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Caspase Substrates and Inhibitors

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Caspases are proteases at the heart of networks that govern apoptosis and inflammation. The past decade has seen huge leaps in understanding the biology and chemistry of the caspases, largely through the development of synthetic substrates and inhibitors. Such agents are used to define the role of caspases in transmitting life and death signals, in imaging caspases in situ and in vivo, and in deconvoluting the networks that govern cell behavior. Additionally, focused proteomics methods have begun to reveal the natural substrates of caspases in the thousands. Together, these chemical and proteomics technologies are setting the scene for designing and implementing control of caspase activity as appropriate targets for disease therapy.

WHAT IT TAKES TO BE A CASPASE

The need to recycle scarce amino acids in early biotic environments required that proteases were among the first proteins to appear in the most primitive replicating organisms. Since that distant origin, proteases have diversified into every biological niche, occupied every cellular and extracellular compartment, and are encoded in all organisms on the planet with the exception of the most primitive viruses (Lopez-Otin and Overall 2002; Rawlings et al. 2008). Diverging from their primeval role in protein degradation and recycling, most of the 600 or so mammalian proteases are involved in very limited and specific proteolytic events—initiating and transmitting signals—and a good example of this is the caspase family (Timmer and Salvesen 2007; Crawford and Wells 2011). The caspases belong to a clan, or structurally related group, known as cysteine protease clan CD that also contains the plant metacaspases (Vercammen et al. 2004), and mammalian and plant proteases of the legumain family (involved in specific processing events), the eukaryotic protease separase (required for sister chromatid separation during mitosis), and the bacterial proteases clostripain and gingipain (Chen et al. 1998). Common to all of these proteases is a shared stringent specificity for one type of amino acid side chain at the primary (P_1) specificity position in substrates (Arg or Lys for metacaspases, clostripain, and gingipains; Asn for legumains; and Arg for separase). Thus, the caspase fold—the clan CD fold—supports unusually stringent specificity at the primary cleavage site. Distinguishing the caspases from each other are different requirements at the extended substrate recognition positions.

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The name "caspase" derives from cysteine-dependent aspartate-specific protease (Alnemri et al. 1996): Catalysis is governed by a critical conserved Cys side chain of the enzyme and by a highly stringent selectivity for cleaving peptides and proteins on the carboxy-terminal side of Asp residues. The use of a Cys side chain as a nucleophile during peptide-bond hydrolysis is common to several protease families. But the primary specificity for Asp is very rare among proteases. Of the currently known human and mouse proteases, only the caspase activator Granzyme B, a serine protease, shares this primary specificity (Odake et al. 1991; Harris et al. 2000). The primary specificity pockets of caspases are almost identical, being formed by the side chains of the strictly conserved residues Arg-179, Arg-341, and Gln-283 (caspase-1 numbering convention). This deep, highly basic pocket (Fig. 1) is perfectly shaped to accommodate an Asp side chain, accounting for the up to four orders of magnitude lower catalytic efficiency for cleavage of peptides with a P_1 Glu residue in most caspases (Stennicke et al. 2000), with the notable exception of the *Drosophila* caspase Dronc, which seems to have some specificity for Glu in addition to Asp (Hawkins et al. 2000; Snipas et al. 2008).

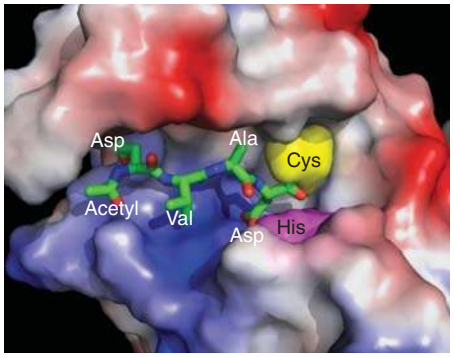

Figure 1. A caspase catalytic cleft. The active-site cleft of caspase-3 bound to the tetrapeptide inhibitor acetyl-Asp-Val-Ala-Asp-fluoromethylketone (Ac-DVAD-FMK), taken from PDB code 1CP3 (Mittl et al. 1997). The caspase is shown as an electrostatic potential surface, where blue hues denote positive potential and red hues negative potential. The deep basic cleft next to the catalytic Cys (yellow) and His (purple) residues of the enzyme occupies the P_1 Asp residue and is conserved in all active caspase structures. To the left of this are the P_2–P_4 residues, some occupying clearly visible specific pockets. The location of residues to the right (the P′ side) are unavailable in this or similar caspase structures because all common peptidyl inhibitors of caspases are designed without P′ substituents.

CASPASE SUBSTRATES

Synthetic Substrates

A very large number of reports of the design, construction, and application of synthetic substrates have led to some pretty clear insights into the catalytic mechanisms and specificity preferences of individual caspases. There are principally two ways to investigate caspase substrates: (1) the inherent preferences in the active-site cleft that distinguish them from each other, and (2) their natural biological substrates. The first tells us a lot about caspase catalysis and is much easier to investigate than the second. This, in turn, has led to a consensus of the "inherent subsite preference" defining the preference of caspases for individual peptide sequences, and to the development of peptide-based and genetically encoded probes that have been used to follow caspase activity in cells and even whole organisms. Importantly, understanding the inherent subsite preference of caspases has not really achieved the expected predictive power of defining optimal in vivo protein substrates, as described below.

Peptide-Based Substrates

Several strategies to identify inherent subsite preference have been applied to caspases, generally incorporating peptides attached to reporter groups (Fig. 2). This allows for definition of preferred sequences on the "unprimed" side—the residues amino-terminal—of the scissile bond. Positional scanning substrate combinatorial library (PS-SCL) methods, first introduced for caspases in 1997 (Rano et al. 1997), are based on synthesis of libraries of substrate mixtures, which are linked with highly fluorescent molecules. These fluorophores, which are called reporter groups, occupy the

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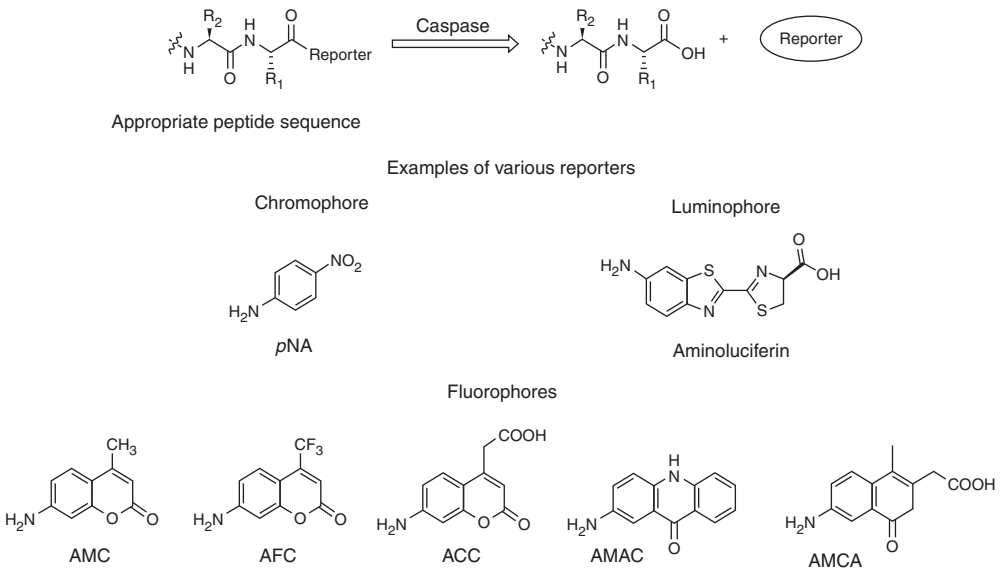

Figure 2. Caspase cleavage of reporter group from substrate. Examples of reporter groups of the three main categories (fluorophores, chromophores, and luminophores).

