extrinsic, intrinsic, or granzyme B apoptotic pathways.[5] Inactivation of the apoptotic intrinsic pathway is often regarded as a 'hallmark of cancer' as it leads to the uncontrolled proliferation of cells.[6] The intrinsic pathway responds to intercellular stress triggers, including oncogene activation and DNA damage, and therefore is often targeted in the treatment of cancer.[7] As caspases 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.[8]

Current methods of caspase-3 detection include the use of caspase specific antibodies[9] and fluorescent inhibitors.[10] In addition, FRET-based fluorogenic substrates, consisting of either small molecule fluorophores[11] or fluorescent fusion proteins,[12] have been developed, typically applying the substrate sequence Asp-Glu-Val-Asp (DEVD).[13] 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-yl)acetyl, 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.[14] 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.[15] 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.[16] 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.[17]

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 (by FRET) substrates was designed incorporating a pentapeptide recognition sequence (X_4-X_3-X_2-X_1-X_1'). Residue X_4 is where the most variability is found within substrates of the caspase family, with preference absolute for aspartic acid for caspase-3 and -7.[18] Caspases are not as selective for the X_3 position, although glutamic acid is by far the most favoured residue.[18] Caspase-3 and -7 both prefer hydrophobic residues at the X_2 position, with caspase-7 having far more specific

[a] EaStCHEM, School of Chemistry, University of Edinburgh, David Brewster Road, EH9 3FJ Edinburgh, United Kingdom. E-mail: annamaria.lilienkampf@ed. ac.uk; mark.bradley@ed.ac.uk † Electronic Supplementary Information (ESI) available: [Supporting figures, synthesis and characterisation of the substrates, and enzymatic assays]. See DOI: 10.1039/x0xx00000x

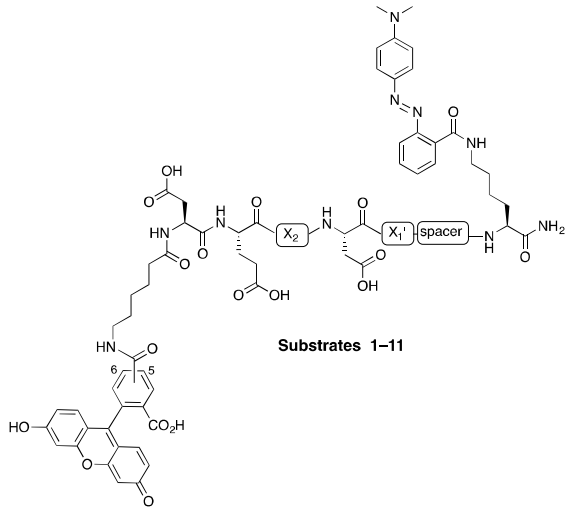

Fig. 1 Design of the internally quenched substrate library, incorporating a pentapeptide recognition sequence Asp-Glu-X_2-Asp-X_1' (caspases cleave between Asp and X_1'). The N-terminus bears a 6-aminohexanoic acid (Ahx) spacer and 5(6)-carboxyfluorescein (mixture of isomers) as the donor fluorophore, and the C-terminus has a methyl red (quencher) coupled to a lysine side chain. See Table 1 for each individual substrate sequences.

Table 1. 11-membered FRET-based substrate library FAM-Ahx-Asp-Glu-X_2-Asp-X_1'-spacer-Lys(MR)-NH_2 (see Figure 1 for structures).[a]

Substrate	X_2	X_1'	Spacer
1	Val	Gly	–
2	Pro	Ala	–
3	Pro	Ser	–
4	Val	–	Ahx[b]
5	Pro	–	Ahx
6	Pro	Gly	Ahx
7	Val	Gly	Ahx
8	Pro	Ala	Ahx
9	Val	Ala	Ahx
10	Pro	Ser	Ahx
11	Val	Ser	Ahx

[a] For the solid-phase synthesis and characterisation of the fluorogenic substrates, see supporting information. [b] Ahx = 6-Aminohexanoic acid.

requirements than caspase-3, retaining a higher preference for valine.[18a] This gives rise to the possibility of improving specificity towards caspase-3 by altering the X_2 position.[18a,19] Similarly, substitution of valine at the X_2 position of Asp-Glu-Val-Asp with proline is known to abolish binding to cathepsin B.[20] The X_1' 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_1' position, natural caspase-3 substrates typically show a high preference for small amino acids.[21] 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 ($\lambda_{Ex/Em}$ 492/517 nm) and methyl red (2-(*N*,*N*-dimethyl-4-aminophenyl)azobenzenecarboxylic acid) (λ_{max} ~480 nm) as the acceptor/quencher (see Fig. 1). In the substrates, the X_2 position contained either valine or proline, and X_1' glycine, alanine or serine. In addition, a 6-aminohexanoic acid (Ahx) spacer was positioned between the X_1' residue and the methyl red motif in to investigate possible steric hindrance from the quencher.

