Selective inhibitors of apoptotic caspases: implications for novel therapeutic strategies

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Caspases are essential for apoptosis. A crucial question regarding the role(s) of these proteases is whether the selective inhibition of an effector caspase will prevent cell death. We have identified potent, selective non-peptide inhibitors of the effector caspases 3 and 7. Apoptosis can be inhibited and cell functionality maintained using an inhibitor selective for caspases 3 and 7. This has important therapeutic implications and the potential to generate novel anti-apoptotic strategies in diseases that involve dysregulated apoptosis.

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▼Apoptosis is mediated by several intracellular proteases known as caspases. Caspases play a critical role in numerous pathways leading to cell death and, to date, at least 14 members of the caspase family have been identified[1,2]. The involvement of caspases in apoptosis has been shown *in vitro* by measuring enzyme activities and evaluating the effects of non-selective inhibitors in cell lysates and tissue extracts[3–5].

Although they are informative, cell-based studies have used peptide inhibitors with limited cell permeabilities, irreversible peptide inhibitors and prodrug peptide inhibitors. The moderate caspase selectivities associated with these reagents have made it extremely difficult to accurately assess the importance of a specific caspase in apoptosis, and the interpretation of results is often ambiguous.

Caspase-null mutants have been used to demonstrate the importance of individual family members in cells derived from these caspase-deficient mice and this has allowed the association of individual caspases with specific morphological changes that occur during apoptosis[6–10]. However, because these animals either die *in utero* or possess short life spans, their usefulness in the development of relevant disease models is severely compromised. In this article, we discuss the usefulness of selective caspase inhibitors in terms of understanding cell- and disease-specific apoptotic processes.

Caspase inhibitors

The optimal consensus tetrapeptide substrate sequences for each of the caspases have been elegantly determined using a positional scanning library[11]. Based on these results and the known cleavage sequences of macromolecular substrates for this family of enzymes, the caspases can be subdivided into three groups: (1) caspases involved in inflammation (caspases 1, 4 and 5); (2) initiators (caspases 6, 8, 9 and 10); and (3) effectors (caspases 2, 3 and 7). The differences in consensus cleavage sequences between the three groups of enzymes suggest that selective inhibitors for a group of caspases (if not individual caspases) should be obtainable.

Studies with macromolecular viral inhibitors of caspases have also suggested that selectivity for a small number of caspases is possible[12]. The cytokine response modifier (CrmA) produced by cowpox virus was the first natural caspase inhibitor to be identified. Mutational studies have shown that it has a substrate-like mechanism of action in which CrmA interacts with the enzyme via the pseudosubstrate peptide sequence LVAD. It most potently inhibits caspases 1, 5 and 8, with K_is of 4–10 pM, and inhibits the remaining family members with K_is of >1 nM. To date, there has been no firm association between an individual apoptotic caspase and a particular disease state. Experimental data are accumulating that

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Box 1. Glossary

Aβ25–35	Active fragment of amyloid β protein
CAD	Caspase-activated deoxyribonuclease
CAM	Camptothecin
COL2A1	Gene encoding type II collagen
FMK	Fluoromethylketone
LDH	Lactate dehydrogenase
OA	Osteoarthritis
RLU	Relative light units

point towards certain caspases playing key roles in the apoptotic signalling cascade.

Caspase 3 has been found to be activated in virtually every model of apoptosis[13]. It belongs to a subfamily of effector caspases that also includes caspases 6 and 7. These caspases are activated downstream of initiator caspases such as caspases 8, 9 and 10. Natural substrates of caspase 3 include many proteins involved in cell maintenance and/or repair[14]. For example, oligonucleosome fragmentation (DNA laddering) is a characteristic feature of apoptosis and is mediated by caspase-activated deoxyribonuclease (CAD; see Box 1 for glossary of abbreviations), whose activation is effected by the caspase-3-mediated cleavage of the CAD inhibitor ICAD[15]. The availability of selective inhibitors of caspase 3 would allow the evaluation of the potential for inhibition of apoptosis at the level of an effector caspase. In this article, we describe the use of selective caspase inhibitors in chronic and acute degenerative diseases and discuss the potential development issues with anti-apoptotic therapies.

