

# Unnatural amino acids increase sensitivity and provide for the design of highly selective caspase substrates

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Traditional combinatorial peptidyl substrate library approaches generally utilize natural amino acids, limiting the usefulness of this tool in generating selective substrates for proteases that share similar substrate specificity profiles. To address this limitation, we synthesized a Hybrid Combinatorial Substrate Library (HyCoSuL) with the general formula of Ac-P4-P3-P2-Asp-ACC, testing the approach on a family of closely related proteases – the human caspases. The power of this library for caspase discrimination extends far beyond traditional PS-SCL approach, as in addition to 19 natural amino acids we also used 110 diverse unnatural amino acids that can more extensively explore the chemical space represented by caspase-active sites. Using this approach we identified and employed peptide-based substrates that provided excellent discrimination between individual caspases, allowing us to simultaneously resolve the individual contribution of the apical caspase-9 and the executioner caspase-3 and caspase-7 in the development of cytochrome-*c*-dependent apoptosis for the first time.

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Apoptosis, the most well-understood form of programmed cell death, is a highly regulated process controlled and executed by proteolytic enzymes called caspases. The apoptotic process is somewhat hierarchical and caspases can be assigned as initiators (2, 8, 9, and 10) and executioners (3, 6, and 7).<sup>1–3</sup> Apoptosis can be triggered extrinsically via ligation of a death receptor by its cognate ligands, leading to the activation of caspases 8 and 10, or intrinsically following the release of cytochrome *c* from mitochondria with formation of a caspase 9 activation complex known as the apoptosome.<sup>3–5</sup> Mechanistically, caspases display a near absolute preference for aspartate at the P1 position of their substrates. In addition, they require a minimum substrate length of four amino acids N-terminal of the scissile bond. Thornberry *et al.*<sup>6,7</sup> used a combinatorial library of fluorogenic substrates to profile nine human caspases at the P4–P2 region, demonstrating that the caspases tended to have specificity profiles that enabled grouping based on substrate preferences.<sup>6,7</sup> This work provided a great insight into caspase recognition patterns and opened the door for others to pursue small molecule probes for caspase investigations.

To date, various types of substrates and inhibitors have been developed and biologically evaluated against caspases.<sup>2,8–11</sup> Unfortunately, most of them lack selectivity and cannot be used for selectively targeting or analyzing particular

enzymes in complex biological environments.<sup>12–15</sup> This is entirely because of the overlapping specificities of the caspases on their preferred *natural* amino acid sequences. To address this problem we designed and synthesized a *Hybrid Combinatorial Substrate Library* (HyCoSuL) containing 19 natural amino acids (omitting cysteine) and 110 unnatural amino acids. We propose that such a large and varied set of chemical structures provides an excellent tool to investigate caspases and distinguish between them. In this work we dissected the kinetic profiles of six human apoptotic recombinant caspases through HyCoSuL screening. We then designed and synthesized new caspase substrates with the ability to discriminate these enzymes within a group. To further test the specificity and utility of the designed hybrid substrates, we performed a series of experiments in a cell-free model of apoptosis where multiple caspases are activated.

## Results

**HyCoSuL synthesis strategy and its application.** Our objective was to exploit a chemical tool that could be used to develop highly selective and sensitive short peptide-based substrates for caspases. We hypothesized that the use of a large number of unnatural amino acids would allow us to explore chemical space encompassed by the caspase-active

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**Abbreviations:** HyCoSuL, Hybrid Combinatorial Substrate Library; ACC, 7-amino-4-carbamoylmethylcoumarin; RFU, relative fluorescence unit; PS-SCL, positional scanning-substrate combinatorial library; Dab, 2,4-diaminobutyric acid; *D*-hPhe, *D*-homophenylalanine; Ser(Bzl), serine benzyl ester; hCha, homocyclohexylalanine; AFC, 7-amino-4-trifluoromethylcoumarin; XIAP, X-linked inhibitor of apoptosis protein; BIR domain, baculovirus inhibitor of apoptosis protein repeat; Phe(2-Cl), 2-chlorophenylalanine; Pip, pipercolinic acid; hTyr, homotyrosine; Oic, octahydroindole-2-carboxylic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; His(Bzl), N(im)-benzyl-histidine; hPhe, homophenylalanine; Thr(Bzl), threonine O-benzyl ester; Phe(F5), 2,3,4,5,6-pentafluorophenylalanine; Aad, 2-Aminohexanedioic Acid; Nle(O-Bzl), 2-amino-6-benzoyloxyhexanoic acid; NptGly, *neo*-pentylglycine

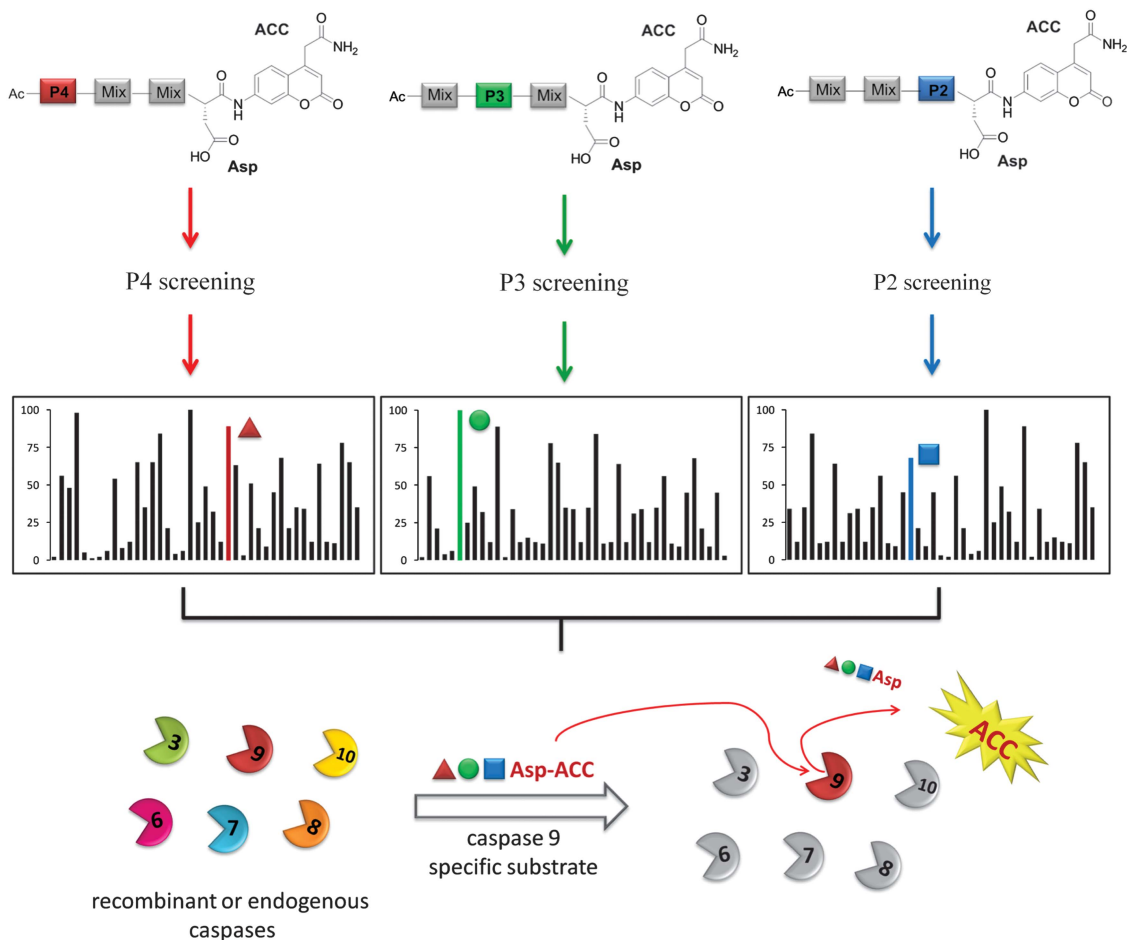
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site cleft, and to this end we synthesized a hybrid combinatorial library with the general formula of Ac-P4-P3-P2-Asp-ACC (Figure 1). Based on this structural scaffold we fixed aspartic acid at the P1 position (an essential requirement for caspases<sup>6,16,17</sup>) and synthesized three sublibraries (P4, P3, and P2) for dissecting caspase preferences at the S4–S2 region. As a reporter group, an ACC (7-amino-4-carbamoylmethyl coumarin) fluorophore moiety was selected for convenience in solid-phase synthesis.<sup>18</sup> Each sublibrary contained one of 129 amino acids fixed at either the P4, P3, or P2 positions. These amino acids consisted of 19 amino acids (18 natural, cysteine was omitted and methionine was replaced with norleucine) and 110 unnatural amino acids, where we define unnatural amino acids as those that are not encoded in proteins. Each pool contained a total of  $129 \times 19 \times 19 = 46\,569$  possible tetrapeptide sequences so that the total number of possible tetrapeptide combinations that can be made using this library is  $129 \times 129 \times 129 = 2\,146\,689$ , and this is significantly greater than in previously published approach ( $20 \times 20 \times 20 = 8000$ ).<sup>6</sup>

