

New Approaches and Therapeutics Targeting Apoptosis in Disease

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Abstract—Apoptosis, the major form of cellular suicide, is central to various physiological processes and the maintenance of homeostasis in multicellular organisms. Presumably, even more important is a causative or contributing role of apoptosis to various human diseases. These include situations with unwanted cell accumulation (cancer) and failure to eradicate aberrant cells (autoimmune diseases) or disorders with an inappropriate loss of cells (heart failure, stroke, AIDS, neurodegenerative diseases, and liver injury). The past decade has witnessed a tremendous progress in the knowledge of the molecular mechanisms that regulate apoptosis and the mediators that either prevent or trigger cell death. Consequently, apoptosis regulators have emerged as key targets for the design of therapeutic strategies aimed at modulat-

ing cellular life-and-death decisions. Numerous novel approaches are currently being followed employing gene therapy and antisense strategies, recombinant biologics, or classical organic and combinatorial chemistry to target specific apoptotic regulators. Convincing proof-of-principle evidence obtained in several animal models confirms the validity of strategies targeting apoptosis and revealed an enormous potential for therapeutic intervention in a variety of illnesses. Although numerous apoptotic drugs are currently being developed, several therapeutics have progressed to clinical testing or are already approved and marketed. Here we review the recent progress of apoptosis-based therapies and survey some highlights in a very promising field of drug development.

I. Introduction

The regulation of cell number is a crucial property of multicellular organisms. The human body is composed of approximately 10^{14} cells. Every day billions of cells die an altruistic death to secure the functionality of the whole organism. Cell death like this is physiological and

has been called programmed or apoptotic cell death. Apoptosis is essential for the regulation of development, the generation of an immune system, and later for maintenance of homeostasis in adult tissues, being a consequence of a balanced cell death versus cell proliferation ratio. Equally, or perhaps even more important, is the role of apoptosis as a cause of disease. Dysregulation of apoptosis can result in severe pathological syndromes. Acute pathologies such as stroke, heart attack, or liver failure are associated with life-threatening sudden death of whole tissue areas or organs, whereas certain neurodegenerative syndromes are the result of a more slowly progressive neuronal cell death. Conversely, an

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Article, publication date, and citation information can be found at <http://pharmrev.aspetjournals.org>.

doi:10.1124/pr.57.2.6.

inappropriately low rate of apoptosis may promote survival and accumulation of abnormal cells that can give rise to tumor formation or autoimmune diseases. A reasonable estimate is that either too little or too much cell death contributes to approximately half of the medical illnesses, for many of which an adequate therapy is lacking.

Apoptosis represents a universal and exquisitely efficient cellular suicide pathway. As our understanding of its importance in normal development has deepened, most of the key players in cellular apoptosis regulation have been identified and can be targeted by therapeutic strategies. These include death receptors triggering apoptosis from the cell surface, Bcl-2 proteins as the gatekeepers of the mitochondrial pathway, caspases as the executioner enzymes, endogenous caspase inhibitors, and various transcriptional regulators. The identification of the major regulators of apoptosis has boosted an intense research in developing therapeutic approaches to intervene with cell death, either in a pro- or antiapoptotic direction. For instance, extracellular death signals sensed by death receptors can be mimicked by recombinant ligands or agonistic antibodies. Caspases can be either inhibited or activated by small molecule drugs. Undesirable proteins, like prosurvival factors in cancer cells, can be specifically down-regulated by antisense or other strategies. Finally, important protein-protein interactions can be interfered with or mimicked by peptides or organic compounds.

During the last decade, substantial advances have been achieved in the field of apoptosis-based therapeutics. First-generation attempts mainly used inefficient gene delivery approaches, recombinant proteins and peptides, or relatively nonselective drugs that were of limited use for clinical application owing to their inherent lack of stability, specificity, limited solubility, and poor cell permeability. Nevertheless, these preclinical experiments performed in cell culture and animal models provided the important proof-of-principle evidence that targeting of apoptosis is a valid strategy for a large number of diseases.

Currently, a great deal of effort is being aimed at improving the prototypic drugs and at replacing them by small molecule organic compounds, which could set the stage for future therapeutics. Oligonucleotide chemistry has expanded the potential use of antisense constructs by enhancing their stability and lowering associated toxic side effects. Furthermore, advances in combinatorial chemistry led to the rapid assembly of chemical libraries containing vast numbers of drug derivatives. Combined with innovative screening techniques, structural biology, and bioinformatics, this has greatly accelerated the development of lead compounds that could progress into clinically applicable drugs. The challenge remains to incorporate the many promising preclinical findings into actual therapeutic practice. Apoptosis-targeting therapies are now advancing from preclinical

trials to actual application. This is a significant progress, given that the field as a whole is only about 15 years old. Without a doubt, many practical therapeutics that modulate apoptosis will appear in the near future.

II. The Apoptotic Machinery

Apoptosis results from a collapse of cellular infrastructure through internal proteolytic digestion, which leads to cytoskeletal disintegration, metabolic derangement, and genomic fragmentation. Members of the caspase family of proteinases form the engine of apoptosis and are involved in initiation, execution, and regulatory phases of the pathway. Based on the order in cell death pathways, caspases are divided into major groups (Fischer et al., 2003; Fuentes-Prior and Salvesen, 2004). The subset of caspases that cleave selected substrates to produce the typical alteration changes associated with apoptosis are known as executioner caspases, which in mammals are caspases-3, -6, and -7. Executioner caspases are activated by apical initiator caspases, including caspase-8, -9, and -10 (Fig. 1).

Among the molecules that regulate apoptosis are several members of the inhibitor of apoptosis (IAP¹) family, which prevent cell death by acting as endogenous suppressors of caspase activity (Salvesen and Duckett, 2002). The IAPs contain one to three copies of a domain known as the baculoviral IAP repeat (BIR), which are involved in the inhibition of caspases. The most thoroughly characterized member of the IAP family is X-chromosome-linked inhibitor of apoptosis (XIAP). Structural studies have revealed that the BIR3 domain of

¹Abbreviations: IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; XIAP, X-chromosome-linked IAP; SMAC, second mitochondria-derived activator of caspases; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DD, death domain; DISC, death-inducing signaling complex; NF- κ B, nuclear factor- κ B; MS, multiple sclerosis; GVHD, graft-versus-host disease; TEN, toxic epidermal necrolysis; CD95L, CD95 ligand; ICE, interleukin-1 β -converting enzyme; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; AQZs, anilinoquinazolines; fmk, fluoromethylketone; SAR, structure activity relationship; FADD, Fas-associated death domain; HTS, high-throughput screening; M-826, 3-(((2S)-2-[5-tert-butyl-3-(((4-methyl-1,2,5-oxadiazol-3-yl)methyl)amino)-2-oxopyrazin-1(2H)-yl]butanoyl)amino)-5-[hexyl(methyl)amino]-4-oxopentanoic acid; CDP571, humicade; MX1122, 2,4-di-Cl-Cbz-Val-Asp-fmk; MX1013, z-VD-fmk; VX-740, pralnacasan; IDN-6556, 3-[2-(2-tert-butyl-phenylaminoxyalyl)-amino]propionylamino-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid; HIV, human immunodeficiency virus; FDA, Food and Drug Administration; NCI, National Cancer Institute; PETCM, α -(trichloromethyl)-4-pyridineethanol; IBM, IAP-binding motif; cIAP, cellular IAP; ML-IAP, melanoma IAP; BH, Bcl-2 homology domain; SAHBs, stabilized α -helix of Bcl-2 domains; TC-A, tetrocarcin A; BH3Is, BH3 inhibitors; MMP, mitochondrial membrane permeabilization; PBR, peripheral benzodiazepine receptor; PK11195, 1-(2-chloro-phenyl)-N-methyl-N-(1-methylpropyl)-1-isoquinoline carboxamide; Ro5-4864, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine-2-one; wt-p53, wild-type p53; Ad-p53, wt-p53-expressing adenovirus; Mdm2, murine double minute 2; HSP, heat shock protein; 17-AAG, 17-allylamino-17-demethoxy derivative of geldanamycin; mAb, monoclonal antibody; Ant, Antennapedia; ANT, adenine nucleotide translocase.