$P_{1'}$ position, and after enzyme cleavage, they are released and emit fluorescence when excited by an appropriate wavelength (Fig. 2).

Measurements of liberated fluorescence yield data on reaction kinetics, allow for identification of optimal inherent subsite preference, and provide a powerful method for rapid and reliable analysis of protease specificity. However, it is worth noting that PS-SCL approaches are only helpful for identification of nonprime residues of a substrate (Poreba and Drag 2010). Interestingly, such studies can yield somewhat unexpected results, as exemplified by scanning of caspase-1. Thus, the WEHD sequence is the most favorable tetrapeptide recognition motif for caspase-1 (Rano et al. 1997), inconsistent with the original optimal sequence YVAD for the enzyme (Thornberry and Molineaux 1995) and divergent from the sequence YVHD found to be cleaved in caspase-1's natural substrate pro-IL-1β. Nevertheless, further investigation with several individual substrates confirmed that the WEHD tetrapeptide was better than the YVAD tetrapeptide because the k_{cat}/K_M value was ~50-fold higher (Table 1).

The pioneering work of Thornberry et al. (1997) using a tetrapeptide PS-SCL approach and Talanian et al. (1997) using sets of individual peptide substrates, subsequently refined by others, led to our current understanding of the inherent subsite preferences summarized in Figure 3. Importantly, caspase-2 seems to be an outlier because its activity on tetrapeptides was very low, and cleaved substrates efficiently only when an S5 residue was provided, with hydrophobic residues favored in the this position (Talanian et al. 1997). A newer strategy that substantially simplified the synthesis of fluorogenic libraries, using the bifunctional fluorogenic group 7-amino-4-carbamoylmethylcoumarin (ACC), was subsequently introduced (Harris et al. 2000), allowing solid-phase synthesis of PS-SCLs (Maly et al. 2002). The advantage of this technique is that any amino acid may be

Table 1. k_{cat}/K_M and K_i values for selected synthetic substrates cleaved by caspase-1

Substrate	$k_{cat}/K_M \times 10^5$ (M^{-1} sec^{-1})
Ac-WEHD-AMC	33.4
Ac-YVHD-AMC	2.81
Ac-YVAD-AMC	0.66
Pro-IL-1β	1.5

From Rano et al. (1997).

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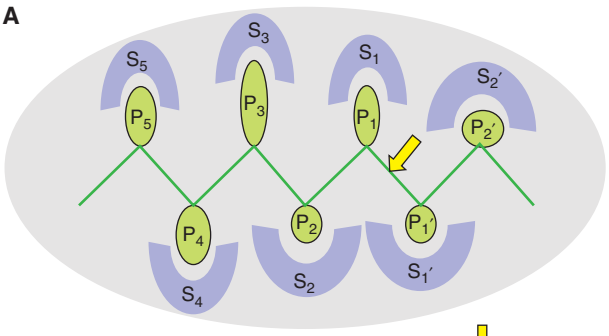

B

Caspase-	P_5	P_4	P_3	P_2	P_1	P_1'
1, 4, 5, 14		W/Y	E	X	D	ϕ
8, 9, 10		I/L	E	X	D	ϕ
3, 7		D	E	X	D	ϕ
6		V	E	X	D	ϕ
2	V/L	D	E	X	D	ϕ

Figure 3. Inherent substrate specificity. The convention in naming substrate residues in protease active sites is to name the residue to the amino terminal of the scissile bond (arrow) P_1 and the one to the carboxy-terminal side P_1'. (*A*) Other residues (green) are numbered consecutively from these origins. Many of these residues will occupy specific pockets on the enzyme, numbered with the matching S designation (blue), each of which may be constructed of several enzyme residues. The objective of this interaction mode, which almost always binds the cleavage region in an extended peptide conformation, is to align the substrate accurately into register with the catalytic machinery. (*B*) Caspases bind substrates through typical interactions of the active-site cleft with amino acids in the cleavage site. Studies with synthetic peptides and peptide libraries have shown that substrate residues P_4–P_1' contribute to binding through association with S_4 through S_1' pockets in the active site. All caspases show exquisite selectivity for Asp in the P_1 position, a preference for Glu in P_3, and intolerance for charged residues at P_1', where Gly, Ala, Thr, Ser, and Asn are preferred (denoted by the ϕ symbol). Distinguishing between them is primarily the nature of the P_4 residue, and the inherent substrate specificities are generally well understood at the atomic level. Importantly, caspase-2 has an additional interaction at S_5 that enhances catalysis at least 30-fold.

placed at any position, and the approach was applied to examine caspase-3 substrate specificity (Walters et al. 2009). The results were consistent with those obtained from AMC-based substrate libraries (Thornberry et al. 1997), confirming that the type of fluorophore used does influence substrate specificity. Microarray techniques using peptide nucleic acid–encoded libraries of rhodamine-based fluorogenic substrates enable identification of individual subsite preferences via hybridization to complementary DNA oligonucleotides. Using this technique for defining optimal caspase-3 substrates, and a library of 192 peptide sequences, revealed agreement with the known enzyme specificity (Winssinger et al. 2004), validating the approach and confirming that inherent subsite occupancy of caspases is independent of the technique used.

The most striking differences among caspases-1, -3, and -8 can be observed in the S_4 pocket. Caspase-1 prefers bulky, hydrophobic residues (tyrosine and tryptophan) (Rano et al. 1997; Thornberry et al. 1997), caspase-3 has a near absolute requirement for aspartic acid (Ganesan et al. 2006a,b), and caspase-8 can liberally accommodate residues, but branched leucine and valine are most preferable (Fig. 3) (Blanchard et al. 1999; Wei et al. 2000).

All methods described above that use fluorogenic peptide substrates are based on measurements of increasing fluorescence after caspase cleavage. However, this is not the only way. 2-Aminoacridone (AMAC) has been used as a reporting group for a caspase substrate (Ac-DEVD-AMAC) and by measuring the decrease of fluorescence after enzyme cleavage (Lozanov

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Table 2. Comparison of kinetic parameters of substrate DEVD for caspase-3 linked with different reporter groups

Substrate	k_{cat} (sec^{-1})	K_M (μM)	$k_{cat}/K_M \times 10^6$ (M^{-1} sec^{-1})	References
Ac-DEVD-AMAC	9.95	4.68	2.13	Lozanov et al. 2009
Ac-DEVD-AMCA	5.86	13.65	0.42	Lien et al. 2004
Ac-DEVD-AMC	9.1	10	1.4	Garcia-Calvo et al. 1999
Ac-DEVD-AFC	—	16.8	1.3	Deveraux et al. 1997; Sun et al. 1997
Cbz-DEVD-aminoluciferin	—	7.66	—	O'Brien et al. 2005
Ac-DEVD-pNA	2.4	11	0.22	Talanian et al. 1997
Cbz-DEVD-SiR600	3.8	1.2	3.1	Kushida et al. 2012

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et al. 2009). In this approach, because the products of hydrolysis are not fluorescent, background correction is not necessary; thus, there are some advantages in this strategy over the commonly used ones (Table 2). Luciferase-based substrates incorporate the same specificity design, but use aminoluciferin as the leaving group. Like all luciferase assays, the luciferin is oxidized by luciferase and light is emitted. This method has lower background levels and higher sensitivity than fluorescent assays and is suitable for examining cell systems (O'Brien et al. 2005), cell lysates (Kindermann et al. 2010), and in vivo assays (Hickson et al. 2010).