The substrates **1–11** (Fig. 1, Table 1) were synthesised on an Fmoc-Rink-amide linker derivatised aminomethyl polystyrene resin (1 % DVB, 100–200 mesh, loading 1.2 mmol/g) using standard Fmoc solid-phase chemistry with DIC and Oxyma as coupling combination (ESI, Scheme S1–S2). Fmoc-Lys(Dde)-OH was coupled onto the Rink-linker followed by coupling of the Ahx linker (when present) and next five amino acids (Asp-Glu-X_2-Asp-X_1'). With the N-terminus Fmoc group still attached, the Dde protecting group was selectively removed with $NH_2OH{\cdot}HCl$/imidazole, followed by coupling of methyl red (in essence Fmoc-Lys(Dde)-OH is a bi-functional spacer utilising the orthogonal nature of the Fmoc and Dde protecting groups).[22] Finally, Ahx spacer and 5(6)-carboxyfluorescein were consecutively coupled. This modular approach allows for the changing of the quencher or conversion of the positions of the quencher and the fluorophore at final stages of the synthesis. After deprotection and cleavage off the resin, the substrates were purified by preparative RP-HPLC and analysed by MALDI-ToF MS.

Peptides **1–11** were initially evaluated for their ability to act as substrates for caspase-3 and -7 at 2.5, 5 and 10 μM allowing the effects of the X_2 and X_1' substitutions to be assessed. All the substrates had low background fluorescence levels and showed time dependent activation with caspase-3 (ESI, Figure S1). Substrates **2** (Asp-Glu-Pro-Asp-Ala) and **3** (Asp-Glu-Pro-Asp-Ser), both of which have proline at the X_2 position, showed reduced activation with caspase-3 compared to the other probes; however, this activity was restored by introduction of an the Ahx spacer (**8** and **10**), suggesting that the reduced cleavage of **2** and **3** arose from steric hindrance by 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_1' position. Substrate **9** showed a 3 and 5-fold higher increase in fluorescence than the two commercial controls, Ac-Asp-Glu-Val-Asp-AFC (**AFC**) and MCA-Asp-Glu-Val-Asp-Ala-Pro-Lys-DNP (**MCA**), respectively (ESI, Figure S2). In the initial screens, caspase-7 showed significantly lower cleavage of substrates containing proline at X_2 position, confirming that selectivity can be achieved by replacing valine in that position (ESI, Figure S3 and S4).[19c]

To evaluate the effectives of the substrates, as well as to further investigate selectivity, the K_M and k_{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 values < 5 μM) compared to **AFC** and **MCA** (K_M values 9.8 μM and 13.5 μM, respectively). Substrates bearing a valine at X_2 position typically showed lower K_M values than the corresponding peptides bearing a X_2 proline, with the exception of substrate **10** (sequence Asp-Glu-Pro-Asp-Ser-Ahx, K_M 1.8 μM), which exhibited equal binding to **11**

Table 2. Kinetic analysis of substrates **1–11** (n = 3) with caspase-3 and caspase-7.

	Caspase-3			*Caspase-7*		
Probe	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1} min^{-1}$)	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1} min^{-1}$)
1	2.3 ± 0.5	23.4 ± 1.4	10.3	1.2 ± 0.2	10.2 ± 0.5	8.6
2	4.6 ± 0.5	8.4 ± 0.3	1.8	n/a[c]	n/a	n/a
3	2.9 ± 0.5	20.1± 1.0	7.0	n/a	n/a	n/a
4	1.7 ± 0.3	13.4 ± 0.5	7.8	2.9 ± 0.5	10.1 ± 0.6	3.4
5	2.7 ± 0.4	36.5 ± 1.7	13.3	2.4 ± 0.5	8.2 ± 0.5	3.5
6	2.6 ± 0.4	58.4 ± 2.4	22.2	2.1 ± 0.2	17.7 ± 0.6	8.4
7	1.4 ± 0.2	20.4 ± 0.8	14.9	5.1 ± 0.9	19.9 ± 1.3	3.9
8	3.5 ± 0.5	11.1 ± 0.5	3.2	27.5 ± 8.0	3.8 ± 0.7	0.14
9	2.2 ± 0.3	53.3 ± 2.4	24.2	3.4 ± 0.3	10.7 ± 0.3	3.2
10	1.8 ± 0.3	30.7 ± 1.2	16.9	5.2 ± 0.8	2.6 ± 0.2	0.5
11	1.7 ± 0.3	19.7 ± 0.9	11.7	2.7 ± 0.4	6.4 ± 0.3	2.4
MCA[a]	13.5 ± 2.6	13.5 ± 1.3	1.0	15.1 ± 3.2	5.9 ± 0.7	0.4
AFC[b]	9.8 ± 1.9	14.7 ± 1.2	1.5	10.8 ± 2.8	11.6 ± 1.4	1.1