We selected commercially available unnatural amino acids with diverse physicochemical attributes to cover a broad

range of possible substrate–enzyme interactions: acidic and basic, small and large, hydrophilic and hydrophobic, with defined stereochemistry (*L* or *D*) or lacking stereochemistry (dehydro amino acids). After the screening of six human recombinant caspases, we were armed with data that allowed us to design and synthesize new substrates with a very high selectivity index. The general architecture of HyCoSuL and its use for discovery of selective and sensitive caspase substrates is presented in Figure 1. The most selective substrates can be used in cell-based assays as well as serve as the scaffold for selective inhibitor design. On the other hand, most active substrates (the highest  $k_{cat}/K_m$  values) can be used in regular kinetic studies on recombinant caspases.

**Substrate specificity determination.** Each of the P4, P3, and P2 sublibraries was screened at  $50\ \mu\text{M}$  final total substrate mixture, with caspase concentrations in the range of 10–200 nM. The concentration of each caspase in the assay was selected in preliminary experiments to provide an optimal fluorescence readout (see Materials and Methods). Initial rates of hydrolysis were recorded as relative fluorescence units (RFUs) over time and normalized by inclusion of



**Figure 1** General scheme for HyCoSuL design and its use for the discovery of selective and sensitive substrates for caspases. HyCoSuL is composed of three sublibraries containing 129 individual natural and unnatural amino acids. Screening results reveal the identity of residues that contribute to activity at a specific position for each caspase. In the example in the colored bars represent the most selective residue when compared with other caspases (not shown in this illustration). Sometimes, but not always, the most selective residue is also the most active. Thus, HyCoSuL is useful not only for the design and synthesis of sensitive caspase substrates, but also for the discovery of caspase substrates with high selectivity

at least three references substrate mixtures. Each caspase was screened at least three times ( $n=3-5$ ) and the average value of RFU/s (expressed as % normalized to the best substrate) is presented as a specificity matrix. All substrate specificity profiles for each tested caspase can be found in the Supplementary Data section; however we have distilled their essence into Table 1.

The selectivity overview presented in Table 1 confirms that some caspases share common preferences at a particular subsite, and that these preferences are substantially extended in several cases by incorporating unnatural amino acids into the peptidyl substrates. However, along with the expected preferences come some unexpected ones, and we have summarized these below. We propose that understanding the amino acid preferences at each position, and perhaps more importantly the restricted amino acids at each position, would allow for the design of highly selective substrates.

**The P4 position.** The P4 position is the most distinguishing characteristic discriminating substrate selectivity in the caspase family.<sup>6,19</sup> In general, our observed P4 preferences followed established rules, with caspases 3 and 7 favoring Asp, and caspases 6, 8, 9, and 10 favoring aliphatic residues.<sup>6,19</sup> Caspase 3 and 7 are very closely related enzymes sharing 57% sequence identity and very similar substrate specificity.<sup>20</sup> At the P4 position, caspase 3 favored Asp ~10-fold over other amino acids but caspase 7 was more catholic, preferring Asp but also tolerating aspartic acid

methyl, cyclohexyl and benzyl esters, and also histidine benzyl ester, thioproline. The broader tolerance of caspase 7, also seen at the P3 position (see below), is the key distinguishing feature between the closely related paralogous caspases 3 and 7. Distinctions between caspases at the P4 position can also be found via negative selection. Caspases 3 and 7 accept very few amino acids from our library, except Asp and a few others mentioned previously. Caspases 6 and 10 also have strict preferences at the P4 position. The only group that is not tolerated by caspase 8 at the P4 position is basic amino acids. The most promiscuous is caspase 9 that recognizes almost all chemical structures at P4. We detected only a few amino acids that were completely ignored by this enzyme.

**The P3 position.** The P3 position provides the least discrimination between caspases. This position is intolerant of *D*-amino acids and secondary amino acids, and generally prefers Glu. Interestingly, Glu-esters were also well tolerated at this position, particularly for caspases 7 and 10. In available crystal structures, the  $\gamma$ -carboxylate of P3 Glu generally forms an ion pair with Arg-341 that is absolutely conserved in all human caspases,<sup>21</sup> and one might therefore expect that the ion pair was necessary for substrate recognition. However, the tolerance for Glu-esters, as well as aliphatic residues by caspases 7 and 9, suggest that this ion pair is not essential for optimal P3 occupancy. Most likely, Arg-341 conservation is required for interaction with P1 Asp, to which it also makes an ion pair. The substrate specificity of

**Table 1** The aggregated substrate specificity profiles of six recombinant human caspases

	Casp-3	Casp-6	Casp-7	Casp-8	Casp-9	Casp-10
<i>P4 position</i>						
Asp	•••••	•	•••••	••	•	•
Asp esters	•	•	•••	•	•	•
Ile, Leu, Val	•	•••••	•	•••••	•••••	•••••
Abu, hLeu, Nva, Tle	•	•••••	•	•••••	•••	•••••
Hyp, Thz, Hyp(Bzl), Oic	•	•	••	•••	•••••	•
<i>D</i> -hPhe, <i>D</i> -Phg	•	•	•	•••	•	•
dhLeu, dhAbu	•	•	•	•••	••	•
Phe(2-Cl)	•	••	•	•	•	•••
Others	•	•	•	•	•	•
<i>P3 position</i>						
Glu	•••••	•••••	•••••	•••••	•••••	•••••
Glu esters	•••	•••	•••••	••	••	•••••
hGlu	•••	••	•••••	•••••	•••	•••••
Val, Tle	••	•	•••••	••	•••••	•••
<i>D</i> -amino acids	•	•	•	•	•	•
Phe-derivatives	•••	•	••	•	•	•
Bulky amino acids	••	••	•••	••	••	••
Basic amino acids	••	•	•••	•	••	••
Small, hydrophobic	••	•	••	••	••	••
<i>P2 position</i>						
Thr(Bzl)	•••••	•••••	•••••	•••••	•	••
Val, Ile	•••	••	••	••	•	••
Oic, Pip, Tic	••	•	••	••	••	•••••
His, His(Bzl)	•	•••••	•	••	•••••	•••••
Ser(Bzl), hPhe, hTyr	•	•••	•	••	•	•••••
<i>D</i> -amino acids	•	•	•	•	•	•
Others	•	•	•	•	•	•••

We present only some of most important information extracted from the full HyCoSuL screen. The code used for substrate specificity description is as follows: •••••, very high activity (81–100%); •••••, high activity (61–80%); ••••, medium activity (41–60%); ••, weak activity (21–40%); •, very weak or no activity (0–20%)

caspase 9 at P3 was unusual, with *tert*-Leucine and valine dominating over glutamic acid.

