From bench to clinic with apoptosis-based therapeutic agents

Donald W. Nicholson

Merck Frosst Centre for Therapeutic Research, Merck Research Laboratories, PO 1005 Pointe Claire-Dorval, Quebec, Canada H9R 4P8
(e-mail: donald_nicholson@merck.com)

A retrospective look at the basis of human disease pathogenesis almost always reveals an apoptotic component that either contributes to disease progression or accounts for it. What makes this field particularly exciting is the breadth of therapeutic opportunities that are on offer. The pace of apoptosis research has raised expectations that therapeutics will follow soon. But many of the organizations that are best placed to take advantage of these discoveries consider the ability to modulate the life or death of a cell for the purpose of disease treatment as perhaps being 'too good to be true'. Nevertheless, practical therapeutics that modulate apoptosis will no doubt appear in the clinic or on the shelf in the next few years.

The human body is composed of approximately 10^{14} cells, each of which is capable of committing suicide by apoptosis. Not surprisingly (although just recently understood), this process has inherent weaknesses that, when compromised, can result in inappropriate cell death (either too little or too much) and disease pathogenesis. Other articles in this issue have highlighted the complex role that apoptosis has in the homeostasis of multicellular organisms; here I describe the immense potential for therapeutic use that exists in modulating apoptosis for the treatment of human diseases.

Technological power meets apoptosis

The springboard for many of these discoveries has been the genetic blueprint of the cell death pathway, which was originally defined in the nematode *Caenorhabditis elegans*[1]. Each of the central components of this pathway were found to have mammalian counterparts that often existed as multigene families. Their rapid discovery was empowered by the birth of computational biology (formerly termed 'bioinformatics'), which evolved alongside the advances made in genomics resources. For example, public-domain expressed sequence tag (EST) collections were used to identify key members of the Bcl-2, caspase, tumour necrosis factor (TNF) receptor and adapter molecule families — often simultaneously in multiple laboratories — and the now-completed human genome sequence will no doubt fill in the gaps. Understanding the function and relationship between these molecules was aided by an ever-evolving set of impressive cell biology tools, including interaction analysis with yeast two-hybrid systems, and knockout mice whose phenotype frequently clarified an otherwise complex biology. In addition, nearly 20 three-dimensional structures for biomolecules involved in apoptosis have been resolved using X-ray or nuclear magnetic resonance techniques, yielding a better understanding of their *modus operandi* and their functional interrelationships, as well as the design of specific inhibitors. The therapeutic landscape has also witnessed major changes. Antisense-based therapies have been made more viable by better oligonucleotide chemistry, resulting in increased metabolic stability, cell penetration and fewer side effects. Injectable recombinant biologicals are demonstrating clinical efficacy and are becoming more broadly accepted as legitimate therapeutic agents. Last but not least, combinatorial chemistry and rapid analogue synthesis — techniques by which large numbers of chemical permutations can be quickly assembled — have greatly accelerated the pace at which potential drug candidates can be generated. These remarkable accomplishments have positioned the apoptosis field for what is hoped will be a smooth transition from bench to clinic.

Opportunities and limitations

Even a quick glance at the molecular components of the cell death pathway (see the other articles in this issue) reveals many opportunities for apoptosis modulation. With this plethora of opportunities unfortunately comes a number of practical limitations, not the least of which is the practical issue that the cell death pathway as currently known contains very few conventional drug targets, such as enzymes and small-ligand receptors. Attention has therefore focused on other strategies to affect the proteinaceous components of the apoptotic pathway.

Modulating the expression of key molecular components of the cell death machinery is an attractive and obvious strategy. But whereas gene and antisense therapy seem the most viable approaches at present to alter gene expression, and antisense Bcl-2 shows promise in the treatment of cancer, these technologies are still in their infancy.

An alternative is to interfere with specific protein–protein interactions inside or outside the cell. But despite intensive research, small molecules that interfere with specific protein–protein interactions are almost unheard of because the surface areas between interacting polypeptides are large and difficult to disrupt. The 'critical interaction' strategy, where small-molecule inhibitors disrupt only key sites of binding between interacting polypeptides, is sound in principal but has been lacking in practice. Even so, small molecules with a high affinity for the binding cleft of the Bcl-2 homology (BH) domain BH3 on the surface of Bcl-2 seem to induce apoptosis in cultured cells[2].

For recombinant protein strategies, a different set of equally complex issues arise. For example, there is always the possibility that the cells of the immune system will develop autoantibodies against recombinant proteins and, furthermore, their use is mostly limited to extracellular targets because large proteins do not readily enter the cell.