[a] **MCA** = MCA-Asp-Glu-Val-Asp-Ala-Pro-Lys-DNP. [b] **AFC** = Ac-Asp-Glu-Val-Asp-AFC [c] Could not be determined.

(Asp-Glu-Val-Asp-Ser-Ahx, K_M 1.7 μM). When the substrates were ranked by their caspase-3 catalytic efficiency (k_{cat}/K_M), more of a division between them was observed. Peptides **2** and **8**, both bearing proline at the X_2 position and alanine at the X_1', were poor substrates for caspase-3 with k_{cat}/K_M values of 1.8 and 3.2 $\mu M^{-1} min^{-1}$, respectively. Within the series, **6** (Asp-Glu- Pro-Asp-Gly-Ahx) and **9** (Asp-Glu-Val-Asp-Ala-Ahx) had the highest catalytic efficiency (22.2 and 24.2 $\mu M^{-1} min^{-1}$, respectively) proving that the enzyme can efficiently cleave substrates with proline in X_2 position. Based on the catalytic efficiency, the X_1' position overall had a slight preference for glycine and serine over alanine, especially with substrates incorporating proline at X_2.

With caspase-7, commercial substrates **MCA** and **AFC** had a K_M values of 15.1 and 10.8 μM, *i.e.*, they did not show notable selectivity for caspase-3 (k_{cat}/K_M values 0.4–1.5 $\mu M^{-1} min^{-1}$ for both enzymes). At the X_1' 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. Figure 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 X_2 position (peptides **5**, **6**, **8**, and **10**) proved 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** also had 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).

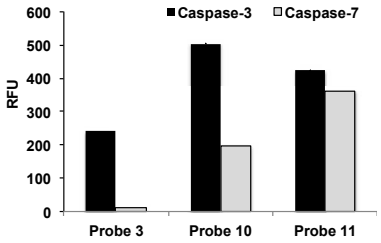

Fig. 2 Comparison of fluorescence increase ($\lambda_{Ex/Em}$ 485/525 nm) with substrates **3** (Asp-Glu-Pro-Asp-Ahx), **10** (Asp-Glu-Pro-Asp-Ser-Ahx) and **11** (Asp-Glu-Val-Asp-Ser-Ahx) at 3.1 μM (n = 3, standard deviation ±1.0–3.1 RFU) incubated with caspase-3 and caspase-7 (15 nM) for 60 min. Proline at the X_2 position yields a caspase-3 selective substrate.

Conclusions

11 FRET-based fluorogenic substrates, having a pentapeptide recognition sequence with two variable positions, were

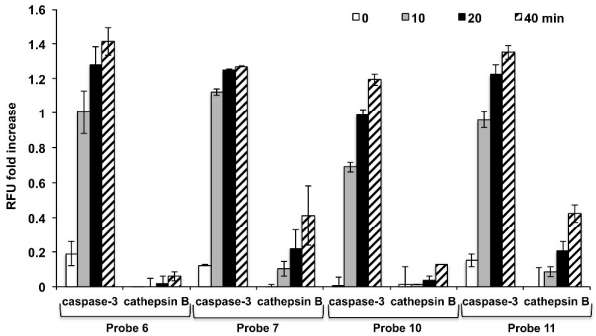

Fig. 3 Comparison of activation by caspase-3 and cathepsin B with substrates containing proline or valine at X_2 position (**6** *vs* **7** and **10** *vs* **11**), with proline yielding a caspase-3 selective substrate. Compounds (10 μM, n = 3) were incubated with caspase-3 (15 nM) or cathepsin B (25 nM), and the increase in fluorescence was recorded as 'RFU fold increase over background' (normalised to zero), to eliminate the effects of reduced fluorescence of fluorescein at low pH due to the pH of the cathepsin buffer (pH 5.0).

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 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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