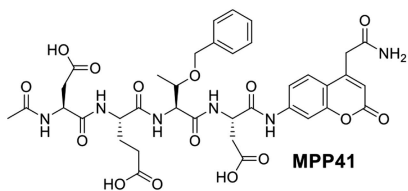
**The P2 position.** The P2 position was not previously thought to provide a high degree of discrimination,<sup>6</sup> yet analysis of the P2 HyCoSuL sublibrary revealed some unexpected preferences. We found that the best recognized amino acid at this position for caspases 3, 6, and 7 was threonine benzyl ester, revealing that the S2 pocket is large enough to accommodate the bulky benzyl group. Other amino acids that were well tolerated at P2 position were proline derivatives (octahydroindole-2-carboxylic acid (Oic), pipercolinic acid (Pip), and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic)), and this is also quite unexpected based on canonical natural amino acid preferences.<sup>6</sup> Subsite preferences suggested that the caspase 6 S2 subsite is large enough to accept even bulkier moieties than the other executioner caspases. Caspase 9 demonstrated essentially exclusive selectivity for His, N(im)-benzyl-histidine (His(Bzl)), and Tic. Caspase 10 recognizes large and hydrophobic amino acids (serine benzyl ester (Ser(Bzl)), homophenylalanine (hPhe), homocyclohexylalanine (hCha)) as well as small and basic amino acids. This preference distinguishes caspase 10 from its close paralog caspase 8. Caspases 3, 6, and 7 do not accommodate basic amino acids and caspase 8 has a very similar 'negative pattern', but it can accommodate basic amino acids. Caspase 9 is ultra-selective at P2 and >95% of amino acids tested are prohibited at this position. Of all the caspases tested, only caspase 10 demonstrated a wide tolerance at P2, failing to accept only *D*- and dehydro-amino acids.

**Design of the most sensitive caspases substrates through HyCoSuL screening.** Armed with substrate specificity profiles of six human caspases, we proceeded to synthesize new substrates displaying enhanced sensitivity over existing substrates. The best substrates for caspase 3 and 7 (MPP41 and MPP42) contain threonine *O*-benzyl ester (Thr(Bzl)) at the P2 position that seemed to enhance exploration of the S2 chemical space. We also noticed that glutamic acid at P3 can be successfully replaced by many other amino acids (like 2-thienyl Ala) with only slight decreases in overall activity. The best substrates for caspases 3 and 7 are twofold better than those based on DEVD sequence (Figure 2). For caspase 6 we found that Val at P4 and Glu at P3 are obligatory for substrates; however, at P2 a few bulky amino acids are tolerated (like Thr(Bzl) or hPhe). These substrates (MPP48 and MPP49) are 4–5-fold better than the reference one (Ac-VEID-ACC). An analysis of caspase 8 substrate specificity revealed that the most active substrate (MPP46) contains three unnatural amino acids: *tert*-leucine (Tle) at P4, homoglutamic acid (2-aminohexanedioic acid (Aad)) at P3, and threonine benzyl ester (Thr(Bzl)) at the P2 position. The  $k_{cat}/K_M$  for this substrate is ~5.5-fold higher than for the reference Ac-IETD-ACC substrate. For caspase 9 we discovered that replacing Glu at P3 with the unnatural *tert*-leucine (Trt) resulted in a threefold increase in activity (MPP47). Caspase 10 is the only enzyme for which we did not obtain improved activity. The replacement of histidine at P2 for serine benzyl ester (Ser(Bzl)) resulted in a

slight increase of activity (~20%) (MPP43). Therefore, overall, although we were able to enhance catalytic rates of peptide hydrolysis by incorporating unnatural amino acids, the degree of enhancement could be considered moderate.

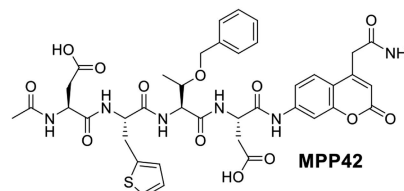
**Design of caspase-selective substrates.** The commonly used caspase substrates containing only natural amino acids lack selectivity (Figure 3). To overcome these limitations, we decided to explore whether a HyCoSuL approach, which extends far beyond traditional positional scanning-substrate combinatorial library (PS-SCL), could deliver enhanced selectivity – that is, could we generate individual substrates that showed dramatically enhanced selectivity for individual caspases. Our initial goal was to discover new fluorogenic substrates with the ability to discriminate caspases in a virtual experiment where all caspases are present at the same concentration. Clearly, it would not be possible to achieve absolute selectivity, and hence we aimed at providing the best possible selectivity, displayed as relative catalytic efficiencies. This '*proof of concept*' experiment demonstrated that for almost all caspases our expectations were met. We could not produce a substrate that efficiently discriminated caspases 3 and 7, but we found that replacing Glu from the DEVD sequence with 2,3,4,5,6-pentafluorophenylalanine, Phe(F5) (compound MPP39) showed excellent discrimination of caspases 3 and 7 compared with other caspases. The most selective caspase 6 substrate is MPP36 that contains threonine at P4, glutamic acid at P3, and *neo*-pentylglycine (NptGly) at P2. MPP30, the most selective caspase 8 substrate, contains three unnatural amino acids, with the *D*-homophenylalanine (*D*-hPhe) at P4, Aad at P3, and Thr(Bzl) at P2. To distinguish caspase 9 from others enzyme, we synthesized MPP8 that possesses Oic (a cyclohexyl proline derivative) at P4, Tle at P3, and His at P2. Finally, for caspase 10 we found several selective substrates, among which MPP52 displayed the highest  $k_{cat}/K_M$  selectivity index among all tested substrates. This substrate contains 2-amino-6-benzyloxyhexanoic acid (Nle(*O*-Bzl)) at P4, 2,4-diaminobutyric acid (Dab) at P3, and norleucine at P2. In Figure 3 we present our best hits, and a detailed kinetic analysis for these (and several other) substrates can be found in the Supplementary Data section. We were delighted to find substrates that would distinguish each of the apical apoptotic caspases (8, 9, and 10) from each other and from other caspases.