Importantly, the methods to determine substrate specificity described above can only be used to identify nonprime substrate specificity; however, they fail to determine the prime site of a substrate. To establish prime and nonprime substrate specificity, other approaches are required. Internally quenched fluorescence peptide substrates overcome this problem (Stennicke et al. 2000). In this technique, a donor (fluorogenic group) and an acceptor (quenching group) are located on the opposite sides of the substrate. Intact substrates do not show a fluorescence signal (or a very weak one), and after protease cleavage, the quenching group is separated from the fluorophore and increased fluorescence emission is observed (Fig. 4). Thus, it was revealed that in the position $P_{1'}$, small residues, like Gly, Ser, and Ala, are favored. Interestingly, substrates

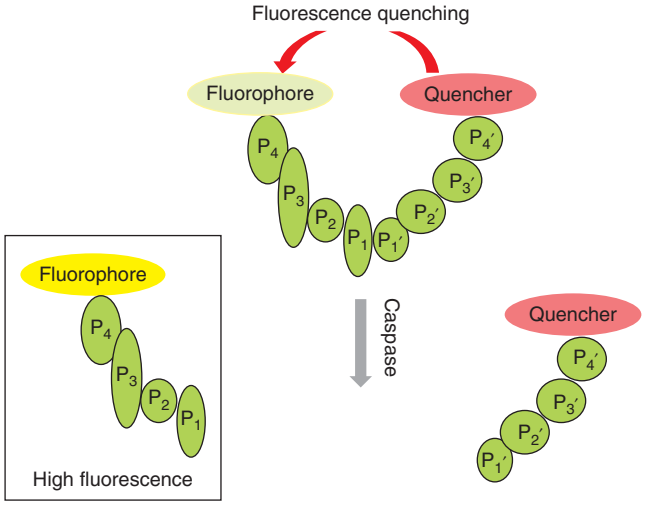

Figure 4. Schematic representation of fluorescence-quenched substrates. Peptide selectivity elements (green) are bracketed by a fluorophore and a cognate quencher. Cleavage between the fluorophore and quencher results in enhanced fluorescence.

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with large aromatic residues such as Phe and Tyr were cleaved efficiently as well. However, peptides with charged residues, branched aliphatic residues, and Pro were poorly tolerated (Stennicke et al. 2000; Petrassi et al. 2005).

Nonchemical synthesis-based techniques have been applied to defining caspase specificity, revealing essentially the same answer as the peptide-based approaches (Boulware and Daugherty 2006). Thus, cellular libraries of peptide substrates (CLiPS) is a biologically based technique that displays peptide sequences genetically encoded in a sacrificial protein on the surface of bacteria. Fluorescence-activated cell sorting (FACS) is used to find optimal substrates and allows for quantitative measurement of whole-cell fluorescence and determination of kinetic parameters. The investigation of caspase-3 substrates using this technique disclosed the DXVD↓G sequence as an optimal sequence, consistent with previous findings. Proteomic identification of protease cleavage sites (PICS) uses mass spectroscopy to interrogate a proteome-derived peptide library treated with a protease to establish the substrate specificity (Schilling and Overall 2008). Both CLiPS and PICS, with caveats, are capable of disclosing the prime-side preference of caspases, aligning well with the fluorescence-quenching chemical synthesis described above.

Overlapping Substrate Specificity

With all the work on optimal subsite preferences of caspases, it might seem that the defined substrates constitute the selective ones. However, there is a problem of cross-reactivity among caspases. It is extremely important to realize that simple peptide substrates, developed to examine the activity of caspases, lack selectivity for individual caspases (McStay et al. 2008). The majority of the substrates are only useful for studies on individual purified caspases but are ineffective for research on complex biological samples such as lysates of apoptotic cells. The consensus sequences of caspases are overlapping; thus, one caspase may cleave a substrate that was designed for other caspases. These findings indicate that caution is needed, especially while studying cell extracts (Timmer and Salvesen 2007; McStay et al. 2008).

Apoptotic caspases-2, -3, -6, -7, -8, -9, and -10 were analyzed with commercially available peptide-based substrates conjugated to the fluorophore AFC (McStay et al. 2008). Interestingly, only caspase-2 and caspase-9 cleaved their putative preferred substrates (VDVAD and LEHD, respectively) the most efficiently and did not recognize other substrates. The findings are in line with reports describing that for efficient cleavage, caspase-2 requires the P5 residue in the substrate (Talanian et al. 1997; Tang et al. 2011). Caspases-8 and -10 preferred the substrate sequence of caspase-9 (LEHD) rather than their assumed substrate of IETD sequence, although both substrates were hydrolyzed well. Interestingly, caspase-6 apart, from cleaving its experimentally determined preferred substrate VEID, also cleaved these two substrates efficiently. Caspase-7 showed activity for its expected DEVD substrate and additionally for the caspase-2 substrate (VDVAD). The most promiscuous turned out to be caspase-3, which cleaved all substrates, albeit with vastly different rates, and often with higher rates than caspases for which the substrates were designed (McStay et al. 2008). Parallel studies using a colorimetric assay to examine the substrate specificity of caspases-1, -2, -3, -6, -7, -8, and -9 revealed similar overlapping specificity (Pereira and Song 2008).

An investigation of the specificity of 10 caspases (1–10) using substrates linked to a 2-aminocridone fluorophore and analyzed by HPLC to establish enzyme activity allowed simultaneous evaluation of a set of substrates in a single sample containing a chosen caspase (Benkova et al. 2009). The substrates used were based on YVAD, VDVAD, DEVD, LEVD, WEHD, VEID, IETD, LEHD, and AEVD, intended for caspases-1, -2, -3/7, -4, -5/1, -6, -8, -9, -10, respectively. As revealed in previous analyses, only caspase-2 cleaved its putative substrate, owing to its strong requirement for P5 residue for cleavage. But, as revealed previously, the caspase-2 substrate was efficiently cleaved by other caspases, caspase-3 being most dominant. Thus, several groups, using different methods, clearly showed that a problem of overlapping substrate specificity

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exists among caspases. None of the investigated substrates showed high specificity toward an individual caspase. However, for each of the three groups of caspases (inflammatory caspases, initiator caspases, and effector caspases), there was some sequence selectivity observed.