Little has been published on selective inhibitors of the individual apoptotic caspases (effector and initiator groups). A conformationally constrained peptidomimetic inhibitor that is selective for caspase 3 over caspase 1 (K_i = 18 nM and K_i = 10,000 nM, respectively) has been disclosed by IDUN Pharmaceuticals (La Jolla, CA, USA) but its profile against the remaining caspases was not reported[16]. There are several other ongoing efforts targeting selective inhibitors of individual members of the apoptotic caspase family. However, in most cases, detailed results have yet to be disclosed. Companies such as Vertex (Boston, MA, USA), Merck (West Point, PA, USA), BASF (Ludwigshafen, Germany) and Pfizer (Groton, CT, USA) established a strong presence in the caspase inhibitor field by first developing inhibitors against caspase 1 (ICE) and, because of the structural homology between all caspase family members, these companies are positioned to use much of what they learned from these studies to develop inhibitors for the apoptotic caspases.

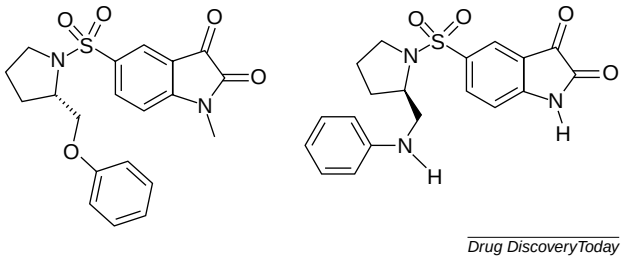

Figure 1. The structures of two isatin sulfonamides, which are caspase inhibitors[17].

Potent and selective inhibitors of caspases 3 and 7 have recently been reported[17,18]. Compound A inhibits caspases 3 and 7 with K_{iapp}s of 15 nM and 47 nM, respectively (Fig. 1). It exhibited 100-fold or greater selectivity for the highly homologous caspases 3 and 7 against all other family members except caspase 9 (Ref. 17). The selectivities for caspases 3 and 7 against caspase 9 were 30-fold and tenfold, respectively (Table 1). Based on its potency and selectivity, this represents an important opportunity to determine the relevance of caspases 3 and 7 in diseases known to be associated with tissue damage and apoptosis such as osteoarthritis (OA). Compound B (Fig. 1) is a structural analogue of compound A with considerably less potency at inhibiting caspases (Table 1).

Table 1. Inhibitor selectivity of the isatin sulfonamides [K_{iapp} (μM)[a]]

Inhibitor	Caspase 1	Caspase 2	Caspase 3	Caspase 4	Caspase 6	Caspase 7	Caspase 8	Caspase 9
A	17	4.9	0.015	33	29	0.047	49	0.46
B	>50	11	12	>50	57	10	>50	>50

Inhibitor studies were performed as described in the experimental procedures.

[a]The K_{iapp} was calculated from the estimated IC_{50} using the following equation assuming competitive inhibition, which is characteristic for this class of compounds:

$$K_{iapp} = \frac{IC_{50}}{1 + \frac{[S]}{K_s}}$$

where [S] is the concentration of the substrate in the assay and K_s is the Michaelis constant of the substrate. The concentrations of inhibitor throughout are micromolar.

Chondrocyte apoptosis

OA is a degenerative joint disease that is histologically characterized by the erosion of articular cartilage. There are no disease-modifying drugs currently available to treat this condition. Elevated levels of apoptosis have been reported in superficial, mid-zone and hypertrophic chondrocytes in OA cartilage[19,20]. In addition, chondrocytes adjacent to OA lesions express high levels of *bcl-2*, a gene involved in the inhibition of apoptosis[21]. Cartilage degradation appears to result from the cleavage of matrix proteins by proteolytic enzymes derived from chondrocytes and/or synoviocytes. As OA progresses, the fibrillar collagen network is degraded and chondrocyte cell death increases[22,23].