**Determination of caspase-selective substrates for cell-based assays.** One of the main driving principles of this study was to develop substrates that could be used to assess the role of individual caspases in complex mixtures, a goal that has previously remained elusive.<sup>12</sup> The HyCoSuL strategy allowed us to design new, very selective caspase substrates, measured by the specificity constant  $k_{cat}/K_M$ . However, in many biochemical experiments to obtain a measurable fluorescent readout, substrates are often used at a concentration significantly above their  $K_M$  values, and thus the rate of their hydrolysis depends only on the  $k_{cat}$  parameter. Such an example is caspase detection in cytosolic extracts. To date, there are no commercial substrates that can demonstrate selectivity in this kind of

**Casp-3 and Casp-7** (reference: Ac-DEVD-ACC: **Casp-3** 474 400 M<sup>-1</sup>s<sup>-1</sup> and **Casp-7** 82 000 M<sup>-1</sup>s<sup>-1</sup>)

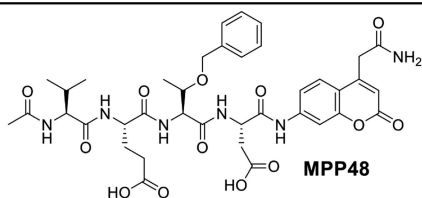
$$\text{casp-3 } k_{\text{cat}}/K_M = 1,050,000 \text{ M}^{-1}\text{s}^{-1}$$

$$\text{casp-7 } k_{\text{cat}}/K_M = 167,000 \text{ M}^{-1}\text{s}^{-1}$$

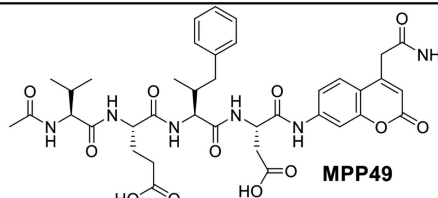


$$\text{casp-3 } k_{\text{cat}}/K_M = 825,000 \text{ M}^{-1}\text{s}^{-1}$$

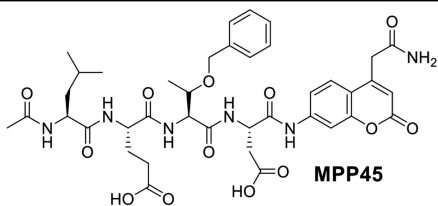
$$\text{casp-7 } k_{\text{cat}}/K_M = 159,000 \text{ M}^{-1}\text{s}^{-1}$$

**Casp-6** (reference: Ac-VEID-ACC 44 800 M<sup>-1</sup>s<sup>-1</sup>)

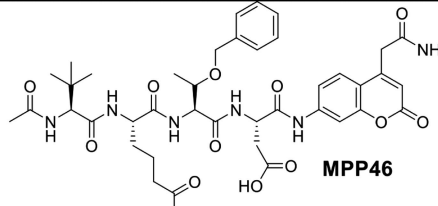
$$k_{\text{cat}}/K_M = 186,000 \text{ M}^{-1}\text{s}^{-1}$$



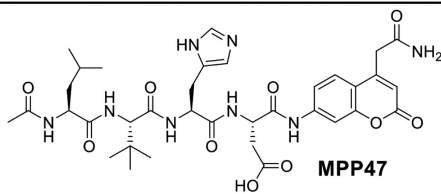
$$k_{\text{cat}}/K_M = 229,000 \text{ M}^{-1}\text{s}^{-1}$$

**Casp-8** (reference: Ac-IETD-ACC 94 900 M<sup>-1</sup>s<sup>-1</sup>)

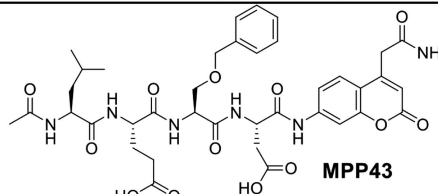
$$k_{\text{cat}}/K_M = 349,000 \text{ M}^{-1}\text{s}^{-1}$$



$$k_{\text{cat}}/K_M = 526,000 \text{ M}^{-1}\text{s}^{-1}$$

**Casp-9** (ref: Ac-LEHD-ACC 12 700 M<sup>-1</sup>s<sup>-1</sup>)

$$k_{\text{cat}}/K_M = 37,000 \text{ M}^{-1}\text{s}^{-1}$$

**Casp-10** (ref: Ac-LEHD-ACC 143 700 M<sup>-1</sup>s<sup>-1</sup>)

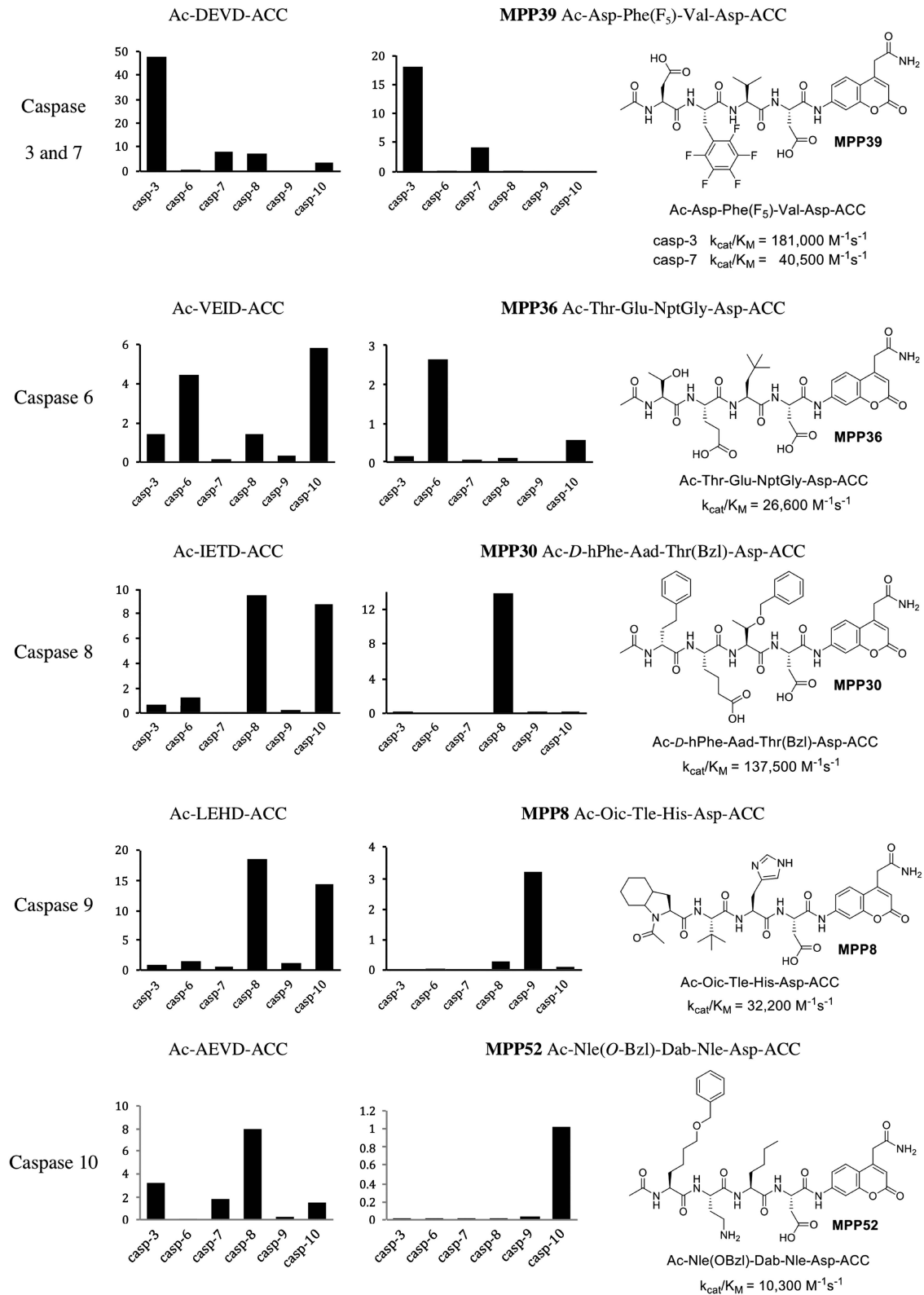
$$k_{\text{cat}}/K_M = 176,000 \text{ M}^{-1}\text{s}^{-1}$$