Opportunities are scarce for the development of

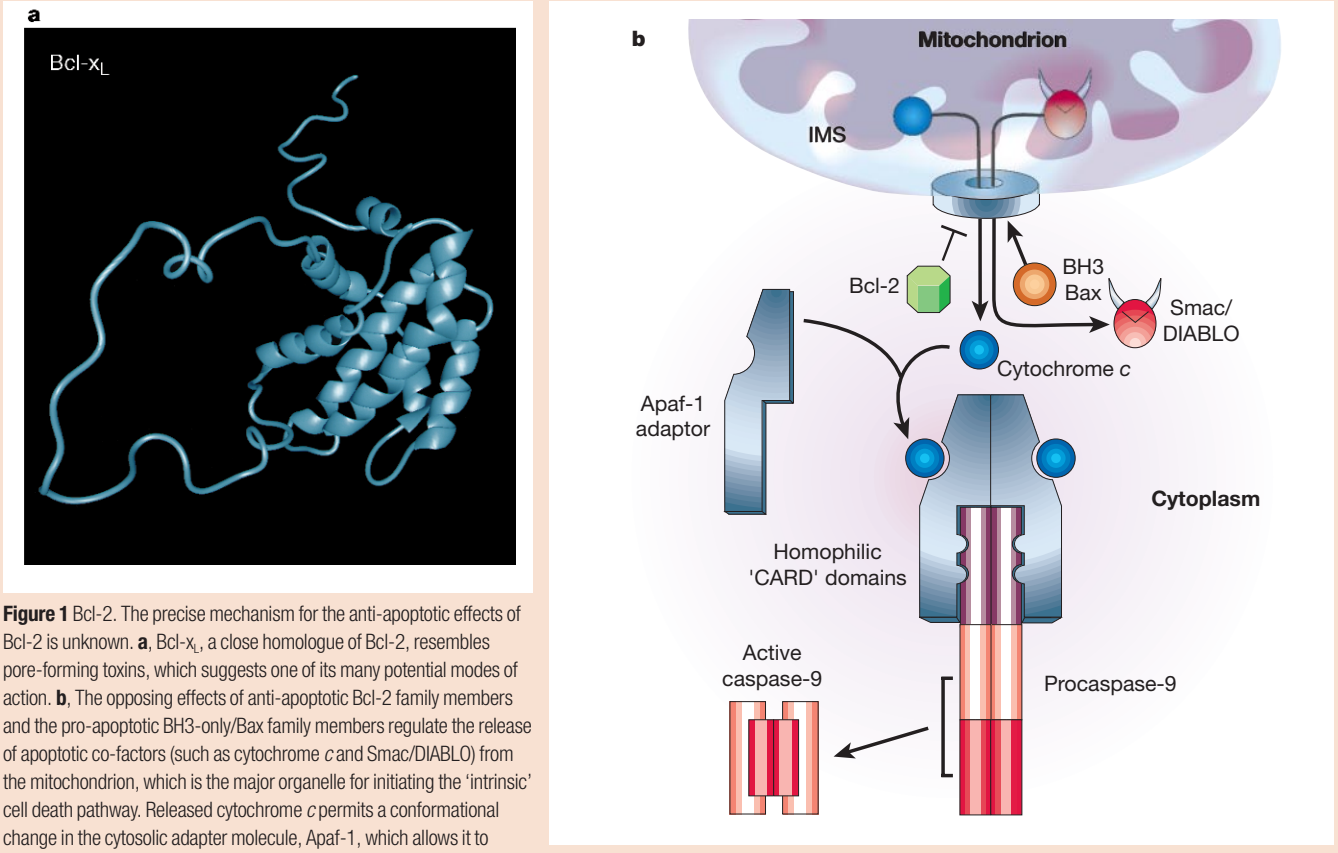


Figure 1 Bcl-2. The precise mechanism for the anti-apoptotic effects of Bcl-2 is unknown. **a**, Bcl-x_L, a close homologue of Bcl-2, resembles pore-forming toxins, which suggests one of its many potential modes of action. **b**, The opposing effects of anti-apoptotic Bcl-2 family members and the pro-apoptotic BH3-only/Bax family members regulate the release of apoptotic co-factors (such as cytochrome *c* and Smac/DIABLO) from the mitochondrion, which is the major organelle for initiating the 'intrinsic' cell death pathway. Released cytochrome *c* permits a conformational change in the cytosolic adapter molecule, Apaf-1, which allows it to recruit and oligomerize caspase-9 via homophilic caspase recruitment domains (CARDs). Caspase-9 becomes activated and launches the apoptotic pathway. Some evidence indicates that Bcl-2 might also sequester Apaf-1 and that it is released or displaced by the pro-apoptotic (BH3-only/Bax) family members. IMS, mitochondrial internenbrane space.

pharmaceutical therapeutics to modulate the apoptotic pathway; the exception being organic small-molecule inhibitors of caspase activity. But there is no precedent of human therapeutics that successfully target cysteine proteases owing to the difficulties in developing electrophiles that are specific enough that for them not to attack other biological nucleophiles.

On top of all these practical limitations come the theoretical ones. For example, can apoptosis be selectively modulated in one organ or cell type without adverse effects on other key systems? The redundancy that exists in several of the multigene families suggests that this might just be possible, but it has yet to be proven *in vivo*. Similarly, if cells are salvaged by inhibiting apoptosis, will they be functional? In all likelihood this will depend on the cell type, its context and the degree of cellular injury inflicted on it. Nevertheless, impressive advances have been made to modulate apoptosis *in vivo* and although modulating apoptosis will not be a panacea for all of humanity's ailments, the wealth of opportunities merit a persistent effort.

Apoptotic-modulating therapies

Apoptosis-modulating therapeutics are now finally in human clinical trials or are on the brink, having shown efficacy in preclinical animal models. This is significant progress, given that the field as a whole is only about a decade old. The following examples are three of the most advanced and promising opportunities which, it is hoped, will set the stage and establish precedents for many future therapeutics. Each targets a different biochemical component of the cell death pathway and, surprisingly, each represents a different therapeutic modality (for example, disruption of gene function with antisense oligonucleotides (Bcl-2), recombinant biologicals (TRAIL, for tumour necrosis factor-related apoptosis-inducing ligand) and classical organic pharmaceuticals (caspases)). Furthermore, these entities tackle the complex issues of how, on the one hand, to activate apoptosis selectively for the treatment of disorders where there is insufficient apoptosis, such as cancer (Bcl-2 and TRAIL), and how, on the other hand, to block cell death selectively for the treatment of diseases in which excessive apoptosis occurs and needs to be attenuated, such as in neurodegeneration (caspases).