In several transgenic models, dysregulated apoptosis has been observed in chondrocytes associated with major structural and developmental abnormalities, thereby suggesting a crucial role for the regulation of this process in articular cartilage[24,25]. Increased numbers of apoptotic chondrocytes have also been observed in animal models with surgically induced OA[26]. Therefore, the evidence suggesting a role for apoptosis in chondrocytes from normal cartilage is both extensive and convincing. Furthermore, during the development of OA, it has become evident that apoptotic events are dysregulated.

Preventing apoptosis by inhibiting caspases 3 and 7 with compound A resulted in the maintenance of the transcriptional activity of the *COL2A1* promoter (from the gene encoding the major extracellular matrix component of cartilage) in a human chondrocyte cell line (Figs 2,3). The prevention of apoptosis was measured by a cell-death ELISA, which measures DNA fragmentation[17]. This had also been reported previously with primary bovine chondrocytes[17,18]. Compound B, which is considerably less active against the caspases (Table 1), was inactive in these experiments (Figs 2,3). Maintenance of type II collagen promoter activity suggests that expression of this major cartilage matrix protein would be maintained in the presence of the inhibitors. Chondrocytes are responsible for the production and maintenance of the extracellular matrix, which allows optimal fluid-joint articulation. In a catabolic state, therefore, an anti-apoptotic agent would allow cells that were destined to die to survive and, more importantly, to continue to synthesize and deposit viable new matrix.

Based on the *in vitro* data, the inhibitors of caspases 3 and 7 might therefore have therapeutic uses in either the prevention or the treatment of OA. The long-term challenge in this endeavour is to develop drugs that inhibit apoptosis, or particularly caspase activity, which is specific for cartilage chondrocytes, thereby allowing general apoptosis, which is required for homeostasis to function normally.

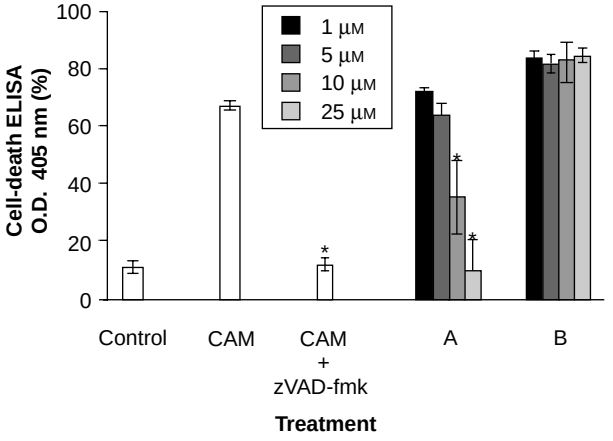

Figure 2. The effect of selective inhibitors of caspases 3 and 7 on camptothecin (CAM) induced apoptosis of human chondrocytes. Immortalized human C20/A4 chondrocytes were treated with CAM (4 μg ml^{-1}) alone or in combination with varying concentrations of compound A or B, and cell death was measured and compared with the death of cells grown in serum containing medium alone (control)[18]. Lysates from approximately 5×10^4 cells were prepared and cell death was measured by the cell-death ELISA. Results are mean ± standard deviation (for three samples). (*Denotes that $P < 0.02$ compared with CAM treatment alone.)