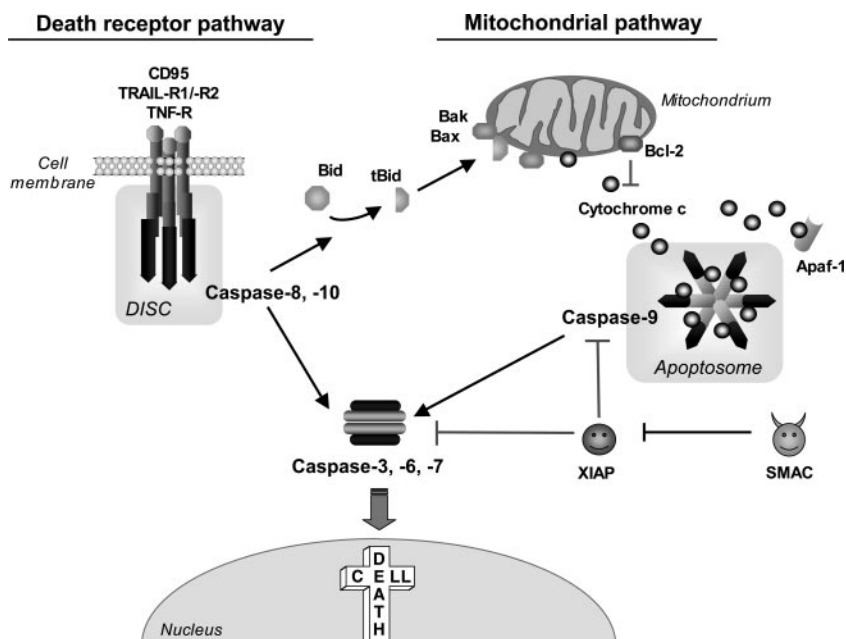


FIG. 1. Signal transduction of apoptosis. Two major pathways of apoptosis exist in mammalian cells. Left, the extrinsic cell death pathway is mediated by a subgroup of the TNF receptor superfamily called the death receptors (CD95, TRAIL-R1/2, and TNF-R1). Receptor-mediated cell death is initiated by the recruitment of adapter proteins, like FADD, via the DD, which then bind to the death effector domain-containing caspase-8 or -10. Formation of this DISC results in the activation of caspase-8, which then directly cleaves and activates caspase-3, -6, or -7, the executioner enzymes of apoptosis. Right, in the mitochondrial or intrinsic pathway, which is initiated by multiple forms of cellular stress, proapoptotic Bcl-2 family members Bax and Bak translocate to the mitochondria. The BH3-only protein Bid activates Bax and Bak to mediate the release of cytochrome *c* in the cytosol. This triggers the assembly of the apoptosome (Apaf-1 and caspase-9) and subsequent activation of caspase-3 and cell death. IAPs bind directly to caspases and inhibit their enzymatic activity. The inhibitory function of IAPs is countered by the SMAC.

XIAP is responsible for inhibition of caspase-9 and that a region adjacent to BIR2 is the major determinant for inhibition of caspase-3 and -7.

Despite overlap of both function and substrate specificity among the many caspases, a hierarchical cascade of activation exists. By examining the patterns of caspase activation following many different apoptotic stimuli, at least two distinct pathways have emerged (Schulze-Osthoff et al., 1998). Death signals originating from cellular stress, including radiation and chemotherapeutic drugs, activate an intrinsic apoptotic program that is mediated largely by the mitochondria. Mitochondrial release of cytochrome *c* into the cytoplasm induces the formation of an oligomeric complex containing cytochrome *c* and Apaf-1. This complex, called the apoptosome, supports the catalytic activation of caspase-9, which further cleaves and activates the effector caspase-3 resulting in the subsequent degradation of cellular death substrates. In addition to cytochrome *c*, the mitochondrially localized proteins SMAC/DIABLO and Omi/HtrA2 are also released into the cytoplasm. Once released, these two proteins bind to XIAP in a manner similar to caspases and thereby promote apoptosis by neutralizing the caspase-inhibitory function of XIAP.

In contrast to the intrinsic pathway, death receptor stimulation activates an extrinsic apoptotic program that often requires no mitochondrial involvement. Death receptors form a subgroup of the tumor necrosis factor

(TNF) receptor superfamily that includes TNF-R1, CD95, and receptors binding to the TNF-related apoptosis-inducing ligand (TRAIL). All death receptors are characterized by an intracellular motif called the death domain (DD). Upon ligand binding, the death receptors interact via their DD with the DD of adapter proteins such as FADD. These adapter proteins also contain a second protein interaction motif, the death effector domain, that facilitates their binding to the initiator caspase-8 (or its relative caspase-10) to form the death-inducing signaling complex (DISC). DISC formation activates caspase-8 through a proximity-induced dimerization mechanism. Subsequently, caspase-8 cleaves and activates caspase-3, resulting in further cleavage of cellular targets. In some cell types, however, the linear progression from DISC formation to caspase-3 activation is insufficient to complete the cell death program, and amplification of the death receptor stimulus by engagement of the mitochondrially mediated cell death pathway is required. One known mechanism by which the mitochondrial pathway is engaged following death receptor activation is through caspase-8-mediated cleavage of Bid, a proapoptotic member of the Bcl-2 family. Once cleaved, truncated Bid translocates to the mitochondria, where it can induce the release of cytochrome *c*, SMAC, and Omi/HtrA2. In this manner, death receptor signals may be amplified through formation and activation of the apoptosome, which can increase the activation of effector caspases.

III. Death Receptors on the Trail to Tumor-Selective Cell Death

The death receptor pathway is initiated by the interaction of cell surface death receptors with their cognate death ligands, such as TNF- α , CD95L, or TRAIL. This event leads to instant intracellular formation of the DISC by the recruitment of the adapter protein FADD and the initiator caspase-8 and -10. Following activation at the receptor, caspase-8 and -10 then start off the caspase cascade by cleavage-mediated activation of downstream effector caspases, such as caspase-3, -6, and -7, culminating in the demise of so-called type I cells. In most cells, however, only low amounts of initiator caspases are activated at the DISC, which is insufficient for cell death. In those so-called type II cells, the extrinsic receptor pathway must be amplified by the intrinsic mitochondrial apoptotic pathway through the caspase-8-mediated cleavage of Bid, a proapoptotic Bcl-2 family protein, which subsequently initiates the release of mitochondrial proapoptotic mediators. The resulting formation and activation of the apoptosome then executes cell death via caspase activation (Los et al., 1999; Hengartner, 2000). Interestingly, for certain receptors the composition of the DISC seems to differ from the classical CD95 DISC (Micheau and Tschopp, 2003). For instance, signaling through TNF-R1 appears to proceed via two sequential signaling complexes. Following TNF stimulation, the first complex is formed at the cell membrane, which contains TNF-R1, the adapter protein TRADD, and components of the transcription factor NF- κ B pathway. Upon dissociation of TNF-R1, a second different complex is formed in the cytosol, which recruits the apoptotic mediators FADD and caspase-8. The formation of the two receptosome complexes, one mediating NF- κ B activation and the other inducing apoptosis, might explain the finding that TNF can induce both survival and cell death, depending on the cellular context.