Bcl-2 antisense

The Bcl-2 family of proteins are among the most studied molecules in the apoptotic pathway[3,4] (Fig. 1a). Although the precise mechanism by which they function as anti-apoptotic molecules remains unclear, the cellular stoichiometry of Bcl-2 family members compared with their pro-apoptotic ('Bax/BH3-only') homologues clearly defines the vulnerability of cells to most, but not all, death stimuli (Fig. 1b). Bcl-2 itself was first identified in B-cell lymphomas in which the genetic lesion was a translocation of the Bcl-2 gene to the control of the immunoglobulin promoter (t(14:18)). The resulting overexpression of Bcl-2 retards the normal course of apoptotic cell death that otherwise occurs to maintain B-cell homeostasis, resulting in B-cell accumulation and follicular lymphoma. This observation showed that cancers do not strictly arise from unrestrained cell proliferation, but could also be due to insufficient apoptotic turnover. It further suggested that a decrease in Bcl-2 levels or the inhibition of Bcl-2 activity might provoke apoptosis or at least sensitize cells to apoptotic death. This has been verified in a large number of studies *in vitro* and is consistent with the phenotype of both Bcl-2-null mice — which develop normally but eventually display marked lymphoid apoptosis, as well as melanocyte, neuronal and intestinal lesions and terminal kidney disease — and Bcl-2 transgenic animals, which accumulate mature B lymphocytes and recapitulate much of the human phenotype[5,6]. In addition to follicular lymphomas, Bcl-2 levels are elevated in a broad range of other human cancers, indicating that this molecule might have a role in raising the apoptotic threshold in a