**Figure 2** Some examples of novel highly sensitive caspase ACC substrates containing unnatural amino acids

assay, as all described substrates exhibit off target reactivity, especially with caspase 3. Unfortunately, we were not able to measure  $k_{\text{cat}}$  values for all substrates for all caspases as some of these substrates, being highly selective of one

caspase, were very poorly recognized by other caspases. Second, for some substrates, their  $K_M$  parameters were above their solubility index. However, as we thought that this analysis would be of significant value, we decided to evaluate





**Figure 3** Comparison of commonly used and HyCoSuL-derived caspases substrates. Six ACC substrates with commercially available sequences were tested toward six recombinant human caspases (left panels). For each substrate, all three kinetic parameters were determined (see Supplementary Data section). Here we present only  $k_{cat}/K_M$  values ( $\times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ). Comparison with the most selective substrates derived from HyCoSuL screening reveals the utility of this approach in developing highly selective tetrapeptide substrates

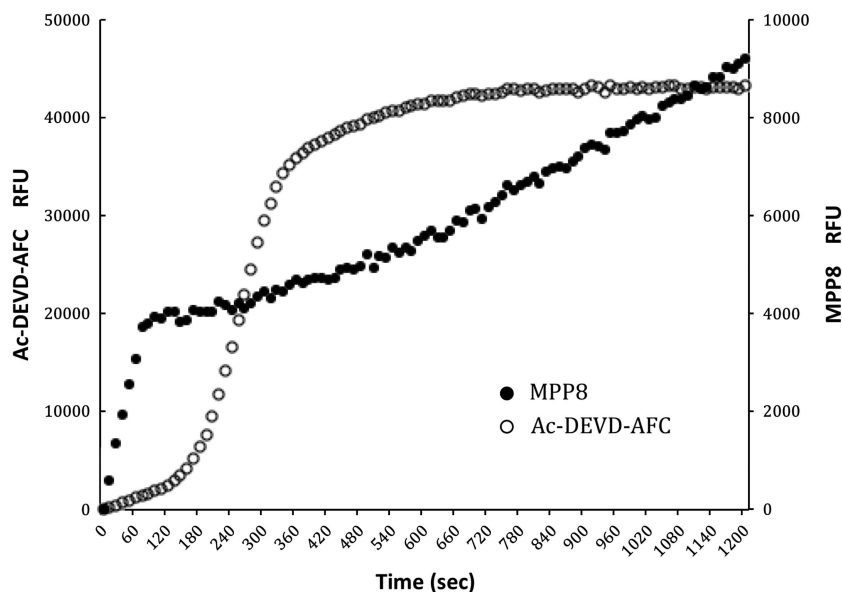
our most selective substrates by screening them at a final concentration of 100  $\mu\text{M}$ , well above  $K_M$  values for the best substrates, and compared them with commercial substrates under the same conditions (Supplementary Table 5). By this experiment we demonstrate that almost all of our substrates with the best  $k_{\text{cat}}/K_M$  selectivity index can also be used for assays running in 'high substrate concentration' conditions, where an individual substrate would exhibit the lowest degree of selectivity for different caspases. Thus, reducing substrate concentrations would result in even more selectivity, but at the price of lower fluorescence yields. Moreover, apart from the substrates described in the previous section, we found some other structures that are promising in this experimental paradigm (see Supplementary Data section).

**Simultaneous detection of caspases 3 and 9 in cell-free apoptosis.** To validate our results and assess the utility of the selective substrates, we tested individual substrates in a complex system where several caspases are active and where they operate in what approaches a natural environment. Because of the uncertainty of cellular penetration, we avoided intact cells, and opted instead for a model of cytochrome *c* programmed *in vitro* apoptosis that has frequently been used in the literature.<sup>22–24</sup> In this model, cytochrome *c* is added to cytosolic extracts of human cells where it triggers the formation of a soluble activation platform known as the apoptosome, driving caspase 9 activation.<sup>25</sup> Active caspase 9 then activates caspases 3 and 7 that are responsible for apoptosis execution. Caspase 9 shows little activity on previously reported peptide-based synthetic substrates, making it impossible to infallibly measure its activity in complex systems where several caspases are

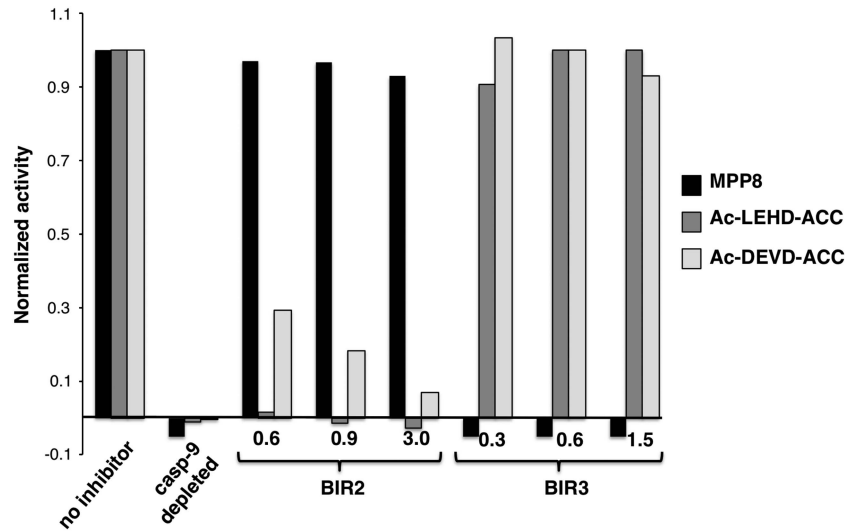
active (especially caspase 3 that is known to hydrolyze existing caspase 9 substrates).

We first compared the cleavage of the caspase 3 reference substrate Ac-DEVD-AFC with the most selective caspase 9 substrate MPP8. A portion of cytosolic extracts pre-equilibrated with 100  $\mu\text{M}$  of both substrates were activated by adding 10  $\mu\text{M}$  cytochrome *c* and 1 mM dATP at 37°C. To avoid unwanted proteolysis disturbing our assay, we also added MG132 (a proteasome inhibitor) to the cytosolic extract. As MPP8 contains the ACC fluorophore, we were able to assay the independent cleavage of each substrate, simultaneously, in the same reaction by leveraging the distinct excitation and emission spectra of ACC (355 and 460 nm) and 7-amino-4-trifluoromethylcoumarin (AFC; 400 and 505 nm; Figure 4).