The impressive apoptosis-inducing properties of death receptors promoted their application in experimental cancer therapy (Daniel et al., 2001). In the early nineties, experiments with agonistic human CD95 antibody showed very promising results. A single injection of an agonistic anti-CD95 antibody could mediate long-term eradication of xenotransplanted human tumors in mice. However, it soon became clear that such antibodies did not cross-react with the murine CD95 receptor. Thus, the considerable toxicity of CD95 triggering on nonmalignant tumor cells was overlooked but became evident when a murine-specific anti-CD95 antibody was injected into mice; these animals rapidly succumbed due to the massive liver toxicity caused by induction of apoptosis in hepatocytes. A similar severe hepatotoxicity has been demonstrated for CD95 ligand and TNF. These findings therefore raised a general skepticism about the feasibility

of cancer therapy involving death ligands without accompanying severe side effects.

New optimism, however, came with the discovery of a novel death ligand TRAIL, which seemed to be most promising for cancer therapy because it specifically killed tumor cells while leaving normal tissues unharmed. Administration of soluble TRAIL demonstrated specific tumoricidal activity against a variety of cancer cell lines (Walczak et al., 1999). Injection of TRAIL into SCID or athymic mice challenged with human mammary carcinoma, glioma, or cholangiocarcinoma induced tumor cell apoptosis, suppressed tumor growth, and moreover, improved survival substantially (Roth et al., 1999; Walczak et al., 1999). TRAIL was not only able to prevent growth of freshly xenotransplanted tumors, but, more importantly, could even eradicate established tumors. Human U87 gliomas grown in athymic mice showed complete regression and ablation of tumor mass upon TRAIL treatment. A combination treatment of low doses of TRAIL with chemotherapeutic agents could also eliminate colon carcinomas in mouse models. Despite an *in vitro* sensitivity of normal human astrocytes, administration of up to 1 mg of TRAIL had no adverse effects on the brain or other tissues of mice. Furthermore, preclinical safety studies in nonhuman primates demonstrated no toxicity of TRAIL treatment, even when doses up to 10 mg/kg/day were applied (Ashkenazi et al., 1999).

Unexpected concerns regarding the safety of applying TRAIL to humans arose when polyhistidine-tagged recombinant TRAIL was found to induce apoptosis of cultured human primary hepatocytes (Jo et al., 2000). Similarly, death of human brain cells was inflicted by trimerized Flag-tagged TRAIL (Nitsch et al., 2000). To date, the cause of these findings seems to be clarified as being due to the preparation and tag version of TRAIL, which alters its biochemical properties. Unlike with the polyhistidine-tagged version, hepatotoxicity was not induced with a nontagged soluble form of TRAIL. Furthermore, intravenous administration of nontagged TRAIL was tolerated in cynomolgus monkeys and chimpanzees with no harmful symptoms in either laboratory parameters or organ histology (Lawrence et al., 2001).

Most drugs and radiation therapy used in the treatment of malignancies lead to apoptosis primarily by engagement of the mitochondrial apoptosis machinery. Upon treatment, tumors often become refractory to conventional antitumor therapy, and in many cases, overexpression of antiapoptotic Bcl-2 members is the reason for the chemotherapy resistance. TRAIL-mediated apoptosis, in contrast, can bypass Bcl-2-like proteins by directly activating the caspase cascade. Indeed, combination treatment of TRAIL with conventional chemotherapeutic agents or ionizing radiation mostly results in strongly enhanced cytotoxic effects. Chemo- and radiotherapy can even sensitize previously nonresponding tumor cells to TRAIL. The observed synergistic effects

are not only due to the activation of both the intrinsic and the extrinsic apoptotic pathways. Other cellular mechanisms potentiate this effect, such as transcriptional induction of the TRAIL receptors R1 and R2, a reduced expression of antiapoptotic proteins such as Bcl-2 or Bcl-x_L, and an up-regulation of proapoptotic molecules such as caspase-8 and FADD (Belka et al., 2001; Held and Schulze-Osthoff, 2001).

Currently, there are several clinical and preclinical studies underway investigating the therapeutic potential and safety of TRAIL agonists as anticancer agents (Table 1). Genentech (South San Francisco, CA) and Amgen (Thousand Oaks, CA) are starting clinical phase 1 trials with soluble TRAIL. An agonistic TRAIL-R2 antibody (TRA-8) was generated by Sankyo Co., Ltd. (Tokyo, Japan) and proved to be cytotoxic against primary hepatocellular carcinoma cells without inducing cell death in normal hepatocytes (Ichikawa et al., 2001). It was suggested that the differential sensitivity of tumor and normal cells to this antibody was due to the low expression of the TRAIL-R2 in untransformed cells compared with elevated levels in cancer cells. Already now, several antibodies against TRAIL-R1 and R2, respectively, are investigated in clinical trials. Human Genome Sciences Inc. has recently enrolled patients in phase 2 clinical trials with the TRAIL-R1-specific human monoclonal antibody HGS-ETR1 for the treatment of nonsmall cell lung cancer, colorectal carcinoma, and non-Hodgkin lymphoma. So far, patients revealed no hematological or hepatic toxicity of the HGS-ETR1 antibody with doses up to 10 mg/kg b.wt. Six of 57 patients enrolled in a preliminary trial even reached stable disease.

Due to the superior tumor killing activity of TRAIL agonists and the toxic side effects associated with systemic treatment of other death ligands, TNF had temporarily sunken into oblivion. Nevertheless, besides its direct antitumor effects, TNF has some features that might be exploited for tumor therapy. TNF namely destroys tumor-supplying blood vessels by apoptosis and, furthermore, improves vascular permeability to cytotoxic drugs. Based on these findings, Lejeune and colleagues developed a regimen to treat locally advanced melanomas and sarcomas; high-dose TNF treatment combined with chemotherapeutic drugs was applied by isolated limb perfusion resulting in high complete response rates in patients (Eggermont and ten Hagen, 2001). Meanwhile, TNF was demonstrated to considerably improve penetration of several chemotherapeutic drugs into tumors in animal models. Interestingly, TNF specifically disrupted tumor-associated blood vessels, whereas leaving normal tissues and blood vessels unharmed. Curnis et al. (2000) have recently exploited this increase of drug penetration induced by very low doses of TNF for the treatment of melanoma and lymphoma with doxorubicin. To target TNF to the vasculature, TNF was coupled to a peptide ligand of aminopeptidase

N (CD13) that binds specifically to endothelial cells. Mice with established xenograft lymphomas could be cured by this approach, demonstrating that targeted delivery of TNF may considerably enhance its therapeutic properties.