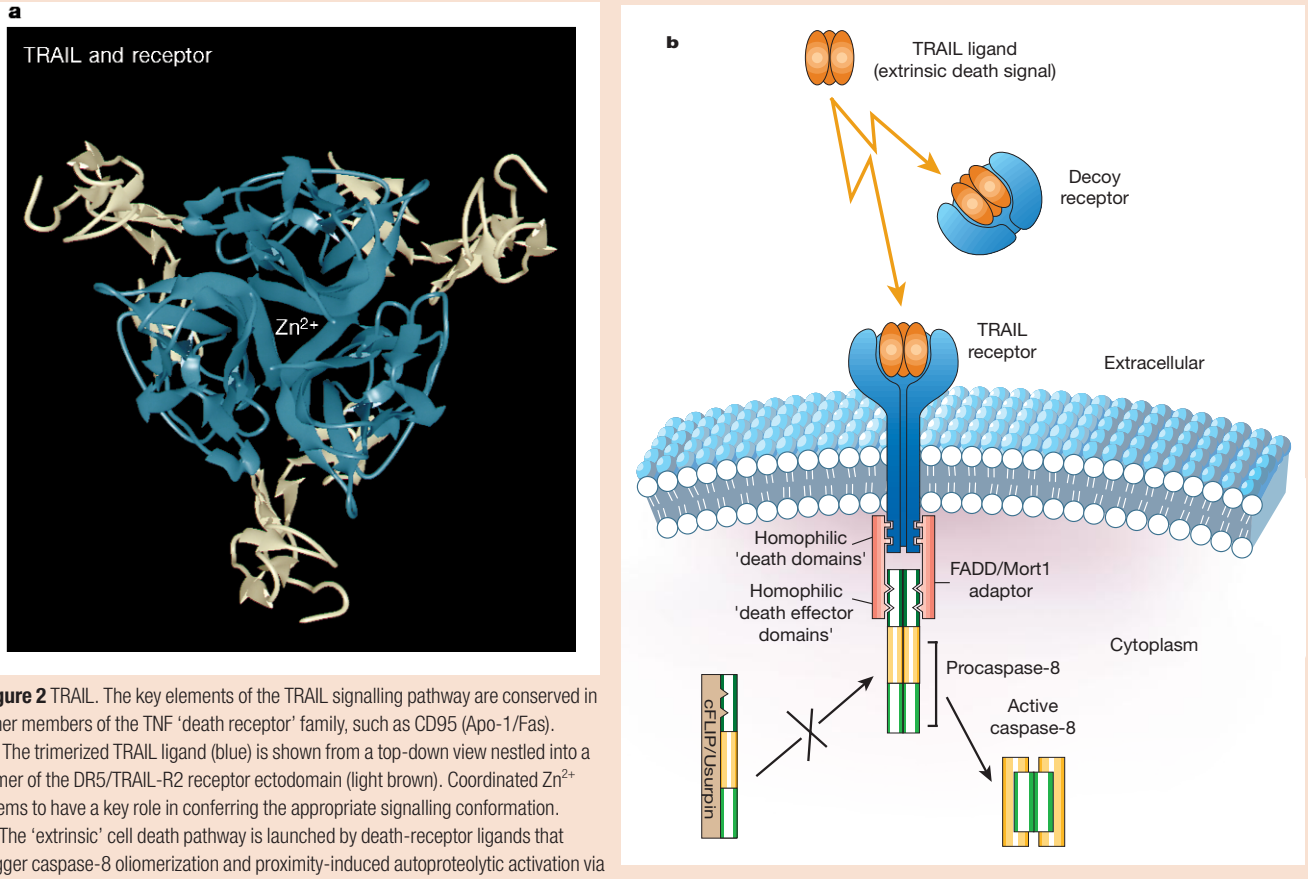


Figure 2 TRAIL. The key elements of the TRAIL signalling pathway are conserved in other members of the TNF 'death receptor' family, such as CD95 (Apo-1/Fas). **a**, The trimerized TRAIL ligand (blue) is shown from a top-down view nestled into a trimer of the DR5/TRAIL-R2 receptor ectodomain (light brown). Coordinated Zn^{2+} seems to have a key role in conferring the appropriate signalling conformation. **b**, The 'extrinsic' cell death pathway is launched by death-receptor ligands that trigger caspase-8 oliomerization and proximity-induced autoproteolytic activation via adapter molecules such as FADD/Mort1. Activation is regulated by decoy receptors, which preclude the binding of TRAIL to the functional receptor, and dominant-negative pseudo-caspases such as c-FLIP/Usurpin, which prevent the recruitment of the caspase-8 proenzyme into the receptor complex.

broad spectrum of cancerous disorders. This would bode well for a Bcl-2-directed therapeutic, although it is likely — given the size of this multigene family — that other Bcl-2 family members will be the dominant anti-apoptotic effector in some types of cancer.

In the absence of a clearly defined biochemical mechanism of action for this family of cell-death regulatory proteins (for which conventional inhibitors could therefore be developed), gene therapy and antisense approaches have become the logical alternative. This has come the furthest for the 18-mer all-phosphorothioate Bcl-2 antisense oligonucleotide, G-3139 (Genta). This molecule (5′-d(P-thio)TCT-CCC-AGC-GTG-CGC-CAT-3′) targets the first six codons of the human Bcl-2 open reading frame. The antisense binds to the Bcl-2 mRNA, thus precluding it from translation into Bcl-2 protein and targeting the message for degradation. Phosphorothioate bonds improve metabolic stability within the antisense oligonucleotide. This tips the balance between pro-apoptotic and anti-apoptotic family members in favour of pro-apoptotic members, resulting in apoptosis.

Preclinical data

In preclinical animal models, xenotransplantations of human tumours into mice with severe combined immunodeficiency (SCID) are markedly affected by continuous subcutaneous infusion of the Bcl-2 antisense G-3139 but not by the appropriate controls such as reverse, scrambled or mismatched oligonucleotides. For example, a marked decrease in tumour growth (more than 90%) of Merkel cell carcinomas (an aggressive neuroendocrine skin cancer with high metastatic potential) that were xenografted into SCID mice was observed after 28 days of treatment, and efficacy was superior to treatment with cisplatin[7]. Importantly, the apoptotic death of tumour cells in other models is enhanced by co-administration with standard chemotherapeutic agents, indicating, as might have been predicted, that lower Bcl-2 levels decrease the apoptotic threshold of these cells and make them chemosensitive (or at least reverse Bcl-2-mediated chemoresistance). For example, human melanomas grown in SCID mice were ablated by a combination of the Bcl-2 antisense G-3139 and dacarbazine (the only single agent currently approved by the US Food and Drug Administration (FDA) for treating metastatic melanoma)[8].

Clinical data

This has translated favourably into the clinic in phase I/IIa studies, which demonstrated that 43% of treated patients (6 of 14) showed clear anti-tumour responses associated with decreased Bcl-2 levels and increased apoptosis in melanoma biopsies[9]. G-3139 is currently in phase III clinical trials for malignant melanoma in the United States, for which it has been awarded fast-track priority by the FDA.

Positive results are also emerging for other human cancers, indicating that this approach might indeed have wide applicability. Encouraging results have been described for the treatment of non-Hodgkin's lymphoma. In a phase I study in relapsed patients with Bcl-2-positive lymphomas, disease stabilization was seen in 43% (9 of 21) and improvements were seen in 14% (3 of 21, including one complete responder)[10]. Phase II clinical studies are now underway to improve efficacy by combining the Bcl-2 antisense oligonucleotide with conventional chemotherapeutics, such as cyclophosphamide. Similarly, combination approaches using chemotherapeutics with antisense Bcl-2 are being examined in the clinic for relapsed small-cell lung carcinoma (paclitaxel), hormone-resistant metastatic prostate cancer (mitoxantrone), breast cancer (docetaxel), colorectal cancer (irinotecan) and relapsed acute leukaemia (fludaribine and cytosine arabinoside).

Bcl-2 is only one of the molecules within the apoptotic cell death pathway that could, in principle, be manipulated for therapeutic

gain. Others that might soon follow include Bcl-2-related proteins, such as Bcl-x_L. This protein can be modulated simultaneously with Bcl-2 by means of a bi-specific antisense oligonucleotide that targets a region of sequence identity between their respective messenger RNAs (which is not contained in the pro-apoptotic splice variant, Bcl-x_S)[11]. In principle, this strategy could further accentuate the chemosensitivity of cancer cells by targeting two key anti-apoptotic proteins instead of just one. Similarly, Bcl-x antisense oligonucleotides that can shift pre-mRNA splicing away from the formation of the anti-apoptotic variant Bcl-x_L to the pro-apoptotic variant Bcl-x_S can sensitize cells to apoptotic stimuli[12,13].

FLIP and survivin antisense

An alternative opportunity for antisense-based therapy may be provided by c-FLIP, which is a naturally occurring dominant-negative antagonist of death-receptor signal transduction (see Fig. 2b). Inhibition of c-FLIP might be useful in the treatment of carcinomas that have acquired resistance to CD95 (Apo-1/Fas)-dependent killing, as has been demonstrated in tissue culture with highly malignant adenocarcinomas originating from cholangiocytes[14].