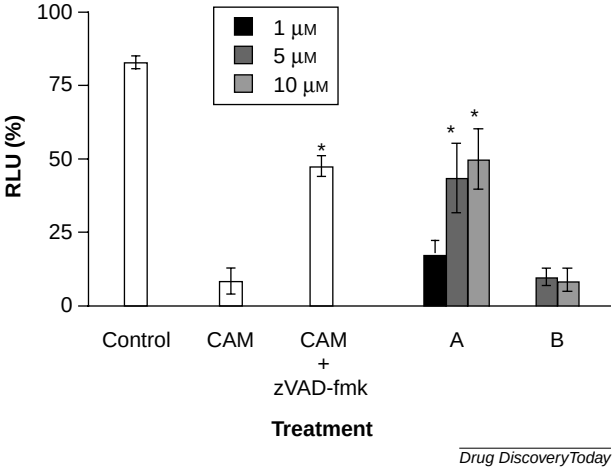

Figure 3. The maintenance of type II collagen promoter activity after apoptotic insult in the presence of the caspase inhibitors and zVAD-fmk. The C20/A4 chondrocyte cells stably transfected with a construct in which the regulatory sequences (–577 to +3426) of the gene encoding type II collagen (*COL2A1*) are driving luciferase reporter gene expression[17,18]. Cells were also grown in serum-containing medium as a control. Cells were treated with 4 μg ml^{-1} camptothecin (CAM) or 4 μg ml^{-1} CAM plus varying concentrations of compound A or B for 24 h. Expression of the *COL2A1* gene was measured as luciferase activity using a luminometer, expressed as relative light units (RLU). Results are mean ± standard deviation (for three samples). (*Denotes that $P < 0.02$ compared with CAM treatment alone.)

Neuronal cell apoptosis

Caspase activation might be involved in both acute and chronic neurodegenerative processes, and treatment with caspase inhibitors might protect neurons from apoptotic cell death[27,28]. In animal models of stroke and traumatic brain injury, caspases (including caspase 3) have been shown to be activated, and treatment with irreversible peptide inhibitors can improve the outcome[29–35]. Similarly, caspase proteins have been implicated in the progression of neuronal apoptosis in chronic degenerative diseases such as Huntington's and Alzheimer's[28,36–38].

Inhibition of apoptosis in primary neuronal cell culture

In our studies, treatment with isatin sulfonamides (compound A) inhibited cell death in primary cultures of cortical neurons. Using lactate dehydrogenase (LDH) release as a marker of plasma membrane disruption, treatment with compound A resulted in an approximatey 50% reduction in cell death induced by the active fragment of amyloid β protein (Aβ25–35) (Fig. 4). Similar results were obtained following treatment with hydrogen peroxide and, in both cases, the protection obtained was equivalent to results with the irreversible peptide inhibitor zDEVD-fmk[39]. These results show that a selective inhibitor for caspases 3 and 7 can protect neurons from the CNS from apoptotic insults.

Inhibition of apoptosis in a neuronal cell line

Other studies were aimed at using selective caspase inhibitors to determine the pathways involved in neuronal apoptosis. One cell system frequently used to investigate mechanisms of neuronal death is the rat PC12 pheochromacytoma cell line. Although these cells are of sympathetic lineage, they have proved to be useful in examining the role of various apoptotic pathways common to neuronal cells. In naive PC12 cells, withdrawal of growth factors results in a well-characterized apoptotic cell death[40–42]. Various caspase proteins have been shown to be activated during this process[43–45] and caspase inhibitors can provide protection in this system[43,45]. However, the precise role of individual caspases has remained elusive. For example, caspase-3-like activity is detected in the first 6 h after trophic factor deprivation in these cells[43] but peptide inhibitors directed against caspase 3 do not rescue these cells[44,45]. These results have led to the conclusion that caspases 3 and 7 are not crucial for this apoptotic system.

Isatin sulfonamides were used after growth factor deprivation of PC12 cells in an attempt to understand the role of caspases 3 and 7 in trophic factor deprived PC12 cells[39]. Using LDH release as a marker of cell death, compound A produced a dose-dependent decrease in cell death (Fig. 5a). The IC_{50} for this response was 309 nM, an approximate order of magnitude increase in potency over the pan-caspase inhibitor zVAD-fmk (the decreased concentrations in this assay compared with the chondrocyte cell cultures were due to the lack of serum in the media, which reduced non-specific protein binding of these compounds). As has been reported previously, zDEVD-fmk did not provide protection in this assay at any dose examined (1–100 μM; J.A. Erhardt *et al.*, unpublished). Further confirmation of these results was obtained by examining mitochondrial dehydrogenase activity as a marker for cell viability. Both compound A and zVAD-fmk provide protection in this assay that is equivalent to the addition of serum (Fig. 5b). By contrast, zDEVD-fmk-treated cultures showed only a slightly higher activity than control serum-deprived cultures.