TNF is involved in a wide range of biological responses and is regarded as a cardinal mediator of inflammation. It is therefore not surprising that dysregulation of TNF responses is linked to severe pathological syndromes. TNF seems to play a major role in the pathogenesis of rheumatoid arthritis and inflammatory bowel diseases. Animals with inflammatory bowel disease benefited significantly from treatment with anti-TNF antibodies or genetic TNF knockout (Neurath et al., 1997). Symptoms in about 60% of patients improved considerably upon single infusions with a chimeric monoclonal anti-TNF antibody (infliximab, remicade) (Targan et al., 1997). Infliximab (Centocor; Schering Plough, Kenilworth, NJ) comprises the variable region of a mouse anti-human TNF antibody fused to the constant region of human IgG₁. It binds and neutralizes TNF, both soluble and membrane-bound forms, and lyses cells expressing membrane TNF (Scallan et al., 1995). CDP571 (humicade), another humanized anti-TNF antibody developed by Celltech (Berkshire, UK) and Biogen (Cambridge, MA), works similar. Although infliximab is already approved for the treatment of rheumatoid arthritis and inflammatory bowel disease, CDP571 has entered phase 3 clinical trials, but so far, with disappointing results for Crohn's disease (Sandborn et al., 2004a). CAT (Cambridge, UK) and Abbott (Abbott Park, IL) developed a next-generation recombinant anti-TNF antibody (adalimumab, Humira) in contrast to the murine chimeric proteins infliximab and CDP571. Some patients treated with infliximab experienced allergic reactions. The fully human antibody adalimumab might therefore substitute previous anti-TNF therapies, particularly in patients with acute hypersensitivity reactions to chimeric antibodies (Sandborn et al., 2004b). Adalimumab is approved for the treatment of rheumatoid arthritis and owing to excellent results in phase 3 trials, will be presumably approved for psoriasis soon.

Another clinically relevant anti-TNF strategy was developed by Immunex (Seattle, WA); Etanercept (Enbrel) is a chimeric TNF-inhibitory molecule composed of the extracellular TNF-binding region of TNF-receptor 2 fused to an IgG heavy chain (Sandborn and Hanauer, 1999; Kam and Targan, 2000). Etanercept, marketed by Amgen and Wyeth (Madison, NJ), is already approved for the treatment of rheumatoid arthritis, psoriasis, spinal arthritis, and Crohn's disease. However, TNF is also involved in the combat against tuberculosis bacteria. Patients harboring latent infections are in danger of severe tuberculosis reactivation upon treatment with TNF inhibitors. Even though the actual risk is low, TNF-blocking therapies have been associated with the occurrence of lymphoma, infections, congestive heart

TABLE 1
Preclinical and clinical therapeutics targeting death receptors

Molecular Target	Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
TRAIL receptors	HGS-E/TR1	Agonistic TRAIL-R1 mAb	HGS/CAT	Apoptosis induction in various tumor cell lines and tumor xenografts, synergistic with anticancer drugs	Phase 2, no hepatic or hematological toxicity with up to 10 mg/kg i.v.
	HGS-E/TR2	Agonistic TRAIL-R2 mAb	HGS/CAT	Apoptosis induction in tumor cell lines	Phase 1
	HGS-TR2J	Agonistic TRAIL-R2 mAb	HGS / KNBWY	Apoptosis induction in tumor cell lines	Phase 1
	PRO1762	Soluble human Apo2L/TRAIL	Amgen/Genentech	Apoptosis induction in tumor cell lines, no side effects in cynomolgus monkeys and mice, synergistic with anticancer drugs	Phase 1
CD95/Fas	TRA-8	Agonistic TRAIL-R2 mAb	Sankyo Co., Ltd.	Apoptosis induction in tumor cell lines	Preclinical
	CD95-Fc	Humanized CD95 Fc-decoy construct	ApoGenix	Inhibition of CD95 signaling	Preclinical, first intended for spinal cord injury
TNF	Recombinant TNF- α	Combination of TNF and chemotherapy	Eggermont and ten Hagen, 2001	Apoptosis induction in tumor-associated blood vessels	Approved for isolated limb perfusion therapy in melanoma
	HUMIRA (Adalimumab)	Recombinant human IgG ₁ κ mAb against TNF- α	CAT/Abbott	Inhibition of TNF- α	FDA approved for rheumatoid arthritis, psoriasis, ankylosing spondylitis, and Crohn's disease
	Infliximab (Remicade)	Mouse/human TNF- α antibody	Centocor/Schering-Plough	Anti-inflammatory, also induces apoptosis in macrophages	FDA approved for rheumatoid arthritis and Crohn's disease
	Enbrel (Etanercept)	Recombinant TNF-R2/IgG fusion protein	Amgen/Wyeth	Anti-inflammatory in rheumatoid arthritis, Crohn's disease, and other inflammations	Approved for United States, some patients with severe side effects (infections, neurologic, and hematologic disorders)
	CDP571	Mouse/human TNF- α antibody	Celltech	Disappointing results in Crohn's disease	Phase 3 for rheumatoid arthritis stopped
	ISIS 104838	2'-O-Methoxyethyl antisense construct against TNF- α	ISIS	Improvement of rheumatoid arthritis-associated symptoms	Phase 2 for rheumatoid arthritis, Crohn's disease, psoriasis

Companies: Abbott Laboratories (www.abott.com), Amgen (www.amgen.com), ApoGenix (www.apogenix.de), CAT (Cambridge Antibody Technology, www.cambridgeantibody.com), Centocor (www.centocor.com), Celltech Group (www.celltechgroup.com), Genentech (www.gene.com), HGS (Human Genome Science, Inc., www.hgsi.com), ISIS Pharmaceuticals (www.isispharm.com), KNWBWY (Kirin Brewery Company Ltd., www.kirin.com), Sankyo Co., Ltd. (www.sankyo.co.jp), Schering-Plough (www.sch-plough.com), Wyeth (www.wyeth.com).

failure, demyelinating disease, lupus-like syndrome, and other systemic side effects (Scheinfeld, 2004).

Also the CD95 receptor/ligand system is most likely a very appealing target for therapeutic intervention. A most severe acute trauma is the injury of the spinal cord. Initial studies on spinal cord injury demonstrated that inhibition of apoptosis by caspase inhibitors improves the regeneration process (Li et al., 2000). In a recent study, Demjen et al. (2004) showed that treatment of mice experiencing spinal cord injury with neutralizing antibodies to CD95L effectively decreases cell death at the lesion site. Only several weeks after injury, mice treated (prior to transection of the spinal cord) with anti-CD95L antibodies could again actively move their hind legs. Recovery was associated with regenerating corticospinal fibers. Although several points clearly need to be further investigated, such as potential immunological side effects or carcinogenic risks, CD95-neutralizing molecules seem to hold much promise for treating spinal cord injuries.

The CD95 death receptor system has been implicated in other severe syndromes including stroke, liver failure, multiple sclerosis (MS), graft-versus-host disease (GVHD), and toxic epidermal necrolysis. For instance, CD95- and TNF-mediated signaling is presumably involved in stroke-associated tissue damage, characterized by massive neuronal death after brain ischemia. Mice deficient in CD95 (*lpr* mice) or CD95 ligand (*gld* mice) are partly protected from stroke and even more so, if TNF is inactivated in addition. Injection of mice with neutralizing CD95 and TNF antibodies after ischemic injury resulted in a marked decrease of both infarct volume and mortality (Martin-Villalba et al., 2001). MS is a progressive inflammatory disease caused by selective death of oligodendrocytes, resulting in demyelination of motoric nerve fibers. Several experimental findings hinted at a possible involvement of CD95 in MS, as patients revealed elevated soluble CD95 levels in cerebrospinal fluids together with a markedly increased expression of CD95 in T-cells, oligodendrocytes, and MS lesions (D'Souza et al., 1996; Ichikawa et al., 1996). Compared with wild-type mice, *lpr* mice are rather resistant to experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein. Also, administration of neutralizing anti-CD95L antibodies significantly reduced inflammation and demyelination in this model (Okuda et al., 2000). Graft-versus-host disease is a complication associated with bone marrow transplantation from allogeneic donors. The transplanted donor lymphocytes attack host organs, especially skin, gut, and liver. In its mild form, this can be useful for the eradication of persistent leukemia; however, aggressive GVHD can severely endanger the patient's life. In animal models, anti-CD95L antibodies could inhibit GVHD (Hattori et al., 1998). It was also demonstrated that even though inactivation of TNF or perforin could reduce GVHD, only targeting of CD95 did

not ablate the beneficial graft-versus-leukemia effect (Tsukada et al., 1999). Toxic epidermal necrolysis (TEN) is a severe and often fatal reaction to drugs, characterized by massive keratinocyte apoptosis and detachment of large areas of the epidermis. Highly increased expression of functional CD95L has been observed in CD95-positive TEN-keratinocytes. Progression of TEN in patients could be blocked by administration of antibodies interfering with CD95/CD95L interaction (Viard et al., 1998).