Finally, one of the most prominent gene products associated with a wide variety of human cancers is survivin, a member of the inhibitor-of-apoptosis (IAP) family of proteins (see Fig. 3b). Antisense oligonucleotides directed against this molecule can induce spontaneous apoptosis in lung cancer cells, malignant melanomas and other cancer cell types. Overall, there are many opportunities to reset the apoptotic threshold of cancerous cells using antisense technology, particularly for acute treatment. In practice, their true utility will depend on many subtleties within the cell death pathway and how exactly it has been corrupted in specific disease pathologies.

Recombinant TRAIL (Apo-2L)

Cell death signals are commuted via two major biochemical routes in mammalian cells. The 'intrinsic' pathway, which responds to most pro-apoptotic signals, is modulated by the interplay between Bcl-2 and Bax/BH3-only family members and involves cues emanating largely via the mitochondrion (see Fig. 1b). The 'extrinsic' pathway, in contrast, is triggered by the ligation of 'death' receptors belonging to the TNF-receptor superfamily[15,16] (Fig. 2a). Activation of these receptors results in recruitment of caspase zymogens into oligomeric complexes and triggers their proteolytic activation[17,18] (Fig. 2b). 'Death receptors' all contain homophilic 'death domains' within their cytoplasmic extensions, which serve to recruit adapter proteins that in turn recruit procaspase-8. CD95, the prototypical death receptor, does so via FADD (for Fas-associated death domain protein), a bipartite adapter that directly bridges the CD95-ligand-ligated receptor with the caspase-8 proenzyme. TNF receptor 1 (which binds TNF-α) uses a similar mechanism except that an additional adapter, TRADD, bridges the receptor with FADD. The signal transduction pathways for the two pro-apoptotic TRAIL receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2/Apo-2/TRICK2/Killer) also seems to use both FADD and procaspase-8 in keeping with their molecular brethren[19–21].

Death-inducing TNF-receptor family members and their cognate ligands serve many crucial physiological functions including tumour killing, lymphocyte culling and establishing zones of immune privilege. It is therefore not surprising that elaborate molecular control mechanisms have evolved to fine-tune this system to prevent either inappropriate or inadequate killing, and that disease pathologies can evolve when these systems fail. Among these controls, for example, are polypeptide modulators that serve largely to deactivate death-receptor activity, including decoy receptors that lack a carboxy-terminal 'death domain' and can function as ligand sinks to prevent engagement of the apoptotic pathway (for example, DcR1/TRAIL-R3/TRID and DcR2/TRAIL-R4/TRUNDD for TRAIL). Alternatively, recruitment of caspase-8 into the successfully ligated death-receptor complex is prevented by c-FLIP, a catalytically incompetent pseudo-caspase that also desensitizes receptor-mediated apoptosis[22,23]. Together, these and other regulatory elements (such as the extent of nuclear factor (NF)-κB activation) determine the relative sensitivity or resistance of cells to ligand-provoked cell death, which clearly differ between normal and cancerous cells.

TRAIL therapeutic agent

The focus on TRAIL as a potential therapeutic agent became obvious as surprising differential sensitivity to TRAIL-stimulated apoptosis was observed between normal and cancerous cells[24,25]. Approximately 80% of human cancer cell lines, representing colon, lung, breast, skin, kidney and brain tumours, are sensitive at least to some extent to TRAIL, whereas most normal cell types are relatively resistant. However, the molecular basis for this differential sensitivity is not clear. TRAIL and its receptors are broadly expressed in most organ systems, thus indicating the importance of the presence or absence of regulatory molecules in determining apoptotic sensitivity. Initially it seemed that the presence of TRAIL decoy receptors in normal cells (but not cancer cells) might confer resistance to TRAIL and thus explain this phenomenon[26], but a correlation of TRAIL sensitivity in cancer cells with reduced decoy-receptor mRNA expression cannot be firmly established[27]. Other studies indicate that the subcellular distribution of the death and decoy receptors might account for the differential sensitivity of cells to TRAIL[28], or that c-FLIP levels dictate vulnerability to death receptor-mediated killing[29]. Regardless of the apparent confusion in being able to explain the molecular basis for TRAIL sensitivity in cancer cells compared with resistance in normal cells, recombinant TRAIL has appealing therapeutic potential for the treatment of a variety of human cancers. An important consideration that distinguishes this approach from targeting Bcl-2 is that death receptor-mediated apoptosis in itself is predicted to be independent of p53 status, which is corrupted in about 50% of all primary human tumours[30]. TRAIL-mediated apoptosis is also largely independent of Bcl-2 itself because it bypasses the 'intrinsic' cell death machinery in favour of a direct caspase activation pathway. In principle this should permit the treatment of chemoresistant cancers with TRAIL. Time will tell whether this is so. Recent evidence, for example, suggests that p53 is a transactivator of TRAIL receptor expression and thus might have a role in governing TRAIL sensitivity *in vivo*[31].