The differences in outcome after inhibitor treatment were not due to poor cell penetrability because both compound A and zDEVD-fmk inhibited caspase-3-like activity, as measured by cleavage of a fluorogenic substrate for caspases 3 and 7 in intact cells (Fig. 6a). This indicated that

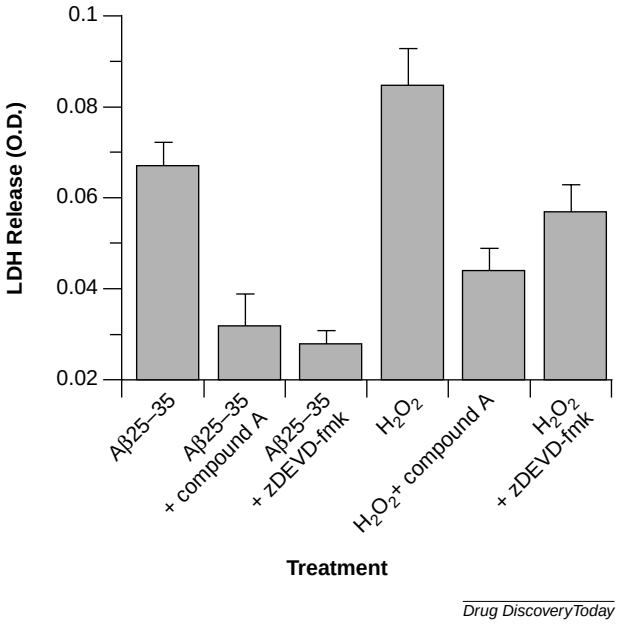

Figure 4. The effect of caspase inhibitors on cell death of primary neuronal cultures. Forebrain neuronal-enriched cultures were prepared from embryonic day 17 rats, and these cells were treated with Aβ25–35 (40 μM for 48 h) or H_2O_2 (10 min exposure to 10 μM H_2O_2, followed by 18–20 h in culture)[39]. Caspase inhibitors were added 1 h before treatment (H_2O_2) or at the time of treatment (Aβ25–35), with incubation continuing for the duration of the experiment. Compound A and zDEVD-fmk were used at 20 μM concentration. Intracellular lactate dehydrogenase (LDH) release into the media was measured to assess neuronal death. Results are mean ± standard error (for three samples).