Activity on Ac-DEVD-AFC exhibited a typical lag phase previously reported,<sup>26</sup> but activity against the optimized caspase 9 substrate MPP8 appeared earlier. This is consistent with the requirement of caspase 9 to activate caspase 3 in a hierarchical manner. Although these data are consistent with selectivity of MPP8 for caspase 9, it is possible that the different sensitivities of the substrates somehow masked nonspecificity. To test this, we assayed caspase activity in the cytochrome *c* activated cytosolic extracts in the presence of highly specific protein inhibitors of the caspases. We chose the BIR2 (baculovirus inhibitor of apoptosis protein repeat) domain of human X-linked inhibitor of apoptosis protein (XIAP), which is stringently selective for caspases 3 and 7, and the BIR3 domain of XIAP, which is stringently selective for caspase 9.<sup>21</sup> We also employed the commercially available LEHD sequence that is sold as a caspase 9 substrate in addition to MPP8 and Ac-DEVD-ACC. Cytosolic extracts were



**Figure 4** Caspase activation time course. Cytosolic extracts were treated with cytochrome *c* and caspase activity was monitored simultaneously at different wavelengths by the caspase-9-selective substrate MPP8 that initiates after  $\sim 1$  min of addition of cytochrome *c*, whereas activity reported by Ac-DEVD-AFC exhibits a lag of  $\sim 2$ –3 min before full activity is obtained. Eventually, Ac-DEVD-AFC substrate is depleted ( $\sim 6$  min) and the curve relaxes to full substrate depletion. Because MPP8 is cleaved more slowly than Ac-DEVD-AFC, substrate is not depleted within the time course. The 'burst' in the first 40–60 s is because of the interaction of the ACC substrate with cytochrome *c* through a mechanism that we do not yet understand, but is independent of any proteolytic activity, as it is observed in controls and is not observed for the AFC substrate. All substrates were used at 250  $\mu\text{M}$ , and we could detect activity at 50  $\mu\text{M}$  with the spectrofluorimeter employed in this study



**Figure 5** Detection of caspase 3 and caspase 9 in cytosolic extracts using synthetic substrates. Cytosolic extracts were incubated with cytochrome *c* for 10 min at 37°C before measuring activity with MPP8, Ac-LEHD-ACC, or Ac-DEVD-ACC. Activated cytosolic extracts were either left untreated (no inhibitor) or treated with the indicated concentrations of BIR2 or BIR3 ( $\mu\text{M}$ ). As a control, caspase 9 was immunodepleted from extracts before addition of cytochrome *c*

pre-activated by adding 10  $\mu\text{M}$  cytochrome *c* and 1 mM dATP at 37°C for 10 min before addition of substrates and BIR domains as appropriate (Figure 5).

The kinetic analysis showed that both Ac-LEHD-ACC and Ac-DEVD-ACC substrates are processed extremely fast, whereas MPP8 is cleaved poorly. This is evidence that LEHD and MPP8 are preferentially cleaved by different enzymes in activated cytosolic extracts as both substrates have comparable kinetic values for recombinant caspase 9. Importantly, MPP8 activity was abrogated by BIR3, and not by BIR2, consistent with an exquisite selectivity of this substrate for caspase 9. In contrast, both activity on Ac-DEVD-ACC and Ac-LEHD-ACC was inhibited by BIR2 and not BIR3. This is strong evidence that the activity against Ac-DEVD-ACC is because of caspases 3 and 7, as expected, and that the activity observed with the supposed caspase 9 substrate Ac-LEHD-ACC in cytosolic extracts is mainly because of caspases 3 and 7, and not caspase 9. The caspase 9 immunodepletion control confirms that activity on the substrates is caspase dependent, because only caspases are expected to be activated in this system – see Supplementary Figure 13B for validation of caspase 9 depletion. Addition of recombinant caspase 9 as well as its cleavage site mutant D330A recapitulated activity on MPP8 (Supplementary Figures 13–15) validating the *in vitro* caspase activation paradigm.

## Discussion

Caspases are key regulatory enzymes in apoptosis and inflammation. The development of selective and sensitive biochemical tools for the investigation of their activation and regulation *in vivo* are obligatory for understanding their biological functions in both normal cells and pathophysiological states. The synthesis and biochemical evaluation of the first combinatorial substrate library targeting caspases over 15 years ago had a great impact on the understanding of

their substrate preferences and predicted functions. Unfortunately, almost all caspase substrates lack specificity and thus cannot be used for monitoring the participation of individual caspases in a complex system. We demonstrate that HyCoSuL is a powerful tool for the discovery of new, more selective and more sensitive caspase substrates. The use of a wide range of unnatural amino acids allowed us to design selective substrates that are able to distinguish between all the apoptotic caspases tested, with the notable exceptions of the closely related executioner caspases 3 and 7. We note a caveat to our approach, because the direct conversion of information obtained through HyCoSuL screening (as well as from traditional PS-SCL) into the design of specific substrates is not straightforward. This is because caspases, akin to many other proteases, likely exhibit subsite cooperativity. Binding of particular amino acid to its cognate subsite is not always an independent process and can be influenced (assisted or disturbed) by neighboring amino acids.<sup>27</sup> Taking this into account, results from both approaches (HyCoSuL and PS-SCL) must be validated by resynthesis and kinetic analysis. Consequently, a selection of best hits from the HyCoSuL screens were used to inform the synthesis of a selection of substrates, and from these individual substrates we determined the optimal and most selective substrates for each caspase.

The biological utility of this approach extends to the ability, for the first time, to observe the activity of individual caspases during cytochrome-*c*-triggered apoptosis. We have not yet attempted to observe the value of the selective substrates in intact cells, mainly because of cell penetration concerns, but this is clearly a venue for future exploration. Moreover, we propose that caspase-selective substrates found through HyCoSuL screening provide an excellent scaffold for the synthesis of highly selective activity-based probes to study caspase activity and activation during apoptosis. We predict that this novel approach can secure a much more thorough understanding of the apoptotic caspase cascade regulation



and possibly guide the design of new compounds with enhanced specificity for therapeutic utility.

### Materials and Methods

**Reagents.** Chemicals were obtained from commercial suppliers and used without further purification. To libraries and individual substrates synthesis, Rink amide RA resin (particle size 200–300 mesh, loading 0.48 mmol/g, Iris Biotech GmbH, Marktredwitz, Germany), Fmoc-protected amino acids (purity >99% Iris Biotech GmbH, Bachem (Torrance, CA, USA), Creosalus (Louisville, KY, USA)), *N*-hydroxybenzotriazole (HOBt monohydrate purity >98%, Creosalus), diisopropylcarbodiimide (DICl, peptide grade, Iris Biotech GmbH), *N,N*-diisopropylethylamine (DIPEA, peptide grade, VWR International, Gdansk, Poland), *O*-benzotriazole-*N,N,N*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, peptide grade, Iris Biotech GmbH), 2-(1-*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphatethanaminium (HATU, peptide grade, Iris Biotech GmbH), 2,4,6-trimethylpyridine (collidine, peptide grade, Sigma-Aldrich Sp. z o.o., Poznan, Poland), *N,N*-dimethylformamide (DMF, peptide grade, Avantor, Center Valley, PA, USA), dichloromethane (DCM, pure for analysis, POCh, Gliwice, Poland), methanol (MeOH, pure for analysis, POCh), acetonitrile (ACN, HPLC gradient grade, Avantor), diethyl ether (Et<sub>2</sub>O, pure for analysis, POCh), piperidine (PIP, purity >99%, Sigma-Aldrich Sp. z o.o.), trifluoroacetic acid (TFA, purity 99%, Iris Biotech GmbH), triisopropylsilane (TIPS, purity 99%, Sigma-Aldrich Sp. z o.o.), acetic acid (AcOH, purity >98%, POCh), and phosphorus pentoxide (P<sub>2</sub>O<sub>5</sub>, purity 98%, POCh) were used. Individual substrates were purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using a semi-preparative Waters Spherisorb S100DS2 column Waters Sp. z o.o. (Warszawa, Polska). The solvent composition was as follows: phase A (water/0.1% TFA) and phase B (ACN/H<sub>2</sub>O 80/20 (% v/v) with 0.1% of TFA). The purity of each individual compound was confirmed by analytical HPLC using a Waters Spherisorb S50DS2 column. Finally, the molecular weight of each substrate was confirmed by high-resolution mass spectrometry on High Resolution Mass Spectrometer WATERS LCT Premier XE with Electrospray ionization (ESI) and Time of Flight (TOF). Data files for these studies can be found in the Supplementary Data section.