Preclinical data

Nevertheless, promising results have been obtained in preclinical animal models involving human tumour xenografts into SCID or nude mice[24,25,32]. These include mammary and colon carcinomas as well as intracranial gliomas. TRAIL was able to prevent the growth of evolving tumours immediately after xenotransplantation and, more importantly, decreased the size of established tumours in this model. For gliomas, injected recombinant TRAIL caused complete disease regression and entirely ablated tumour mass. For colon carcinomas the combination of subthreshold doses of recombinant TRAIL with existing chemotherapeutic agents resulted in a substantial positive interaction, completely eliminating the tumours in some animals. Taken together, these results look very promising, although in the absence of human clinical testing there is no certainty that the positive results achieved in animal models will translate to the complexities of the true human disease. One caveat, for example, is the potential adverse events that might accompany TRAIL injection into human patients. This has been largely addressed by preclinical safety studies in non-human primates (cynomolgus monkeys) that do not show adverse reactions to substantial doses of recombinant human TRAIL (10 mg kg^{-1} d^{-1} for 7 days)[24]. One of the appealing features of TRAIL as a pro-apoptotic receptor ligand is that it does not seem to have the extreme liver toxicity that has precluded the testing *in vivo* of related death-inducing ligands such as CD95 ligand and TNF-α, which both cause massive haemorrhagic necrosis of various tissues including the liver. Although this has not been observed with TRAIL in diverse species from rodents to primates, a recent study indicates that human hepatocytes in culture might in fact be responsive to TRAIL and would thus predict TRAIL toxicity in humans[33,34]. This is being cautiously evaluated by proponents of TRAIL therapy

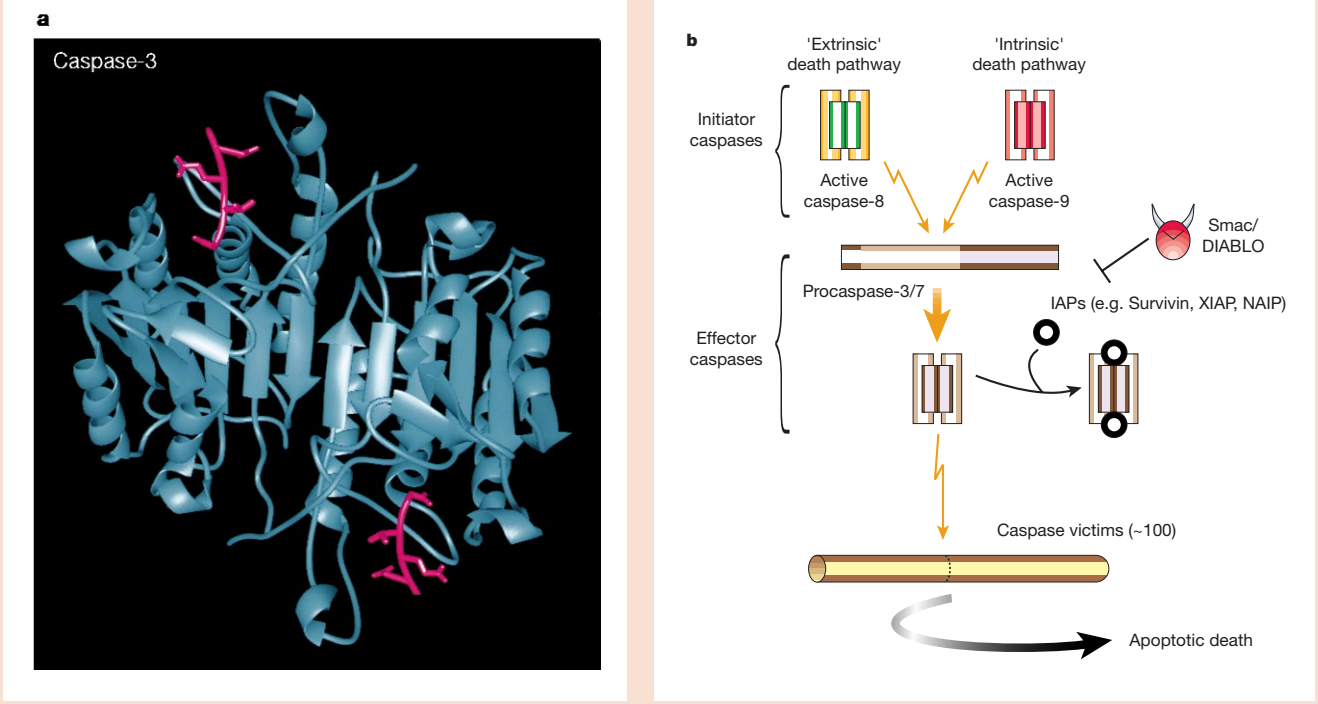


Figure 3 Caspases. Caspases are cysteinyl proteases that mediate most events that culminate in the apoptotic phenotype. Members of this protease family have common structural elements and are functionally redundant in some cases. **a**, The caspase-3 tetramer (blue) is composed of two large and two small subunits (derived from two proenzymes) generating two independent active sites that can be bound with inhibitors (magenta) to block catalytic activity. **b**, Apoptotic cell death is mediated by 'effector' caspases, such as caspase-3 and caspase-7, which cleave a limited subset of critical cellular polypeptides to manifest the apoptotic phenotype. These 'effector' caspases can be activated through proteolytic processing by upstream 'initiator' caspases such as caspase-8 and caspase-9. Two major activation pathways for these 'initiator' caspases are known, the 'intrinsic' pathway (see Fig. 1b) and the 'extrinsic' pathway (see Fig. 2b). Some degree of cross-talk between the two pathways seems to be mediated by tBid, a caspase-8-truncated form of the Bcl-2-related protein Bid (not shown). Active effector caspases are regulated by inhibitor-of-apoptosis (IAP) proteins, which block their catalytic activity and destine them for degradation. The mitochondrial cofactor protein Smac/DIABLO relieves this inhibition to facilitate full engagement of the proteolytic pathway.