These studies clearly show that the currently available peptide substrates for caspases show high cross-reactivity and are not appropriate for investigations of individual caspases in complex mixtures. This is further complicated when once considers that some caspases have higher intrinsic catalytic efficiency and some have higher concentrations than the others. When the activity is measured in cell lysates, the high caspase-3 concentration masks the activity of other caspases, even if a so-called preferred artificial substrate is used. Therefore, it constitutes a complicated task. However, the commercially available substrates are still valuable for examination of individual purified caspases (Timmer and Salvesen 2007; McStay et al. 2008; Benkova et al. 2009). This somewhat complex scenario could be simplified in knockdown cells, in which depletion of specific caspases could be used to draw conclusions of the participation of other caspases in selective substrate cleavage. A caveat here would take into account the hierarchical organization of caspase pathways, and thus experiments would be restricted to knockdown of effector, rather than apical, caspases.

Substrates for Cell and In Vivo Imaging

Although there is no real hope of defining the contribution of individual caspase activation in complex mixtures by using currently available simple peptide substrates, there has been reasonable progress in showing overall caspase activity in situ. Single-cell imaging techniques using caspase reporters have been very useful in observing the kinetics of activation in cell populations, but, of course, the same caveats apply as noted above regarding specificity. Rhodamine-based dyes coupled to tetrapeptides have shown utility in uncovering caspase activity, primarily in apoptotic models in cell culture. Thus, compounds such as Ac-DEVD-*N*′-octyloxycarbonyl-R 110 (Cai et al. 2001), *N*-Ac-DEVD-*N*′-(poly-fluorobenzoyl)-R 110 (Zhang et al. 2003), and Ac-DEVD-*N*′-morpholinecarbonyl-R 110 and the bis-peptide substrate $(Cbz\text{-}DEVD)_2$-R 110 have been used for biological studies in living cells (Liu et al. 1999). Another fluorescent dye based on rhodamine, 2-Me SiR600, where the oxygen in the xanthene moiety was replaced by silicon, resulted in red fluorescence of instead of green. Such probes will be valuable in multicolor imaging, because they provide an additional color window. Furthermore, they appear to be more suitable for in vivo imaging because tissues are more transparent to red than to green light (Kushida et al. 2012). NucView488 may also be an excellent reporter group for biological studies on cells, because the substrate DEVD-NucView488 is reported to be highly cell-permeable, efficiently cleaved by caspases, and is very sensitive (Cen et al. 2008).

In attempts to enhance cell penetration of chemical probes, a cell penetrating Tat peptide can be coupled to a far-red quencher, QSY 21, fluorophore Alexa Fluor 647, joined to caspase recognition sequence DEVD (Bullok and Piwnica-Worms 2005). Upon caspase cleavage, the quencher and fluorophore are liberated. The main advantage of this probe is its far-red fluorescence, so there is a little interference from autofluorescence of cells. Subsequently, the same group developed a second-generation apoptosis imaging probe, KcapQ, in which the penetrating peptide was replaced by insertion of D-amino acids to prevent adventitious cleavage and reduce toxicity (Maxwell et al. 2009).

Genetically encoded probes based on Förster (fluorescence) resonance energy transfer (FRET) have been applied to analyze caspase activity in single cells. Using a CFP–YFP pair connected by a flexible linker that contains the caspase cleavage sequence DEVDG, it is possible to monitor the rate of caspase activation following apoptotic induction (Rehm et al. 2002). When the linker is cleaved, energy transfer diminishes and the CFP signal increases, an event that can be monitored by live-cell microscopy. Thus, the time to caspase activation varies among individual cells, depending on the type of treatment and concentration of apoptosis inducer used. Once initiated, disruption of the

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FRET signal tends to be rapid (<15 min) and largely independent of the apoptotic inducer, attesting to the fast nature of caspase activation. Importantly, FRET probe cleavage is substantially slower in caspase-3-deficient cells, revealing the importance of caspase-3 cleavage of the reporter. As a means to partly distinguish between apical and effector caspases, the DEVD/G sequence can be replaced by DEVD/R (Albeck et al. 2008), based on the differential preference for Arg in $P_{1'}$ of caspase-3 and caspase-8 (Stennicke et al. 2000). An additional attempt to provide for simultaneous imaging of differential caspase specificities led to the construction of a tripartite FRET probe with the general architecture CFP-YFP-mRFP (mRFP, monomeric red fluorescent protein). The sequence DEVD was inserted between CFP and YFP, whereas VEID was inserted between YFP and mRFP (CFP-C3-YFP-C6-mRFP). After protease cleavage, flow cytometry was used to distinguish activities of DEVDase and VEIDase, allowing for separate detection of two FRET signals (Wu et al. 2006).

Natural (Endogenous) Substrates

Several hundred proteins have been reported to be cleaved in a caspase-dependent manner during apoptosis in vivo (or at least in cellulo), and the list is still growing. It has been proposed that the sum total of these proteolytic events defines the complex cellular morphology known as apoptosis (Martin and Green 1995; Salvesen and Dixit 1997). To test this prediction, it would be necessary to separate the cleavage events that cause apoptotic function/morphology from those innocent victims that are inevitable given the complexity of animal proteomes. It is not so easy to do this in a scientifically rigorous manner, especially given the continual proliferation of reported proteins that are cleaved by caspases, deriving from the sensitive and high-throughput focused proteomics techniques that have recently become available.

Only a small group of these proteins is likely to be biologically significant, whereas the others are just cleaved as bystanders (Fuentes-Prior and Salvesen 2004; Crawford and Wells 2011). To validate whether a suspected protein is an important caspase substrate, one could consider whether it obeys the following criteria, which are related to the confidence in their assignment as naturally occurring events (Timmer and Salvesen 2007):

- Proteins shown to be cleaved by recombinant caspases in a purified system (preferably including rate—k_{cat}/K_M—calculations)
- Cleavage products identified in cells
- Cleavage-site mutants generated, showing abrogation of cleavage
- Cleavage-site mutants introduced into deficient cells
- Cleavage-site mutants introduced transgenically into whole animal

So far, only a handful of the hundreds of reported events have been confirmed to be of significance in vivo, and readers interested in more detailed discussion of this topic are referred to reviews (Timmer and Salvesen 2007; Crawford and Wells 2011). Just as for any post-translational modification, caspase cleavage can have a gain-of-function or loss-of-function consequence for the protein. For loss-of-function events, one would expect a substantial amount of the protein to be cleaved, but for a gain-of-function event, there need only be a small portion of the total molecules cleaved to allow the product(s) to signal. Currently, there is virtually no knowledge of how much cleavage of any caspase substrate there should be for a biological or pathological outcome. Ultimately, in the transgenic models in which a caspase cleavage site has been replaced by a noncleavable one, usually an Asp–Ala mutation, there is the formal, albeit low-level, likelihood that a function of the protein other than proteolysis has been modified by the mutation. The list of caspase substrates that have been somewhat validated as important in driving part of the apoptotic phenotype includes proteins with diverse functions in cell structure, cell signaling, and cell motility (for review, see Crawford and Wells 2011).