**Synthesis of NH<sub>2</sub>-ACC-Rink Amide resin.** Preparation of ACC was carried out as described previously according to Maly *et al.*<sup>18</sup> To glass reaction vessel, 1 eq (6.24 mmol, 13 g) of Rink AM resin was added and stirred gently once per 10 min in DCM for 1 h, and then filtered and washed 3 times with DMF. Fmoc-protecting group was removed using 20% piperidine in DMF (5, 5, and 25 min), filtered each time and washed with DMF (six times). Next, 2.50 eq of Fmoc-ACC-OH (15.6 mmol, 6.9 g) was preactivated with 2.50 eq HOBt monohydrate (15.6 mmol, 2.34 g) and 2.50 eq DICl (15.6 mmol, 2.0 ml) in DMF and mixture was added to the resin. Reaction was stirred gently for 24 h at room temperature. Resin was washed four times with DMF and reaction was repeated using 1.5 eq of above reagents to improve yield of ACC coupling to the resin. After reaction, resin was washed with DMF and Fmoc group was removed using 20% piperidine in DMF (5, 5, and 25 min), filtered and washed with DMF (six times).

**Synthesis of NH<sub>2</sub>-Asp(*t*-Bu)-ACC-Rink Amide resin.** Next, 2.5 eq Fmoc-Asp(*t*-Bu)-OH (15.6 mmol, 6.42 g) with 2.5 eq HATU (15.6 mmol, 5.93 g), 2.5 eq collidine (15.6 mmol, 2.03 ml) in DMF were activated for 2 min and added to filter cannula with 1 eq (6.24 mmol) NH<sub>2</sub>-ACC-resin and reaction was carried out for 24 h. Next, resin was washed four times with DMF and reaction was repeated using 1.5 eq of above reagents. After washing with DMF, Fmoc-protecting group was removed using 20% piperidine in DMF (5, 5, and 25 min). Resin was additionally washed with DCM (3 times) and MeOH (3 times) and dried over P<sub>2</sub>O<sub>5</sub>.

**Synthesis of P2, P3, and P4 sublibrary exemplified in detail with the P2 sublibrary.** Each library consisted of 129 sublibraries in which each of the natural amino acids (minus cysteine) and a pool of unnatural amino acids were used at a defined position and an isokinetic mixture of 19 amino acids (without cysteine and with norleucine mimicking methionine) was coupled. Equivalent ratios of amino acids in the isokinetic mixture were created based on their reported coupling rates. A fivefold excess (over resin load) of mixture was used. For fixed positions, a 2.5 eq of single amino acid was used. Reactions were carried out with coupling reagent DICl and HOBt. For P2 coupling, the synthesis of the library was performed using a MultiChem 48-wells synthesis apparatus (FlexChem from SciGene, Sunnyvale, CA, USA). To wells of the reaction apparatus, 1 eq of dry

NH<sub>2</sub>-Asp(*t*-Bu)-ACC-resin (0.04 mmol, 80 mg) was added and stirred gently for 1 h in DCM, and then washed with DMF (four times). In separated vials, 2.5 eq (0.1 mmol) Fmoc-P2-OH was preactivated with 2.5 eq HOBt (0.1 mmol, 15 mg) and 2.5 eq DICl (0.1 mmol, 14 μl) in DMF. Next, preactivated amino acids were added to wells of the apparatus containing NH<sub>2</sub>-Asp(*t*-Bu)-ACC-resin, followed by 3 h agitation. Then, reaction mixture was filtered, washed with DMF (4 times), and a ninhydrin test was carried out. Subsequently Fmoc-protecting groups were removed using 20% piperidine in DMF (5, 5, and 25 min). For P3 and P4 positions coupling, an isokinetic mixture for 48 portions was prepared of 18 Fmoc-protected natural amino acids (omitting cysteine) and with norleucine mimicking methionine (19 amino acids in total). Then, 5 eq of isokinetic mixture, 5 eq HOBt (9.22 mmol, 1.4 g), and 5 eq DICl (9.22 mmol, 1.2 ml) were diluted in DMF and preactivated for 3 min. To each of 48 wells containing 1 eq of NH<sub>2</sub>-P2-Asp(*t*-Bu)-ACC-resin, the activated isokinetic mixture was added. After 3 h of agitation, the slurry was filtered and washed with DMF (4 times). A ninhydrin test was carried out and Fmoc-protecting group was removed using 20% piperidine in DMF (5, 5, and 25 min). The same procedure was applied for the remaining substrates. In the same manner the isokinetic mixture was added to sample the P4 position. For *N*-terminus acetylation, to a vial 5 eq AcOH (9.22 mmol, 527 μl), 5 eq HBTU (9.22 mmol, 3.5 g), and 5 eq DIPEA (9.22 mmol, 1.60 ml) in DMF were added. After stirring for 1 min, the mixture was added to each well in the reaction apparatus, containing NH<sub>2</sub>-X-X-P2-Asp(*t*-Bu)-ACC-resin, followed by gentle agitation for 30 min. Next, the resin was washed with DMF (5 times), DCM (3 times), and MeOH (3 times) and dried over P<sub>2</sub>O<sub>5</sub>. For cleavage from the resin, after completing the synthesis, peptides were cleaved from the resin by treating for 2 h (shaking once per 15 min) with a mixture of TFA:TIPS:H<sub>2</sub>O (% v/v/v 95:2.5:2.5; 2 ml/well). The solution from each well was collected separately and the resin was washed once by a portion of cleavage solution (1 ml), followed by addition of diethyl ether (Et<sub>2</sub>O, 14 ml). After precipitation (0.5 h in –20°C) the mixture was centrifuged and washed again with Et<sub>2</sub>O (4 ml). After centrifugation, a white precipitate was dissolved in ACN:H<sub>2</sub>O (v/v, 1:1.4 ml) and lyophilized. The final products were dissolved in DMSO to a concentration of 20 mM and used without further purification. The synthesis of P3 and P4 sublibraries was in the same manner as described above, and P3 and P4 sublibraries were synthesized by coupling fixed amino acid residues to P3 (isokinetic mixture to P2 and P4) and respectively P4 position (isokinetic mixture to P2 and P3).

**Preparation of recombinant caspases.** The detailed description of caspases expression and purification can be found elsewhere.<sup>28</sup>

**Enzymatic kinetic studies.** These were performed using a fMax fluorimeter (Molecular Devices, Sunnyvale, CA, USA) operating in the kinetic mode in 96-well Corning (Corning, NY, USA) plates. The excitation wavelength for ACC-based substrates was 355 nm, and the emission wavelength was 460 nm with a cutoff of 455 nm. For HyCoSuL screening the assay buffer was 10% w/v sucrose, 20 mM Pipes, 10 mM NaCl, 1 mM EDTA, 10 mM DTT, pH = 7.2–7.4 (the buffer for caspases 8, 9, and 10 was supplemented with 1.0 M sodium citrate to ensure maximal activity<sup>29,30</sup>). Buffers were prepared at 23°C, screening of the libraries and assays were performed at 37°C. Caspases 3, 6, and 7 were preincubated for 15 min and caspases 8, 9, and 10 were preincubated for 30 min at 37°C before adding to the wells containing substrate.