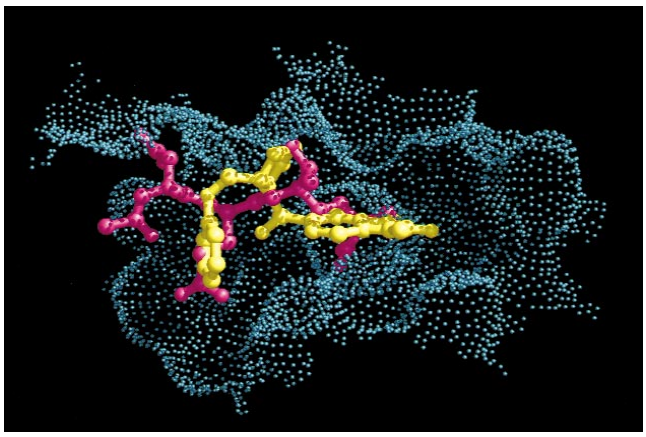
(Genentech/Immunex) under conditions that would be representative of those planned for the clinic. It seems, for example, that not all recombinant TRAIL ligands are equal, as is predicted by the unexpected importance of Zn^{2+} ions in coordinating the trimeric organization of the functional ligand[35,36]. Similarly, appropriate therapeutic windows need to be established, and all potential adverse events predicted by preclinical studies will require careful monitoring once TRAIL therapy is brought to the clinic. However, at the same time the balance of pros and cons needs to be considered because the benefits for cancer patients could potentially outweigh the disadvantages by a wide margin.

Caspases as therapeutic targets

Every mammalian cell contains a compliment of different caspase proteases that suitably equip it for apoptotic death (Fig. 3a). Although there is emerging evidence that sustained pro-apoptotic stress results in the transcriptional upregulation of these proteases, most cell deaths are dependent on the pre-existing caspase armament within each cell. In this way, cells can engage the cell death pathway at will without requiring elaborate biochemical procedures (for example, transcription and translation) that might be subject to interference (for example, by metabolic deficiency or viral pathogens). That cells do not spontaneously commit suicide is because these proteases are dormant when in their unprocessed proenzyme state. Caspase catalytic activity is associated with proteolytic maturation, which is launched through either the 'extrinsic' or 'intrinsic' pathways (see Figs 1b, 2b). However, as might be expected, this poised apoptotic system sometimes misfires, leading to premature cell death. This seems to happen often after acute cellular injury but is also prevalent in chronic disorders where the pro-apoptotic stimulus is obscure. In these cases, caspase inhibition has shown extraordinary promise in multiple disease models, particularly for acute indications.

Caspases were probably one of the first obvious therapeutic targets for modulating apoptosis and they remain the most viable approach to blocking apoptotic cell death as opposed to the death-inducing capacities of the preceding examples, Bcl-2 antisense and TRAIL. In fact, caspase inhibitor programmes were well underway in pharmaceutical companies before the discovery that these proteases had a central role in apoptosis. Their target was the interleukin-1β-converting enzyme (ICE, now designated caspase-1 (ref. 37)), a novel cysteine protease that is responsible for the proteolytic maturation and concomitant activation of pro-inflammatory cytokines, including pro-interleukin-1β and IGIF/interleukin-18 (refs 38, 39). Soon after the discovery that one of the gene products that was essential for apoptosis in *C. elegans* was an ICE-related protease (CED-3)[40], the role of caspases in mammalian apoptosis was rapidly unveiled with the use of the knowledge accumulated with ICE as a template. For example, inhibitors that were designed to target one of these proteases (caspase-3) prevented apoptosis as gene disruption did for neurons in caspase-3-null mice[41,42]. So far, 14 mammalian caspases have been identified, of which 12 human orthologues are known[43,44]. Functionally, these proteases divide into two major subfamilies: those related to ICE (caspase-1, caspase-4 and caspase-5) function predominantly, if not exclusively, in cytokine maturation; the remainder perform an elaborated CED-3 function to mediate apoptosis. Among these latter CED-3-like mammalian caspases, a further subdivision exists: 'initiator' caspases (for example, caspase-8, caspase-9 and caspase-10) respond to pro-apoptotic stimuli and subsequently catalyse the activation of more abundant, and catalytically robust 'effector' caspases (for example caspase-3 and caspase-7) that largely perform the proteolytic cleavage events necessary to mediate the apoptotic phenotype (Fig. 3b). This division of duties is consistent with the proteolytic specificity of individual

Box 1
Caspase inhibitor design



Caspase inhibitor design has taken advantage of key features found within the active-site substrate-binding cleft as revealed by X-ray crystallography (the active site 'bite' of caspase-3 is shown in the figure opposite with overlayed peptoid (magenta) and isatin (yellow) inhibitors). It was recognized early on that extremely potent inhibitors for these enzymes could be made by coupling an appropriate P_1-Asp tetrapeptide (tetrapeptides are sufficient for specific recognition by these proteases and Asp in the P_1 position is a near absolute requirement) with an appropriate C-terminal electrophile, such as an aldehyde or ketone, to attack the thiol side chain of the active-site cysteine residue (at the right side of the magenta inhibitor). From this, inhibitors have evolved along at least two routes to circumvent the disastrously poor cell permeability that preclude these molecules from utility except *in vitro*. Peptoid inhibitors retain the physical characteristics of the original substrate counterpart, including high potency and specificity, but can bypass many of the liabilities of their peptide predecessors. Non-peptide inhibitors, such as isatins[63], do not contain peptide equivalents (not even the Asp carboxylate or a P_1 equivalent) but their highly electophilic nature and metabolic instability place major limitations on their current utility. Overall, major gains have been made in the cell permeability of these next-generation peptoid and isatin inhibitors, making them 100–1000-fold more potent than z-VAD-like inhibitors in cell-based apoptosis assays and yielding substantial improvements *in vivo* as a consequence. So far, isatins are effective only against the effector caspases (caspase-3 and caspase-7).

caspase family members[45] and indicates that the functional redundancies built into each of the three caspase subgroups, coupled with the cell-type specificity that has evolved in complex multicellular organisms, might permit the specific targeting of individual caspases for therapeutic purposes.