A major objective of defining the inherent substrate preferences of caspases, using chemi-

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cal scanning methods described earlier, has been to use this information to predict natural substrates—hence, the concept of predicting caspase natural substrates from consensus sequences (Timmer and Salvesen 2007). However, it turns out that this is not the only requirement for protein to become a caspase substrate. Protein substrates of caspase-3 show such discrepancies, a lot of them are cleaved at nonoptimal sites (Earnshaw et al. 1999; Nicholson 1999; Fischer et al. 2003; Fuentes-Prior and Salvesen 2004). This suggests that caspase exosite(s), regions involved in substrate binding that are remote from the active site, distinguish specific tertiary motifs in substrates (Fuentes-Prior and Salvesen 2004; Timmer and Salvesen 2007; Yin and Dong 2009). This additional condition sometimes annuls the requirements based on predictions from short synthetic peptides (Fuentes-Prior and Salvesen 2004; Timmer and Salvesen 2007). An interesting example of this propensity is seen in a biological diversity approach that sought to define the substrate preference of caspase-3 by phage display. An M13 bacteriophage gene III display fusion approach using fully randomized libraries of four and six residues was mostly in line with previously determined substrate specificity for caspase-3 and -8 using chemistry-based approach (Lien et al. 2004). Interestingly, some variations on the canonical motifs were discovered, such that peptide DLVD was cleaved by caspase-3 up to 170% faster than peptide DEVD. This method may generate much more conformational variability in peptide sequences, and also longer peptide sequences, possibly revealing the importance of structure and extended sequence in defining real caspase preferences that go beyond the residues lining the catalytic cleft, and this is discussed in more detail below. Currently there seems to be only one report of a defined caspase exosite, one in caspase-7 that distinguishes its preference over caspase-3 for the cleavage of PARP (Boucher et al. 2012). The existence of additional exosites is highly likely given that members of the inhibitor of apoptosis protein (IAP) family interact with regions distant from the caspase active-site cleft exosites (Srinivasula et al. 2001; Shiozaki et al. 2003; Scott et al. 2005; Timmer and Salvesen 2007).

The second issue to be considered is cleavage-site conformation of protein substrates. Unstructured and flexible loops are more readily accessible to caspases (Timmer and Salvesen 2007). As an example illustrating such a phenomenon, caspase-1 and its substrate pro-IL-1β may serve. The enzyme cleaves the substrate, pro-IL-1β, efficiently in a nonoptimal sequence regardless of whether the substrate is heat-denatured or folded (Sleath et al. 1990; Timmer and Salvesen 2007). Hence, it indicates that additional interactions during substrate cleavage might be unnecessary for some caspases, but important for others.

Several groups have deployed variations on a theme known as *N*-terminomics (and sometimes degradomics) by using focused proteomics technologies to interrogate natural caspase substrates (Van Damme et al. 2005; Enoksson et al. 2007; Dix et al. 2008; Mahrus et al. 2008; Timmer et al. 2009; Xu et al. 2009; Agard et al. 2010). Such technologies, reviewed in detail in Crawford and Wells (2011) and Impens et al. (2010), are capable of disclosing portions of the universe of substrates that have been cleaved by caspases in vivo, or at least in cellulo, and all have very high sensitivity and allow analysis of the caspase *N*-terminome in a very detailed way. Techniques used to identify natural substrates have been divided into two groups: forward (in vivo) and reverse (in vitro) (Crawford and Wells 2011). The first method allows definition of substrates that are cleaved in a specific biological process, whereas the second determination of substrates of a particular caspase—with the caveat that the natural context is lost. During investigations of an important cleavage, it is valid to apply these two kinds of techniques, because there may occur some discrepancies between cleaving substrates by caspase in vivo and in vivo. Moreover, in some cases, it may be obscure which caspase cleaves a specific substrate that is known to be cleaved during a certain process (Crawford and Wells 2011). Overall, there is still little confidence yet for the biological importance of cleavage events disclosed by these emerging technologies, although as

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data sets and validation procedures improve, we expect substantial progress in understanding the relevance of specific protein cleavages. Readers interested in the results of this line of research are pointed to the following database resources that capture most of the known cleavages of natural caspase (and other protease) substrates as well as predictive resources: wellslab.ucsf.edu/degrabase/; cutdb.burnham.org; clipserve.clip.ubc.ca/topfind; merops.sanger.ac.uk; bioinf.gen.tcd.ie/CASBAH/; sunflower.kuicr.kyoto-u.ac.jp/~sjn/Cascleave/links.html.

SYNTHETIC CASPASE INHIBITORS

Biologists, chemists, and pharmaceutical scientists probably disagree about what it takes to be a good and selective enzyme inhibitor. The words "potent" and "specific" are overused in the literature, and this is probably because a chemist may consider a compound to be potent even though it doesn't do much in vivo. Likewise, a biologist may consider a compound to be specific because it blocks a biological pathway of interest, even though it is broadly reactive with what a pharmaceutical chemist may consider "off targets." The ideal inhibitor would have high potency (tight binding: $K_i < 10$ nM; or rapid reaction: $k_{obs}/I > 100{,}000\ M^{-1}\ sec^{-1}$) and show selectivity for its target over all possible off-targets with a factor >1000 (and, of course, be bioavailable and pharmacologically active in vivo). Such a compound does not yet exist for any caspases. Why is this? The most likely reason is that the catalytic center and associated substrate binding cleft in proteases are designed to bind peptides, and thus any peptide or peptidomimetic is likely to bind more-or-less to every caspase. The catalytic center is just too good a thermodynamic sink for inhibitors that are competitive with substrate—so-called orthosteric inhibitors (Mattos et al. 2006; Drag and Salvesen 2010). However, less-than-ideal inhibitors have been highly informative in helping to define biological pathways and as lead compounds for anti-caspase therapy. Moreover, a rapidly growing interest in "allosteric" inhibitors—those that target highly specific sites *away* from the active site—is likely to achieve higher selectivity and specificity, although at the expense of potency (Drag and Salvesen 2010).

Orthosteric Inhibitors

The starting point for conventional, orthosteric, inhibitor design is to profile the inhibitor using substrate combinatorial library screening, caspase crystal structure analysis, and/or inhibitory structure–activity relationship studies. Detailed analyses of crystal structures of caspases bound to various peptide inhibitors have been used widely to help define whether there are any structural attributes dedicated to a particular caspase that can be useful for development-specific caspase inhibitors. Although the simple answer has been yes, in principle and on a coarse scale, the more important finding in comparing structures of inhibited and free caspases is that the structural plasticity and mobility of the active site have restrained the impact of structure-based probe design. The active sites and regions of caspases that direct inherent subsite occupancy are just too mobile for current design methods to deliver specificity based only on structural information; this is discussed further below.

To address this problem, researchers have developed more tailored peptide and peptidomimetic backbones for caspase binding. An analysis of the literature reveals that caspase inhibitor structures can be divided into three regions: the amino-terminal P_4 position, the dipeptide P_3–P_2 region, and a $P_{1'}$ electrophilic group, sometimes called a "warhead" or "cysteine trap" (Fig. 5). In typical approaches, a series of inhibitors is synthesized in which a particular region is varied while the rest of the scaffold is held the same.