**Characterization of caspases specificity using HyCoSuL.** Standard enzyme assay conditions for P2, P3, and P4 positions were as follows: reaction volume 100 μl; total final substrate mixture concentration 50 μM; enzyme concentration caspase 3: 10–20 nM, caspase 6: 100–200 nM, caspase 7: 50–100 nM, caspase 8: 50–100 nM, caspase 9: 100–150 nM, and caspase 10: 50–100 nM. The total assay time was 30 min, and the linear portion of the progress curve (generally 15–30 min) was used to calculate velocity. All experiments were repeated at least three times and the results presented are means. The difference between individual values was <10% in every case. Analysis of the results was based on total RFUs for each sublibrary, setting the highest value to 100% and adjusting the other results accordingly.

**Synthesis of individual optimized substrates.** The 2.5 eq Fmoc-P2-OH was preactivated with 2.5 eq HOBt and 2.5 eq DICl in DMF and added to cartridge with 1 eq NH<sub>2</sub>-Asp(*t*-Bu)-ACC-resin (all substrates contained Asp at P1 position) and followed by gentle agitation for 3 h. Then, it was filtered and washed with DMF (six times). Fmoc-protecting group was removed using 20%

piperidine in DMF (5, 5, and 25 min). Ninhydrin test was carried out each time after coupling and deprotection. A solution of 2.5 eq Fmoc-P3-OH, 2.5 eq HOBt, and 2.5 eq DIPI in DMF was added to the resin and the slurry was agitated for 3 h. After removal of the solution, the resin was washed with DMF (six times), and coupling and deprotection of Fmoc-P4-OH was carried in identical conditions like P2 position. N-terminus was protected with acetyl group using 5 eq AcOH, 5 eq HBTU, and 5 eq DIPEA in DMF as previous described. After solvent removal, the resin was washed with DMF (six times), DCM (three times), and MeOH (three times) dried over P<sub>2</sub>O<sub>5</sub> and cleaved from the resin with a mixture of TFA/TIPS/H<sub>2</sub>O (% v/v/v 95:2.5:2.5). The crude product was purified by HPLC and lyophilized. Its purity was confirmed by analytical HPLC. Each optimized substrate was analyzed using HRMS. Optimized substrates were dissolved in peptide grade DMSO to 20 mM concentration and stored at -80°C until use.

**Comparison of kinetic parameters ( $k_{cat}$ ,  $K_M$ , and  $k_{cat}/K_M$ ) for individual substrates.** Substrates were screened against human recombinant caspases at 37°C in the above assay buffers. Buffers were prepared at 23°C. Caspase 3, 6, and 7 solutions in buffer were preincubated for 15 min (caspases 8, 9, and 10 were preincubated for 30 min) at 37°C before adding to the substrate in the wells of a 96-well plate reader operating in the kinetic mode. Total assay time was 10–20 min. ACC concentrations were calculated by the hydrolysis of eight independent ACC-coupled substrate solutions at known concentration, and an average value was determined. Enzyme kinetic assay conditions were as follows: 100  $\mu$ l total reaction volume, eight different substrate concentrations, and enzymes at appropriate concentrations. Release of ACC fluorophore was monitored as above. Each experiment was repeated at least three times, and the results are presented as an average with the error bars describing the S.D. Final substrate concentrations for  $k_{cat}/K_M$  determination ranged from 0.05 to 500  $\mu$ M. Concentration of DMSO in the assay was <2% (v/v). Calculation of kinetic parameters was carried out using GraphPad Prism software (La Jolla, CA, USA).

**FreeStyle 293-F cell cytosolic extracts assay.** Hypotonic lysis of FreeStyle 293-F (Life Technologies, Grand Island, NY, USA) cells was performed, and cytosols collected as described previously.<sup>24</sup> Each 100  $\mu$ l portion of FreeStyle 293-F cytosolic extract was activated by addition of 1  $\mu$ l of dATP (12 mM in water), and 2  $\mu$ l of horse cytochrome *c* (1 mM in water) in the presence of the proteasome inhibitor MG132 (10 mM in DMSO), 2  $\mu$ l. The total reaction volume was 80  $\mu$ l. The proteolytic activities of caspase 3 and caspase 9 were measured using four short peptide-based fluorogenic substrates (MPP8, Ac-LEHD-ACC, Ac-DEVD-ACC, or Ac-DEVD-AFC) at the final concentration of 250  $\mu$ M (the excitation wavelength for AFC-based DEVD substrate was 400 nm, and the emission wavelength was 505 nm). Release of ACC or AFC fluorophore was monitored as above. Each experiment was repeated at least three times, and the results are presented as an average RFU/s value. The S.D. for each value was calculated from at least three independent measurements.

**Caspase inhibition by XIAP-BIR2 and XIAP-BIR3.** Caspase 3/7 activity was inhibited in cellular extracts by using XIAP-BIR2 domain (concentration range: 0.6–3.0  $\mu$ M), and caspase 9 was inhibited by addition of XIAP-BIR3 domain (concentration range: 0.3–1.5  $\mu$ M). Cytosolic extracts were preincubated with BIR2 or BIR3 domains for 30 min at 37°C before cytochrome *c* stimulation. The XIAP fragment BIR2 (124–237) and BIR3 (262–348) were cloned into a modified pET15b (EMD Millipore, Billerica, MA, USA) vector containing 8  $\times$  His residues at the N-terminus and expressed in BL21 (DE3) *E. coli* by induction with 0.2 mM IPTG at 30°C for 4 h.

**Immunoblotting and caspase 9 immunodepletion.** To monitor caspase 9 processing after cytochrome *c* activation, cytosolic extracts were stimulated and fractions were collected at the corresponding time points. Each fraction was mixed with SDS loading buffer, boiled, subjected to Laemmli SDS-Page (12% gel), and transferred to a nitrocellulose membrane for western blotting. Primary antibodies were detected by fluorophore-coupled secondary antibodies (LiCOR, Lincoln, NB, USA), and signal acquired using the Odyssey system. For caspase 9 immunodepletion, 100  $\mu$ l of Protein A/G-Plus agarose resin (Santa Cruz Biotechnology, Paso Robles, CA, USA) was mixed with 50  $\mu$ l of anti-caspase 9 antibody in PBS and incubated overnight at 4°C to couple the antibody on the beads. Resin was extensively washed with PBS, and subsequently mixed with 1 ml of cytosolic extracts and incubated for 5 h at 4°C. Immunodepletion efficiency was evaluated by western blotting, and the immunodepleted extracts

were subsequently used for kinetic studies. The following antibodies were used for immunoblots: anti-caspase 9 rabbit antisera (a gift from Dr. Douglas R Green, Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, USA), anti-Caspase 3 rabbit polyclonal (Cell Signaling Technology, Beverly, MA, USA), anti-cleaved caspase 3 (D175) rabbit polyclonal (Cell Signaling Technology), and anti-HSP90 mouse monoclonal (BD Transduction Laboratories, San Diego, CA, USA).

## Conflict of Interest

The authors declare no conflict of interest.

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