Because death receptor pathways are involved in a wide variety of human diseases, they are certainly very interesting targets for the development of new therapeutic regimens. Nevertheless, disadvantages associated with protein-based therapies clearly exist; proteins have to be administered systemically, in high doses, and regularly for chronic diseases. Furthermore, recombinant proteins are not very stable, cannot cross the blood-brain-barrier, and are expensive to produce. In this regard, nonpeptidic agents such as caspase inhibitors might turn out as the superior treatment for at least certain apoptosis-associated diseases.

IV. Drug Design of Caspase Inhibitors

Caspases, a family of cysteinyl aspartate-specific proteases, are the core components of most, if not all, apoptotic pathways. In humans, 12 different members have been identified, all of which recognize tetrapeptide motifs (P4-P1) and require an aspartic acid residue at P1 in the substrates (Fischer et al., 2003; Fuentes-Prior and Salvesen, 2004). Caspases are synthesized as inactive single-chain polypeptides and, in most cases, are activated by proteolytic processing. According to their biological function, caspases can be classified into two groups. The first caspase discovered was the interleukin-1 β -converting enzyme (ICE, caspase-1), which along with caspase-4 and -5, is predominantly involved in maturation of proinflammatory cytokines (Thornberry et al., 1992). Most other caspases play key roles in programmed cell death, some of them acting as initiator (caspase-8, -9, and -10) and others as executioner caspases (caspase-3, -6, and -7). Additional nonapoptotic functions of caspases in cellular proliferation and differentiation are subject of current research (Los et al., 2001; Schwerk and Schulze-Osthoff, 2003).

Experiments using caspase knockout and transgenic mice expressing dominant-negative caspase mutants had suggested that the inhibition of caspases has an enormous therapeutic potential in inflammatory and degenerative diseases, including rheumatoid arthritis, liver injury, myocardial infarction, and various neurodegenerative disorders. For instance, caspase-1-deficient mice demonstrated marked resistance to endotoxic shock (Li et al., 1995). Transgenic mice expressing a dominant-negative mutant of caspase-1 had reduced tissue damage and behavioral changes after ischemic in-

jury (Friedlander et al., 1997). Inhibition of caspase-1 also slowed down disease progression in a model of Huntington's disease (Ona et al., 1999).

The design of caspase inhibitors is currently at the forefront of apoptosis-based drug development (Table 2). The enormous potential of caspase inhibitors has been demonstrated with prototype inhibitors in several animal models. Liver diseases like alcoholic liver disease or hepatitis B and C virus infection are associated with accelerated apoptosis. In animal models, the broad irreversible caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was protective and efficiently blocked death receptor-mediated liver injury (Rodriguez et al., 1996; Kunstle et al., 1997). In arthritis models, repression of proinflammatory cytokine release (IL-1 β , IL-18) by blocking its caspase-1-dependent maturation led to efficient reduction of disease severity (Miller et al., 1995; Ku et al., 1996). Myocardial infarction and the resulting death of myocytes could be ameliorated by z-VAD-fmk and related peptide inhibitors in animal models (Yaoita et al., 1998). Also, sepsis that is associated with massive apoptosis of lymphocytes and lethal in approximately 29% of human cases was efficiently reduced in a mouse model by z-VAD-fmk, resulting in increased survival (Hotchkiss et al., 1999). In addition, caspase inhibitors reduced neuronal death and infarct size in stroke models (Cheng et al., 1998). Last but not least, after spinal cord injury activation of extrinsic and intrinsic apoptotic pathways has been demonstrated in animal models, which was efficiently blocked by z-VAD-fmk, leading to reduced lesion size and improved motoric function (Springer et al., 1999).

Caspase inhibitors typically consist of an electrophilic group targeting the active cysteine residue, termed the "warhead", the P1 aspartic acid, and the P2-P4 peptidomimetic region. Depending on the nature of the groups flanking the warhead, the inhibitors can be either reversible or irreversible. Caspase inhibitors with reversible warheads are often substituted with aldehyde, nitrile, ketone groups, whereas irreversible inhibitors bear halo-, acyloxy- or diazo-methylketone residues. The relative advantages of reversible versus irreversible inhibitors as well as of specific versus pan-caspase inhibitors are often debated. Reversible inhibitors are effective in inflammation, but less if apoptosis is to be inhibited. Irreversible inhibitors exhibit a greater potential in cellular assays compared with reversible compounds, however, they are in some cases less specific and also affect other cellular proteases (Wu and Fritz, 1999; Rozman-Pungercar et al., 2003). For instance, the fmk group of the irreversible inhibitor z-VAD-fmk was shown to interact with nonrelated cysteine proteases such as cathepsins (Schotte et al., 1999).

A related issue is the preservation of functionality rather than the survival of cells treated with caspase inhibitors. Are cells rescued from apoptotic death still

functional, or are functionally dead cells kept alive? Caspase inhibitors are able to rescue, for instance, renal and neuronal functions in animal models of ischemic kidney and brain injury (Daemen et al., 1999; Farber et al., 1999). However, some investigations raised concerns with respect to the loss of function after inhibitor treatment. In a model of Parkinson's disease, for instance, z-VAD-fmk inhibited neuronal death, but could not prevent the loss of neurites and the reduction of dopamine uptake (von Coelln et al., 2001). Therefore, the disturbed physiology of cells observed in some cases after inhibition of apoptosis might be a result of interference with other proteases or with other essential caspase functions. Clearly, more studies are required to elucidate the effect of caspase inhibitors on normal cell function as well as possible roles of caspases beyond that of cell death. This is especially important for chronic diseases where prolonged suppression of caspase activity is required but could also result in side effects.

The therapeutic efficacy of the first caspase inhibitors developed has been rather limited due to the poor cell permeability caused by the peptidyl structure and carboxylic acid residues, a limited activity in intact cells, and the lack of stability. To overcome these problems, several approaches are being employed: 1) the stepwise reduction of the peptidic nature of the inhibitor, 2) the use of compounds mimicking the peptide backbone, or 3) the modification of the active warhead. The P1 aspartic acid is crucial for the activity of the inhibitor, and substitution in most cases results in loss of activity. Only a few classes of inhibitors without aspartate at P1 have been reported so far. One example was developed by Okamoto et al. (1999) who replaced the P1 aspartate carboxyl group with an acyl-sulfonamide. The most potent compound of this series exhibited effective caspase inhibition not only in vitro (IC₅₀ 38 nM), but also comparably in vivo (IC₅₀ 230 nM). Other developed inhibitors with an aspartate in P1 position comprise isatin sulfonamides, which are potent reversible, nonpeptidic inhibitors of caspase-3 and -7 (Lee et al., 2000). However, their reactive warhead compromises the stability of isatin analogs. Unspecific interactions with cellular thiols or amines occur and might account for the observed shift in reactivity when comparing their activity in inorganic buffers with the poor activity in cellular extracts.