Warheads

Various types of synthetic inhibitors have been described in the literature, including peptides, peptidomimetics, and nonpeptidic compounds. The guiding principle for many of these studies has been to generate therapeutic leads to modulate apoptotic and inflammatory

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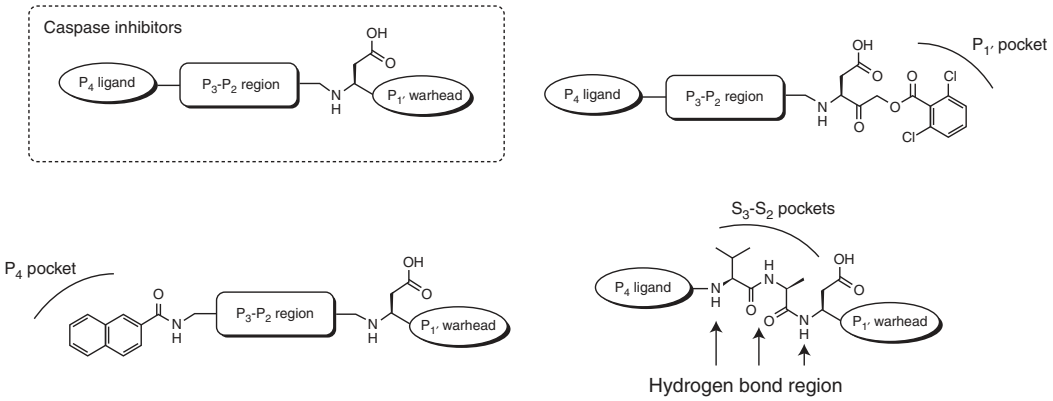

Figure 5. General outline of caspase inhibitor architecture.

pathways in disease. An orthogonal, sometimes overlapping, goal has been to generate probes that can be used to dissect the role of individual caspases, or caspases as a group, in biochemical events and networks that define cellular phenotypes and pathologies. Most synthetic caspase inhibitors are designed to use an electrophilic group (warhead) to modify the active-site cysteine residue covalently, leading to enzyme inactivation (Powers et al. 2002), and can be assigned as reversible (if the enzyme is inactivated through formation of reversible thiohemiketal) or irreversible (if the enzyme is permanently inactivated via formation of the thioether complex) (Fig. 6) (Talanian et al. 2000; Graczyk 2002). Some compounds display a bimodal pattern of inhibition, when a thiohemiketal is slowly transformed into a thioether adduct (Brady 1998; Brady et al. 1999).

The main feature for efficient proteolysis is that caspases require an aspartate residue at P_1 and recognize at least four amino acid residues to the left of the cleavage site (Thornberry et al. 1992; Pop and Salvesen 2009). As in the case of substrates, truncation of a tetrapeptide to a tripeptide or dipeptide leads to a dramatic decrease in inhibitor peptide-based activity (Graybill et al. 1997; James et al. 2004). Thus, inhibitor design for caspases has generally adhered to this consensus, with introduction of varied unnatural amino acid derivatives and variation of the chemistry of the warhead.

At the $P_{1'}$ position, several different electrophiles have been tested among which aldehydes and ketones with their derivatives (halomethylketones, aryloxymethylketones, or acyloxymethylketones), warheads of high utility for design of inhibitors selective to cysteine proteases in

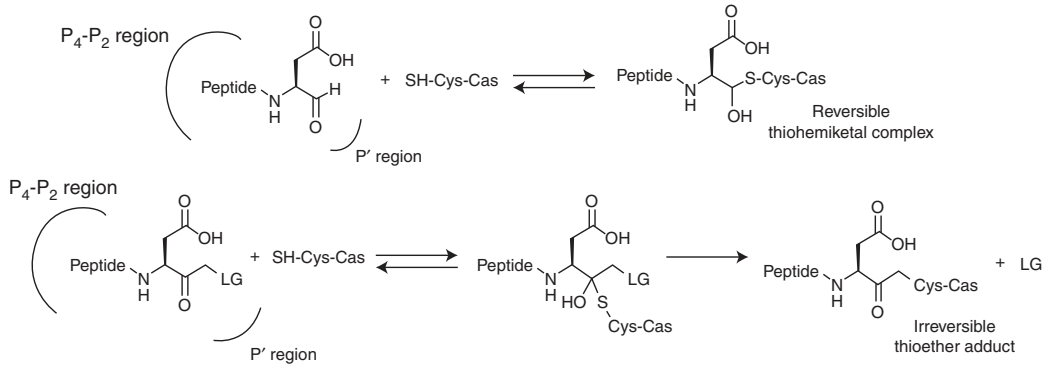

Figure 6. Reversible and irreversible caspase inhibition modes.

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general (Powers et al. 2002), have been heavily investigated. Interestingly, some electrophiles that are reactive with other families of cysteine proteases are very poor for caspases. Vinyl sulfone warheads, Michael acceptors that are highly reactive with cathepsins, and deubiquitinating enzymes (members of the protease clan CA) react very poorly with caspases (Newton et al. 2010). A different class of warhead, aza-epoxide and aza-Michael platforms, show excellent caspase inhibition and are suitable for being extended in the $P_{1'}$ direction (Asgian et al. 2002; Ekici et al. 2004, 2006; James et al. 2004). There is no relevant reason that aza-expoxides or aza-Michaels acceptors could not be useful as therapeutic leads, although this appears not to have been followed up on. Exploration of the $S_{1'}$ pocket of caspase-3 using aza-peptide epoxide inhibitors (Ganesan et al. 2006a) uncovered the basis for inhibition specificity on the right to the scissile bond. The $S_{1'}$ pocket is large and can accommodate bulky, hydrophobic warheads, explaining in part the high reactivity of aryloxymethylketones or acyloxymethylketones with caspases.

Therapeutic Leads

Although the substrate specificity of a particular caspase cannot be used to discriminate it within the family, for reasons of overlap that also pertain to the substrates explained above, there are certain preferences that can be exploited. For instance, the finding that the caspase-1 inhibitor Ac-YVAD-CHO prevents the release of interleukin 1-β from monocytes was part of the initial evidence that caspase-1 is responsible for interleukin 1-β maturation, upon specific proteolysis (Thornberry et al. 1992). Unfortunately, most of these inhibitors cannot play therapeutic roles because of their toxic metabolites. A good example is Cbz-VAD-FMK, a broad-spectrum caspase inhibitor. Cbz-VAD-FMK (a.k.a. ZVAD) is an excellent probe for enzyme kinetic analysis and ablation of apoptosis and inflammation paradigms owing to its rapid inhibition of most caspases in vitro (Table 3), but is useless as a therapeutic lead because of production of highly toxic fluoroacetate (Van Noorden 2001).

Table 3. Kinetic parameters of the widely used and heavily cited Cbz-VAD-FMK (often called ZVAD)

	k_{obs}/I (M^{-1} sec^{-1})	$t/2$ (sec) for $I = 50$ μM	$t/2$ (sec) for $I = 1$ μM
Caspase-1	280,000	0.05	2.48
Caspase-2	290	47.79	2389.66
Caspase-3	16,000	0.87	43.31
Caspase-4	5500	2.52	126.00
Caspase-5	130,000	0.11	5.33
Caspase-6	7100	1.95	97.61
Caspase-7	18,000	0.77	38.50
Caspase-8	280,000	0.05	2.48
Caspase-9	180,000	0.08	3.85

The k_{obs}/I values are adapted from Garcia-Calvo et al. (1998), and the half-time of inhibition ($t/2$) in seconds is calculated according to the relationship $t/2 = \ln 2/k_{obs}$, for the inhibitor concentration given at the head of the column (Bieth 1984).