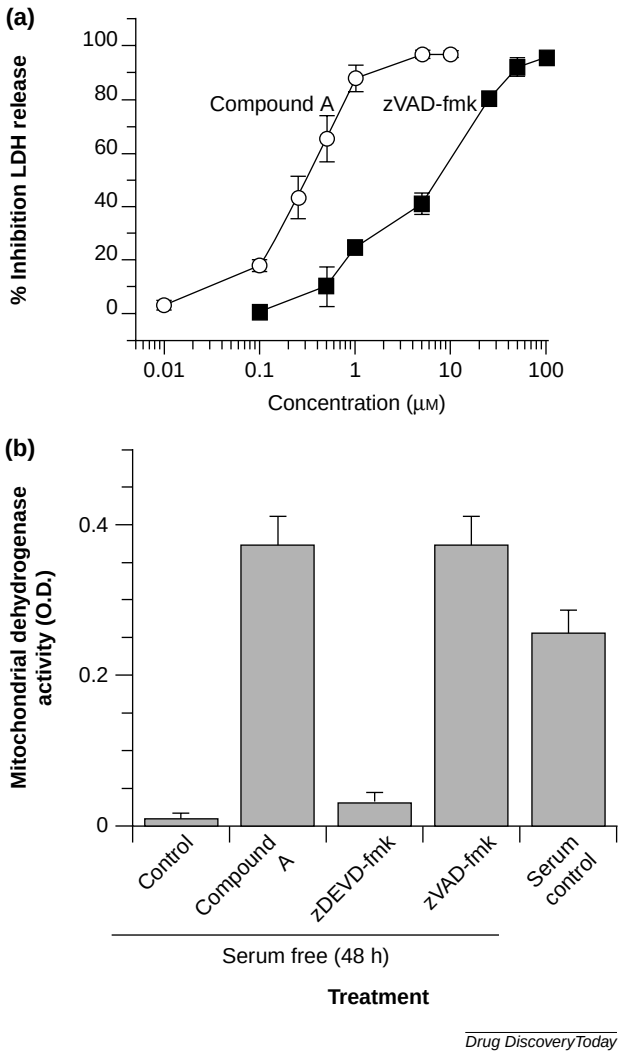

Figure 5. The effect of caspase inhibitors on PC12 cell death. **(a)** Undifferentiated PC12 cells were deprived of serum to induce apoptosis in the presence of caspase inhibitors (compound A at 10 nM–10 μM and zVAD-fmk at 100 nM–100 μM). Lactate dehydrogenase (LDH) levels were measured in the media 24 h after serum removal. Data are presented as inhibition of LDH release relative to serum-free control[39]; results are mean ± standard error (for three samples). **(b)** Mitochondrial dehydrogenase activity was determined in cultures deprived of serum for 48 h in the presence or absence of various caspase inhibitors; this activity indicates the number of viable cells in culture. Treatments were as follows: compound A, 1 μM; zDEVD-fmk, 50 μM; zVAD-fmk, 50 μM; serum-free control; and serum control (2% serum). Results are mean ± standard error (for three samples).

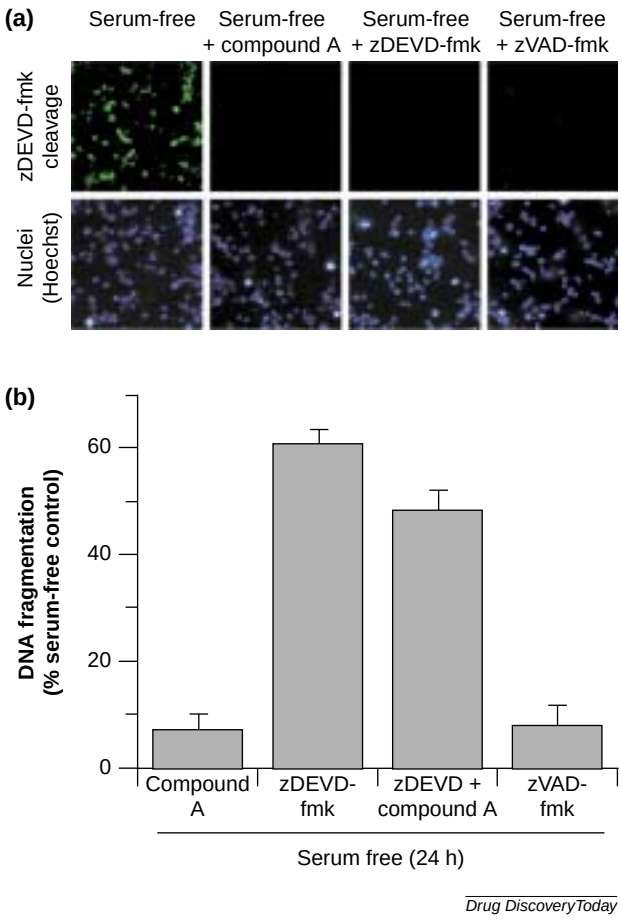

Figure 6. Inhibition of caspase-3-like activity and DNA fragmentation after treatment with caspase inhibitors. **(a)** Fluorescent imaging of serum deprived PC12 cells. A DEVD-based substrate for caspases 3 and 7 that fluoresces upon cleavage was added to serum-deprived PC12 cells at 22 h. After a 2h incubation with this substrate, Hoechst 33342 was added to label nuclei and fluorescent images were captured of intact (unfixed) cells. Nuclear labelling is presented to indicate the relative number of cells in the given fields. Concentrations of inhibitors were as follows: compound A, 1 μM; zDEVD-fmk, 50 μM; zVAD-fmk, 50 μM. Caspase-3 activity could not be analysed from cellular lysates owing to the reversible nature of compound A inhibition. **(b)** Extent of DNA fragmentation relative to control serum-deprived populations. PC12 cells were deprived of serum for 24 h, and DNA fragmentation was measured via ELISA. Cells were treated with caspase inhibitors or a combination of inhibitors at the time of plating: compound A, 1 μM; zDEVD-fmk, 100 μM; zVAD-fmk, 50 μM. Results are mean ± standard error (for three samples).