GlaxoSmithKline (Uxbridge, Middlesex, UK) further disclosed a series of similar piperidinyl substituted isatins and related 5-alkylaminosulphonyl-3,3-dichloro-oxindoles. Also, Pfizer (New York, NY) has developed isatin-based caspase inhibitors, from which the compound MMPSI, a (2-methoxymethylpyrrolidinyl)-sulfonylisatin, was able to reduce ischemic injury in isolated rabbit hearts or cardiomyocytes with an IC₅₀ of 200 nM (Chapman et al., 2002). AstraZeneca (Wilmington, DE) developed a series of nonpeptidic anilinoquinazolines (AQZs) (Scott et al., 2003). Similar to isatin sulfon-

TABLE 2
New drugs and approaches targeting caspases

Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
IDN-6556	Peptidomimetic irreversible caspase inhibitor	Idun	Antiapoptotic, anti-inflammatory and antifibrotic in models of liver damage	Phase 2 started for chronic HCV infection, Phase 2 opened for HBV infection and ischemia/reperfusion injury of liver transplants
IDN-6734	Caspase inhibitor	Idun	Reduces heart muscle damage in animal models of heart attack	Phase 1, acute myocardial infarction
VX-799	Small molecule caspase inhibitor	Vertex/Serono	Effective in sepsis and neuronal cell death models	Phase 1, started in 2003 for septic organ failure
MX-1013	Dipeptide pan-caspase inhibitor	Maxim	Prevents apoptosis in animal models of myocardial infarct, stroke, and acute liver failure	Preclinical, developed for myocardial infarct, stroke, acute liver failure
MX-2060 derivatives	Small molecule caspase activators	Maxim	Found in a cell-based caspase screen, growth inhibition in a rat tumor model	Preclinical
M-920	Broad spectrum caspase inhibitor	Merck Frosst	Effective against septic shock in a mouse model	Preclinical
Small molecule compounds	Caspase activators	Gemin X	Caspase activators in cancer but not normal cells	Preclinical
RGD peptides	Caspase activators	Merck Frosst, Maxim	Apoptosis induction in tumor cell lines	Preclinical
IDN-11104	ICE inhibitor	Idun	Anti-inflammatory in rheumatoid and osteoarthritis models	Preclinical
VX-740 (Pralnacasan)	ICE inhibitor	Vertex/Aventis	Anti-inflammatory in animal models	Phase 2, rheumatoid arthritis patients showed anti-inflammatory effects
VX-765	ICE inhibitor	Vertex	Protects mice against neonatal hypoxia-ischemia, rescues neurons in a Huntington's disease model	Phase 2a planned for psoriasis
M-826	Reversible caspase-3 inhibitor	Merck Frosst	Effective against septic shock in a mouse model	Preclinical
M-791	Caspase-3 specific inhibitor	Merck Frosst	Effective against septic shock in a mouse model	Preclinical
Immunocasp-3, Immunocasp-6	Cell-permeable HER2 mAb fused to caspase-3 or -6	Jia et al., 2003; Xu et al., 2004	Growth inhibition of HER2-positive tumors in a mouse xenograft model	Preclinical
Ad-G/Casp3	Adenoviral chemically inducible caspase-3	Shariat et al., 2001	Reduction in tumor growth in a prostate cancer mouse model	Preclinical
PEF-F8-CP3	Caspase-3 fusion construct with sc-antibody	Tse and Rabbitts, 2000	Antigen-dependent induction of apoptosis	Preclinical
FKBP12/casp-9 fusionprotein	Chemically inducible caspase-9	Nor et al., 2002	Antiangiogenic in mouse models upon induction of caspase-9 dimerization	Preclinical

Companies: Aventis (www.aventis.com), Gemin X Biotechnologies (www.geminx.com), Idun Pharmaceuticals, Inc. (www.idun.com), Maxim Pharmaceuticals (www.maxim.com), Merck Frosst Canada & Co. (www.merck-frosst.ca), Serono (www.serono.com), Vertex Pharmaceuticals, Inc. (www.vpharm.com).

amides, AQZs contain an electrophilic carbonyl that probably functions as the warhead. Interestingly, AQZs, like isatins and disulfiram, exhibit a decisive selectivity for caspase-3 (Nobel et al., 1997). Other caspase inhibitors comprise so-called aza-peptide epoxides, in which the carbonyl group of the epoxide ring is placed identical to the carbonyl of the scissile bond in a caspase substrate (Asgian et al., 2002; James et al., 2004).

As mentioned above, a critical part of a caspase inhibitor is the active warhead, which not only determines the reversibility of caspase inhibition, but might also cause side effects due to the interference with other cysteine proteases. ICN/Enzyme Systems Products, Inc. (Livermore, CA), holding the patent rights on z-VAD-fmk, has recently launched a new broad caspase inhibitor, which is increasingly taken for laboratory use; Q-VD-OPH (quinoyl-Val-Asp(OMe)-[2,6-difluorophenoxy]-methylketone lacks the often unspecific fmk group, but has increased potency, stability, and reduced toxicity. Q-VD-OPH inhibited various caspases with an IC_{50} of 25 to 400 nM and was nontoxic in doses up to 1 g/kg after i.p. administration in mice (Caserta et al., 2003). In vivo studies demonstrated significant reductions of virus-induced myocardial injury and protection of neurons against mitochondrial toxin-induced death (DeBiasi et al., 2004; Yang et al., 2004).

The Merck Frosst Center for Therapeutic Research (Kirkland, Quebec, Canada) has designed tetra- and dipeptides in which the active aldehyde warhead was replaced by a γ -keto group, resulting in faster binding kinetics. The most potent tetrapeptide inhibitor identified inhibited caspase-3 in vitro efficiently ($IC_{50} = 48$ nM), whereas it was only weakly effective ($IC_{50} = 10,000$ nM) in cellular assays (Han et al., 2004). Becker et al. (2004) therefore reduced the peptide nature of caspase-3 inhibitors by employing a peptidomimetic 5,6,7-tricyclic system or a pyrazinone at P2-P3, by replacing the negatively charged P4 aspartyl with neutral groups and by exploiting a hydrophobic binding site C-terminal to the cleavage site. Merck recently also developed nicotinyl aspartyl ketones. The 5-bromonicotinamide derivative of the aspartate aldehyde was a weak inhibitor specific for caspase-3, which could be improved by conversion of the aldehyde to ketones and further substitution at the 5-position of the pyridine ring (Isabel et al., 2003). Interestingly, the nicotinic acid replacements produced conformational changes in the S2 and S3 subsites of caspase-3 and revealed a previously unrecognized binding region. The patent claims a strong and specific inhibition of caspase-3 without the rapid inactivation associated with previous inhibitors.

Several Merck inhibitors are currently in preclinical trials. M-826, a small reversible caspase-3 inhibitor, blocked brain tissue damage in an animal model of hypoxia-ischemia when injected intracerebroventricularly 2 h after ligation (Han et al., 2002). In a mouse model of Huntington's disease, M-826 also prevented cell death of

striatal neurons (Toulmond et al., 2004). The broad spectrum caspase inhibitor M-920 as well as the caspase 3-specific inhibitor M-791 decreased lymphocyte apoptosis in thymus and spleen of mice subjected to polymicrobial sepsis induced by cecal ligation-puncture and rescued 80 to 90% of animals from lethal septic shock (Hotchkiss et al., 2000).