Peptide to Peptidomimetic

A classic example of inhibitor development from peptide substrate specificity knowledge to a peptidomimetic that achieved clinical trial status is Pralnacasan (VX-740). The most common P_3–P_2 motif of early caspase-1 inhibitors is Val–Ala, but it was also reported that replacing the P_2 Ala residue by other amino acids including secondary piperidine or proline does not affect inhibitory potency (Dolle et al. 1994). Such an example is VX-043198 with an unnatural *tert*-leucine in the P_3 position and a secondary proline in P_2 (Fig. 7). This inhibitor is an active metabolite of VX-765, an orally absorbed prodrug. VX-765 is a rare example of a peptide-based caspase inhibitor possessing a good pharmacokinetic profile and displaying utility for treatment of inflammatory diseases (Wannamaker et al. 2007). To address potency issues of VX-765, a new, more flexible pyridazinodiazepine scaffold was described (Dolle et al. 1997). This moiety reduced the peptide character while maintaining a high affinity against caspase 1. The use of a pyridazinodiazepine group for mimicking the Val–Ala sequence resulted in the development of Pralnacasan (VX-740) (Fig. 7), which was tested in late clinical trials as a drug candidate for rheumatoid arthritis (unfortunately failing because of liver toxicity issues) (Dolle et al. 1997; Siegmund and Zeitz 2003;

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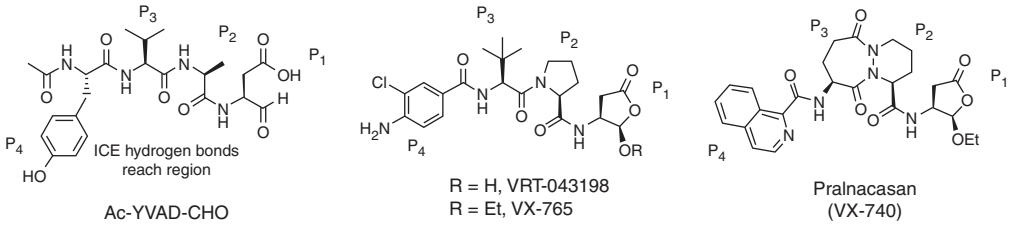

Figure 7. Structures of prototypic peptide-based (Ac-YVAD-CHO) and two peptidomimetic caspase-1 inhibitors.

Linton 2005). The drug possesses a 2-naphthyl hydrophobic moiety in the P_4 position, a pyridazinodiazepine-based bicyclic core mimicking the Val–Ala region (P_3–P_2 residues) and a lactone moiety at the carboxyl terminus, masking an aspartate residue at P_1 (Fig. 7).

Although the most potent caspase inhibitors occupy the S_1–S_4 subsites, it appears that the chemical nature of the $P_{1'}$ leaving group can be a key determinant in the design of inhibitors. Simple dipeptide-based compounds can display nanomolar potency toward caspase-1 (Galatsis et al. 2010). The dipeptide-based IDN-6556, containing a 2,3,5,6-tetrafluorophenoxymethyl ketone warhead, was the first irreversible pan-caspase inhibitor to enter Phase II clinical trials targeting liver diseases. Taken together, it appears that inhibitors with appropriate $P_{1'}$ leaving groups can act as broad spectrum irreversible inactivators targeting inflammatory, initiator, and apoptotic caspases. Such inhibitors, showing little selectivity, may be appropriate therapeutics in acute pathological situations, and that irreversibility per se may not be a barrier to development of drugs—depending on the therapeutic indication.

To enhance selectivity by exploring additional chemical space, screens using nonproteinogenic (unnatural) amino acids have been used to synthesize caspase peptide inhibitors by exploring libraries equipped with acyloxymethylketones (AOMKs) (Berger et al. 2006b) and 2,3,5,6-tetrafluorophenoxymethyl ketone warheads (Leyva et al. 2010). Although these screens have shown enhanced selectivity compared with natural amino acid libraries, there is still a way to go with this approach to achieve more optimal specificity.

Caspase Activity-Based Probes

Activity-based proteomics (ABP) is a relatively new field on the border of chemistry and biology. The main goal of ABP is to design small-molecule chemical tools that will be able to tag and monitor the active status of proteins within a complex proteome, providing links to their functions. The most commonly used probes possess an appropriate peptide or peptidomimetic sequence equipped with an electrophilic warhead that is used to modify the enzyme's active site covalently (Serim et al. 2012). These sequences can be derived from substrate or inhibitor library screening or by iterative chemical synthesis approaches. Characteristic of activity-based probes is an amino-terminal tag, which reveals whether a probe binds to the active site or not. The most popular tags for this purpose are radioisotopes, fluorescent (or luminescent) groups (all three groups give direct readouts), and biotin (which is detected in a semidirect manner) (Fig. 8). All ABPs have this composition and are generally divided by the type of reporter. This concept was pioneered for proteases by James C. Powers and used at first to target serine proteases (Kam et al. 1993). Now this discipline has significantly evolved, con-

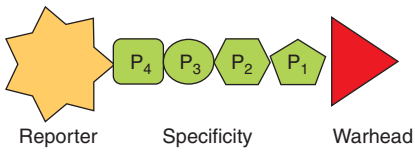

Figure 8. A generalized activity-based probe. The probe is designed to bind in the same manner as a synthetic substrate, but the red warhead binds irreversibly to the caspase catalytic residues.

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firmed by the number of recent review papers concentrating on distinct ABP approaches (Evans and Cravatt 2006; Fonovic and Bogyo 2008; Heal et al. 2008, 2011; auf dem Keller and Schilling 2010; Edgington et al. 2011; Serim et al. 2012).

Biotin Labels

In the early stage of the discovery of activity-based probes (ABPs) for caspases, commonly used inhibitors with appropriate peptide sequences were biotinylated and tested toward their protein targets (Thornberry 1994; Nicholson et al. 1995). Biotin is the tag of choice for ABPs because it displays diffusion-limited binding to streptavidin, allowing for high stringency and rapid probing (Sadaghiani et al. 2007). Because these probes label only active caspases, the principle is extremely useful for distinguishing active enzyme from the bulk of inactive precursors in cells and tissues. The use of biotin for affinity labeling has some limitations, the main problem being that biotin is not suitable for in vivo and in situ studies because of its poor cell permeability and nondirect visualization protocol and the presence of endogenous biotinylated polypeptides (Sadaghiani et al. 2007).