the ineffectiveness of zDEVD-fmk probably resulted from an activity distinct from its interaction with caspases 3 and 7. To explore this, combinations of inhibitors were added to serum-deprived cells, and the extent of DNA fragmentation measured. Similar to zVAD-fmk, compound A dramatically inhibited this apoptotic process. By contrast, zDEVD-fmk treated cultures exhibited a significantly higher level of DNA fragmentation, as did cultures treated with a combination of compound A and zDEVD-fmk (Fig. 6b). By itself, zDEVD-fmk was not toxic to PC12 cells because this compound did not induce cell death when added to control (serum containing) cultures.

Overall, these results show the usefulness of selective caspase inhibitors in protecting neuronal cells and determining apoptotic mechanisms. Isatin sulfonamides improved PC12 cell survival whereas zDEVD-fmk did not. This lack of protection with zDEVD-fmk did not predict the role of caspases 3 and 7, because cell death proceeded in serum-deprived cells treated with a combination of inhibitors, suggesting that zDEVD-fmk possesses an additional proapoptotic activity in this system[39].

Anti-apoptotic therapies

Development of an anti-apoptotic therapy for chronic administration carries with it concerns of potential toxic effects caused by the abrogation of normal (homeostatic) apoptosis in the human adult, which accounts for over 10^{11} cell deaths per day. As a consequence of this concern, the first generation of anti-apoptotic drugs (caspase inhibitors?) are likely to be used only in acute settings such as salvaging cells destined to die by apoptosis following stroke, myocardial ischaemia or acute liver degeneration[46].

A major goal in the treatment of chronic degenerative diseases is the prevention of unwanted cell death while not allowing the proliferative diseases (e.g. cancer, rheumatoid-arthritis-associated synovial hypertrophy[47]) to flourish. Therefore, understanding the role of the specific apoptotic proteases and their aberrant function in these highly proliferative cells is an important challenge. If cancer cell survival is indeed enhanced by non-selective caspase inhibition then therapeutic approaches will need to be selective for the target cell so that general cellular hypertrophy is not stimulated. For the inhibition of apoptosis to be therapeutically beneficial, rescued 'undead' cells must function in a normal manner. A recent report by Davidson and Steller[48] described residual cell function in *Drosophila* retinal degeneration mutants that have a condition similar to human retinitis pigmentosa. The cell survival protein p35 blocked apoptosis in the mutant photoreceptor cells and the flies retained more visual function than mutant, untreated flies. This model suggests that late-stage (mature) anti-apoptotic therapeutic strategies might be effective against chronic degenerative diseases.

There is a clear unmet medical need for the diseases discussed in this article, including OA and neurodegenerative diseases, and there is mounting evidence that targeting undesired apoptosis by inhibiting caspase activity might be beneficial. A key challenge will be determining which caspase is crucial for each disease and whether there is redundancy between caspase family members. This will help lead to the development of a wider range of small-molecule inhibitors that are selective for each caspase. The first clinical trials of apoptosis inhibitors will probably be in acute diseases (stroke, liver degeneration) because the therapeutic window is short and so global apoptosis inhibition is unlikely to be an issue. A more tissue-specific apoptosis inhibitor, in terms of activity against a specific caspase, limited tissue distribution or cell- or gene-targeted activity, will probably be required for chronic neurodegenerative disease or OA, which will ultimately make these more difficult targets.

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A discussion of the impact of company mergers
An overview of the eyeforpharma conference

Reviews **Library design for NMR-based screening**
by Christopher Lepre
Using model system genetics for drug-based targeting
by David Matthews and Jenny Kopczynski
Efflux transport systems for drugs at the blood–brain and blood–cerebrospinal fluid barrier (Part 1)
by Hiroyuki Kusuhara and Yuichi Sugiyama

Monitor New bioactive molecules and combinatorial chemistry