Whereas the C-terminal warhead is responsible for the reactivity of an inhibitor, N-terminal modifications also alter its effectiveness by influencing cell penetration. Most inhibitors therefore carry a hydrophobic N-protecting group. Cytovia, a Maxim subsidiary (San Diego, CA), developed a series of dipeptides with a fmk (or similar) warhead and different N-terminal substituents for inhibition of apoptosis and suppression of IL-1 β secretion. The potent, irreversible inhibitor z-VD-fmk, termed MX1013, inhibited several caspases with IC_{50} values ranging from 5 to 20 nM in vitro and 500 nM in cellular assays. z-VD-fmk effectively blocked endotoxemia in a rodent model (Jaeschke et al., 2000) and, moreover, prevented experimental liver damage caused by CD95 activation at doses of 1 mg/kg i.v. (Yang et al., 2003b). z-VD-fmk also demonstrated neuroprotective effects in a model of transient focal ischemia/reperfusion injury. Cai et al. (2004) further optimized the N-protecting group of dipeptidic inhibitors. Structure activity relationship (SAR) revealed that large groups like benzoyloxycarbonyl exhibited superior caspase-3 inhibiting activity compared with small groups. The most potent compound identified in this analysis was MX1122 (2,4-di-Cl-Cbz-Val-Asp-fmk) with a caspase-3 IC_{50} of 25 nM in vitro and a cell-protecting IC_{50} of 100 nM. In a mouse model of liver failure, 1 mg/kg MX1122 administered after injection of anti-CD95 achieved complete rescue of mice. A similar SAR study of the P2 amino acid in dipeptidyl aspartyl-fmk compounds by Maxim and Co-Censys (Irvine, CA) confirmed valine as the optimal P2 amino acid (Wang et al., 2004a).

The P2-P4 peptidomimetic region is the most variable part of the inhibitor and can determine the selectivity for individual caspases. Starting from the structure of the substrate recognition tetrapeptide, inhibitors are often designed by a stepwise substitution with nonpeptidic components. Sunesis (South San Francisco, CA) has recently developed a novel combinatorial screening method for the identification of suitable P2-P4 backbones, called extended tethering (Choong et al., 2002; Erlanson et al., 2003). The approach allowed the identification of ligands that bind to discrete regions of caspase-3 and helped to direct the assembly of these ligands into small molecule inhibitors. First, a small molecule "extender" that irreversibly alkylates the cysteine residue of caspase-3 and also contains a thiol group was designed. The modified caspase-3/extender protein complex was then screened against a library of disulfide-containing small molecule fragments. Compounds with favorable contacts to caspase-3 were further stabilized

by formation of stable covalent disulfide bonds. Compared with other high-throughput screening (HTS) technologies, this method also allowed weakly interacting compounds to be isolated, which might serve as leads for the development of more efficient inhibitors (Allen et al., 2003).

Already before the discovery of the role of caspases in apoptosis, several companies had started drug programs aimed at screening for caspase-1 inhibitors to suppress IL-1 β production. Pfizer/Abbott prepared a series of caspase-1 inhibitors and tried to improve its potency by rigidifying the caspase-bound inhibitor through an intramolecular hydrogen bond with an amino moiety of sulfonamides (Harter et al., 2004). Further substitution of the benzyloxycarbonyl (z) warhead by structurally similar sulfonamide derivatives resulted in an approximately 10-fold reduced caspase-1 IC₅₀, the most potent compound displaying a remarkable caspase-1 IC₅₀ of 3.4 nM. Pfizer has also evaluated arylsulfonamides as P2-P3 backbone by replacing His-Val of the native caspase-1 substrate (Shahripour et al., 2002).

Pralnacasan (VX-740), a caspase-1-specific and reversible inhibitor developed by Vertex (Cambridge, MA) for the treatment of rheumatoid arthritis, significantly reduced joint symptoms and inflammation in patients of a phase 2a trial. The compound is administered orally as a prodrug that undergoes *in vivo* hydrolysis to the active inhibitor possessing an aldehyde warhead. Unfortunately, pralnacasan was reported to induce abnormal liver toxicity upon long-term treatment in animal studies and was recently withdrawn from phase 2b. Further phase 1 trials are therefore underway, and a new generation inhibitor VX-765 is currently tested for the treatment of inflammatory diseases. Together with Serono (Rockland, MA), Vertex has developed VX-799, a pan-caspase inhibitor for the treatment of sepsis, which demonstrated efficacy in different models of organ failure.

Pioneering work in the area of caspase inhibitor design has been done by Idun Pharmaceuticals (San Diego, CA). They developed C-DEVD-H, a conformationally constrained peptidomimetic caspase inhibitor, serving as a lead for the further development of acyl dipeptide compounds (Karanewsky et al., 1998; Linton et al., 2002a,b). Idun Pharmaceuticals recently designed potent caspase inhibitors based on oxamyl dipeptides (Linton et al., 2004). As electrophilic warheads, polyfluorophenoxy, dichlorobenzoyloxy, or diphenylphosphinyl derivatives gave the best results. At P2 valine was superior to leucine, whereas at P4 compounds with substituted phenyl oxamides were most active. One of the synthesized compounds was able to reduce the infarct size in a rat middle cerebral artery occlusion model by 18%. However, the *in vitro* and *in vivo* activities of the compounds did not correlate well, owing probably still to peptide-associated problems.

A successful compound that has entered clinical trials is the irreversible peptidomimetic pan-caspase inhibitor

IDN-6556. Preclinical studies with IDN-6556 demonstrated selective and irreversible inhibition of several caspases with low to subnanomolar IC₅₀ *in vitro* and submicromolar range *in vivo*. Mouse models of CD95- or galactosamine/lipopolysaccharide-mediated liver injury already indicated that IDN-6556 could be a prime candidate for the treatment of liver diseases because it efficiently reduced serum levels of liver transaminases, irrespective of the mode of administration. IDN-6556 also exhibited marked postinsult efficacy, as it blocked lethality completely, even when administered 4 h after anti-CD95 treatment (Hoglen et al., 2004). The potency of this inhibitor was also proven in the bile duct ligation model (Canbay et al., 2004). Administration of IDN-6556 significantly reduced hepatocyte apoptosis and, importantly, also liver inflammation and fibrosis.

In a first clinical study, healthy volunteers and patients with impaired hepatic function were treated with *i.v.* infusions of IDN-6556. All but one patient exhibited significant falls in serum transaminase levels. Doses up to 1.5 mg/kg for 7 days were well tolerated, and moderate side effects were phlebitis and inflammation at the site of infusion (Valentino et al., 2003). Furthermore, it was demonstrated that apoptosis of sinusoidal cells, a major problem in liver transplantation caused by cold ischemia-warm reperfusion liver injury, could be effectively reduced by IDN-6556 in an animal model (Natori et al., 2003). In contrast to prior experiments with the caspase inhibitor IDN-1965, which absolutely required coadministration to the organ donor, the preservation solution as well as to the liver recipient (Hoglen et al., 2001), IDN-6556 was effective even when added only to the preservation solution. Therefore, in 2003, the FDA granted orphan drug status to IDN-6556 for the treatment of patients receiving liver transplants. Currently, phase 2 clinical trials are underway involving transplant centers in the United States and Europe. Recently reported data from a phase 2a study show that IDN-6556 given orally was well tolerated and significantly improved markers of liver damage in patients infected with the hepatitis C virus, an infection that affects up to 170 million patients worldwide. Thus, IDN-6556 may represent a new class of drugs that protect from inflammation and cellular injury induced by viral infections and other causes.