Although not suitable for use for intact cell imaging, biotin ABPs have been extremely useful in visualizing caspase activation in vitro following cell lysis and ligand blotting, usually with streptavidin conjugates. Thus biotin-VAD-CMK, biotin-YVAD-AOMK, Cbz-EK(biotin)D-FMK, and biotin-DEVD-CMK have been used to detect which caspases are being activated in response to various apoptosis-inducing triggers (Fearnhead et al. 1997; Cardone et al. 1998; Martins et al. 1998), heat shock (Tu et al. 2006), or nitrosylation (Li et al. 1997; Mannick et al. 2001). Although much of the earlier work was performed with FMK warheads, today AOMK warheads are preferred owing to their greater selectivity. Indeed, FMK warheads have lost popularity because of the substantial cross-reactivity with other families of cysteine proteases (Rozman-Pungercar et al. 2003). The sequence elements of early probes were based on prototypic caspase substrates and inhibitors. Unfortunately, these probes lack the specificity and produce high background labeling during crude proteome assays. Because of this, more specific caspase activity-based probes have been produced. For example, work from the Bogyo laboratory demonstrated that the commercially available biotin-VAD-FMK probe is widely reactive with caspases, but also produces high background when assayed with a caspase-3 mutant (C285A). This is not good news if the objective is to detect active enzyme. The tripeptide biotin-EVD-AOMK labels caspases well, with far less background (Kato et al. 2005). But replacing elements in the P_3 and P_2 positions with nonproteogenic (unnatural) amino acids begins to achieve a degree of selectivity for individual caspases. Although the absolute probe specificity has not been obtained, this work is an example of how selective labeling of specific caspases could be developed (Berger et al. 2006a,b).

Radioisotopes

Radioactive isotopes, especially ^{125}I, have several advantages in ABPs: They can be easily introduced into an inhibitor structure, their relatively small size does not change the overall biochemical properties of a parent inhibitor, they can be easily detected by simple autoradiography methods, and they are sometimes used for whole-body imaging. The dipeptidyl general caspase inhibitor ^{125}I-M808 (iodotyrosyl-VD-FMK) was used to show radiolabeling of caspases in tissues from animals following apoptotic challenges (Methot et al. 2004). A set of isatin-based analogs that are able to inhibit caspases, labeled with ^{18}F or ^{11}C, has been used in microPET imaging for detecting apoptosis in animal models, showing that caspase ABPs can act as radiotracers in living organisms (Zhou et al. 2006, 2009; Nguyen et al. 2009). A disadvantage of the isatin-based probes must be synthesized and used rapidly following synthesis. To overcome this limitation, a probe allowing introduction of a ^{64}Cu atom via chelation via a 1, 4, 7, 10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) group has been shown to be a good tracer of caspases in a mouse model (Lee et al. 2009). The probe is bifunctional, be-

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cause it also possesses a fluorescence-quencher pair that produces fluorescence upon proteolysis. Because the probe is really a substrate, not an inhibitor, the radio signal is always "on" and used to trace the compound, whereas the fluorescence generation reports caspase activity. Perhaps a combination of DOTA with a cell-penetrating caspase inhibitor would make an excellent, more widely available, probe for PET imaging.

Fluorescent (Luminescent) Dyes

The great advantage of fluorescent tags is their high water solubility and good cell-penetration properties. Likewise, some of them are pH insensitive, stable to solid-phase peptide synthesis, and give fast and high readouts (Sadaghiani et al. 2007). One of the first approaches in the use of fluorescently labeled probes for caspases, coined FLICA (for fluorochrome-labeled inhibitors of caspases), used carboxyfluorescein (FAM) as the tag in FAM-VAD-FMK (Bedner et al. 2000; Smolewski et al. 2001; Grabarek et al. 2002). FLICA offers a promising assay for studying caspase biology, but lack of specificity and background effects of other proteases with the FMK group limit its utility. To date, various types of FLICAs have been described in the literature. The commercially available FLICAs are usually tagged with FAM or fluorescein isothiocyanate (FITC), green dyes, or sulforhodamine B (SR), a red dye. Likewise, currently used FLICAs are designed for detection of individual caspases or a group of caspases separately (Smolewski et al. 2002; Teodori et al. 2002; Valero et al. 2005; Luthra et al. 2006; Jadhav et al. 2007; Kuzelova et al. 2007).

A developing fluorescent dye for caspase inhibitor labeling is Cy5, which belongs to the cyanine family. This fluorophore is also referred as a near-infrared fluorescent tag (NIRF) because of its spectral characteristic (maximum of absorbance at 649 nM, maximum of emission at 670 nM). Cy5 has been widely used to tag caspase inhibitors with the AOMK warhead, thereby improving selectivity. Caspase probes labeled with Cy5 can be used for in vivo apoptosis imaging by a noninvasive manner (Edgington et al. 2009, 2012). These initial studies with the use of Cy5 show that caspase activity can be detected in many biological assays, from cell-free apoptotic lysates, to tissues, to whole organisms. Moreover, potent and cell-permeable caspase fluorescent activity-based probes possess diagnostic and therapeutic potential. Enhancements in selectivity are likely to use nonproteinogenic amino acid strategies, and some success in this direction has been seen with a use to explore the role of pyroptosis in response to *Salmonella* infection in macrophages of primary mouse bone marrow, revealing the importance of apoptotic and inflammatory caspases (Puri et al. 2012).

Allosteric Inhibitors

Unlike orthosteric inhibitors, in which the starting point is to mimic substrate-binding modes, allosteric inhibitors are more difficult to design rationally. Screening with protein-based recombinant DARpin (designed ankyrin repeats) libraries revealed a highly selective and potent inhibitor of caspase-2. The caspase-2-specific DARpin targets a region that connected the active site and influenced the catalytic conformation of the protease (Schweizer et al. 2003). Although it would be difficult to render a small molecule from the DARpin, this showed that allosteric inhibitors can deliver exquisite specificity. Sometimes, experiments of nature often give reasonable starting points, and to this end, the dimer interface provides a point of leverage. Thus, the BIR3 domain of the endogenous caspase-9 inhibitor XIAP acts by preventing the activating dimerization of the enzyme (Shiozaki et al. 2003), and to this end, peptides mimicking this interaction have been described as allosteric caspase inhibitors (Huber et al. 2012). The dimerization interface of caspases-3 and -7 contains pockets that can be used to block the active conformation, by an allosteric mechanism (Hardy et al. 2004). Zinc has long been known as a caspase inhibitor (Perry et al. 1997; Stennicke and Salvesen 1997), and it has been revealed that this metal targets an allosteric site on caspase-6 (Velazquez-Delgado and Hardy 2012). Design and optimization of allosteric

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caspase inhibitors is in its infancy, but the promises in terms of selectivity are huge (Drag and Salvesen 2010).

CONCLUSION

The explosion of synthetic caspase substrates and inhibitors over the last few years has revealed the promise of defining the fundamental properties of caspases as enzymes, their role in promoting apoptosis and inflammation, and provided several excellent scaffolds for therapeutic leads. Innovative focused proteomics methods have disclosed a huge universe of potential natural protein caspase substrates in vivo. But these advances are not without caveats. Currently there seem to be no caspase inhibitors in advanced clinical trials, and this may be because of overlapping effects—due to the lack of appropriately selective inhibitors—or it may simply be because of the lack of confidence in targeting caspases for therapeutic ends. More selective inhibitors and a better understanding of the final outcomes of caspase inhibition in human disease are needed. The vast array of caspase natural protein substrates so far identified needs to be triaged into those that directly affect the outcome of apoptosis or inflammation from the innocent bystander substrates, and this will require substantial protein engineering and reconstitution experiments. We look forward to the emerging technologies that will provide resolution to these matters.

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