Caspase inhibitors

Although a caspase inhibitor has yet to reach the clinic for the inhibition of apoptosis (ICE inhibitors have done so recently for the treatment of rheumatoid arthritis), preclinical studies are compelling, and the plethora of diseases in which they might have efficacy makes their therapeutic potential enormous. Therapeutics are being developed for various caspase family members, with the most attention being paid to caspase-3 as a major contributor to the apoptotic machinery in many cell types.

With few exceptions, all of the proof-of-concept preclinical studies with caspase inhibitors in animal models of human diseases have been performed with active-site mimetic peptide ketones (for example, benzyloxycarbonyl (z)-VAD-fluoromethylketone (fmk), z-YVAD-fmk/chloromethylketone (cmk), z-DEVD-fmk/cmk and z-D-cmk)[46]. These molecules are all relatively non-selective caspase inhibitors and although they are not appropriate tools for dissecting out the contributions made by individual caspase family members to the apoptotic response that occurs in disease models, they have provided extremely valuable preclinical insight into the potential that caspase inhibition might eventually have in humans. For example, in at least five different models of ischaemia–reperfusion injury (liver, cardiac, renal, intestinal and cerebral), caspase inhibition has shown remarkable efficacy[47–51]. In addition to decreased apoptosis, caspase inhibition improved survival (for example threefold in liver ischaemia), decreased infarct volumes (by 50% in both cardiac and focal cerebral ischaemia) and, ultimately, markedly improved organ function (for example renal function and neurodeficit in models of kidney and focal cerebral ischaemia, respectively). These findings partly address the critical question of whether cells that are saved by anti-apoptotic therapies retain function. Importantly, many of these inhibitor studies (for example, cardiac, renal and cerebral ischaemia) demonstrated that caspase inhibitors that were administered after the ischaemic insult, and coincident with reperfusion, retained their efficacy owing to the natural delay in the apoptotic response, an important practical issue that distinguishes this therapeutic strategy from many that have preceded it. Taken together, these studies demonstrate that caspase inhibition should have protective value in organ transplantation, cardiac arrest and stroke.

In addition, caspase inhibition has shown promise in preclinical animal models of other disorders of the by decreasing apoptotic cell death, including traumatic brain injury, status epilepticus, amyotrophic lateral sclerosis (ALS) and Parkinson's disease[52–55]. In traumatic injury, improved neurological recovery accompanied apoptosis inhibition and in a mutant-superoxide-dismutase-1 model for ALS there was a significant delay in disease onset and mortality. Caspase inhibitors have also shown efficacy in animal models of infectious diseases, including bacterial meningitis and sepsis[56–58]. In the former, inhibitors blocked the apoptotic loss of hippocampal neurons, decreased spongiform encephalopathy and markedly improved behavioural scores. In the latter, septic animals more than doubled their survival probability. It therefore seems likely that the acute use of caspase inhibitors will have measurable clinical benefits in addition to a general inhibition of apoptosis. For chronic use, the issues will be much more complex. Although somewhat controversial, it seems that the neuronal loss in chronic neurodegenerative disorders is apoptotic; moreover, caspases might themselves have a role in exacerbating disease pathogenesis, as has been suggested for Huntington's and Alzheimer's diseases[59–61]. Thus, caspase inhibition shows immense preclinical promise, which now requires usable therapeutics that can be taken forward into human clinical trials.

Potent specific caspase inhibitors that are able to cross the membrane of cells are being developed by various pharmaceutical companies (Merck, SmithKline Beecham, BASF, Idun/Novartis and Vertex) (see Box 1), although very little is known about their preclinical status or clinical agendas. Regardless of this apparent information blackout, the remarkable efficacy that 'old generation' caspase inhibitors have in animal models indicates that the emerging crop should indeed be impressive and will no doubt be viable in the clinic. It is also clear that the first entries will probably target acute apoptotic injuries such as cerebral stroke, trauma-induced neurodegeneration, cardiac ischaemia–reperfusion injury, transplantation, acute liver injury and sepsis. Finally, a novel gene-therapy approach has been suggested for the treatment of HIV-mediated AIDS[62]. In this approach, the maturation sites within the caspase-3 polypeptide are replaced with HIV protease recognition motifs. Cells that are infected by the virus undergo apoptosis when the protease clips the engineered caspase, thereby selectively deleting infected cells.

Whether this strategy will add value to the current armament of AIDS therapies is unknown, but it is representative of the many prospects for caspase-modulating therapeutics.

Future prospects

Is the ability to modulate the life or death of a cell — whether it be by using small organic molecules, biological agents, antisense oligonucleotides or gene therapy — indeed 'too good to be true'? Although these are still early days, it is difficult not to get excited about the significant advances that have already been made. The true therapeutic benefit of apoptosis modulation for the treatment of some of the most devastating human diseases remains to be discovered. As the biochemical and molecular complexities of the apoptotic pathway are elucidated, new therapeutic strategies will no doubt arise and more obvious paths forwards for the modulation of chronic apoptosis will appear. Apoptosis-based therapeutics are clearly within our grasp, but the emerging crop are likely to be just the tip of the iceberg. □

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