V. Caspase Activators

In addition to approaches aimed at inhibiting caspases, their selective activation might be a valuable strategy, especially for the therapy of human cancers. Several strategies to trigger caspase activation specifically in tumor cells are currently being designed (Table 2). Inducible caspases that can be activated “on demand” have been engineered by fusing them to chemical dimerization domains. After delivery of these chimeric “death switches” by adenoviral gene transfer, caspases can be

activated to trigger apoptosis in tumor cells by cell-permeable dimerization drugs (MacCorkle et al., 1998; Shariat et al., 2001). Inducible caspase-9 (iCasp9) under the control of a prostate-specific, androgen-responsive promoter was targeted to prostate cancer cells (Xie et al., 2001). In vivo, iCasp9 induced apoptosis in xenografted prostate tumors, but not in hepatoblastoma after chemical induction. A simple i.p. injection of the dimerization agent suppressed prostate tumor growth in nude mice and significantly increased host survival. Chemically inducible caspases have also been proposed as antiangiogenic tumor therapy, delivering caspases to neovascular endothelial cells (Nor et al., 2002). Several related gene therapeutic approaches to deliver caspases to tumor cells have been investigated and confirmed their antitumor activity in both in vitro and in vivo studies. For instance, caspase-6 under control of the human telomerase reverse transcriptase promoter was transferred into glioma cells (Komata et al., 2001). This approach is assumed to trigger tumor-specific apoptosis, since telomerase expression is reactivated in tumor cells, whereas normal cells hardly express the enzyme.

Tumor-selective cell death might also be induced by other chimeric proteins, such as immunocasp-3 and -6, which consist of a single-chain anti-erbB2/HER2 antibody (e23sFv) and the translocation domain of *Pseudomonas* exotoxin-A fused to an active caspase (Jia et al., 2003; Xu et al., 2004). Tumor-specificity of this strategy is provided by erbB2/HER2, which is usually not expressed in adult tissues, but is overexpressed in 20 to 40% of a variety of human neoplasms including breast, ovarian, endometrial, gastric, bladder, prostate, or lung cancer. The antibody used in this approach binds to the extracellular domain of HER2 and is internalized by endocytosis. The translocation domain of *Pseudomonas* exotoxin-A causes the release of the chimeric protein from internalized vesicles and subsequent autoactivation of the caspase. Death-inducing activity of the construct could be demonstrated in HER2 overexpressing tumor cells as well as in a xenograft mouse model. Immunex/Amgen are currently evaluating recombinant caspase-3 linked to the HER2 antibody Herceptin (Genentech) as a clinical treatment.

Another genetic approach of intracellular caspase activation was developed by Tse and Rabbitts (2000). They constructed a single-chain antibody-caspase-3 fusion gene that conferred toxicity in an antigen-specific manner because concentration of the fusion protein after binding to the multivalent antigen led to autoactivation of caspase-3. This is combined with antibodies against tumor-specific antigens, a promising strategy if efficient gene transfer techniques are available. An intriguing novel strategy to target HIV-infected cells in AIDS patients was published by Vocero-Akbani et al. (1999). The caspase maturation sites in procaspase-3 were replaced by HIV protease recognition motifs. Upon transduction of this construct, HIV-infected cells processed pro-

caspase-3, thereby, leading to caspase-mediated apoptosis selectively in infected cells.

Because the success of gene delivery approaches is still limited, pharmacological activation of cellular caspases by small cell-permeable drugs might provide a more efficient venue to target cancer cells. Procaspase-3 is held in an inactive, dormant state in healthy normal cells by an intramolecular electrostatic mechanism. This so-called "safety catch" consists of a triplet of aspartate residues located within a flexible loop near the junction of the large and the small caspase subunits. To activate caspase-3 from the inactive precursor, the junction between the large and small subunits has to be cleaved. Genetic removal of the safety catch loop resulted in increased autocatalytic maturation and susceptibility to caspase-9 (Roy et al., 2001), suggesting that release of the caspase-3 safety catch may be an important determinant of apoptotic competency. Furthermore, cellular acidification, which is typical in early apoptosis, promotes the disruption of electrostatic interactions and results in caspase-3 activation. In this context, screening for specific drugs that disrupt the safety catch might be a promising approach to lower the threshold of caspase activation.

That such direct activators of caspases might be found is supported by the observation that RGD peptides can directly bind to and activate caspase-3. Usually, tripeptides composed of arginine-glycine-aspartate are recognized by integrins in extracellular matrix proteins and can block integrin-mediated signaling and cell adhesion. In several cell types, it was previously shown that soluble RGD peptides induce apoptosis, which was first attributed to the loss of cell attachment and survival signaling. However, Buckley et al. (1999) demonstrated a direct intracellular activation of caspase-3 by RGD peptides that was independent of integrins. Indeed, caspase-3 has an RGD tripeptide sequence near its active site, which was proposed to keep the enzyme in a quiescent state. RGD peptides might therefore disturb this intramolecular interaction leading to the activation of the protease. RGD peptides are already in clinical use as antithrombotic drugs. Merck, in collaboration with the Scripps Research Institute (La Jolla, CA), developed a promising RGD-based candidate drug, inhibiting the new formation of tumor blood vessels. Although several issues concerning the mechanism of action of RGD peptides remain unclear, caspase-activating properties would clearly contribute to the established therapeutic potential of RGD peptides.

Several institutions and companies are performing HTS assays for compounds that could induce caspase activity either in living cells or in vitro. Jiang et al. (2003) identified a small molecule drug, α -(trichloromethyl)-4-pyridineethanol (PETCM), that could activate caspase-3 in cell extracts. PETCM relieved the inhibition of apoptosome formation imposed by the oncoprotein prothymosine- α . Furthermore, down-regulation of

prothymosine- α by RNA interference sensitized cells to apoptosis induction. However, even though a novel role of prothymosine- α has been identified in this study, PETCM is an unlikely therapeutic agent because high concentrations (200 μ M) are required to achieve caspase-3 activation in vitro. In a similar approach, Nguyen and Wells (2003) (Sunesis) screened a chemical library for caspase-3 activating or inhibiting compounds in HeLa cell cytoplasmic extracts supplemented with cytochrome *c*. SAR of the most potent agents led to the identification of dichlorobenzyl carbamates and indolones as strong caspase activators. The activating effect of these compounds turned out to be due to the induction of Apaf-1 oligomerization promoting apoptosome formation. An indolone compound was the most potent in cellular assays activating caspase-3 and cell death with an IC_{50} of 4 to 50 μ M. At 10 μ M, the compound was cytostatic on 40 and cytotoxic on eight cell lines from 48 tumor cell lines of the NCI panel.

Maxim Pharmaceuticals are currently evaluating the potential of a series of small molecule caspase activators MX-2060 isolated by a similar HTS approach for caspase-activating drugs in cell-based assays. Maxim's MX-2060 series of caspase-activating compounds are derivatives of gambogic acid, a natural product from the resin of the tree *Garcinia hurburyi* (Zhang et al., 2004). MX-2167 has been shown to induce apoptosis in multiple cancer cell lines including prostate, breast, colorectal, lung cancer, and leukemia cells. It suppressed tumor growth up to 90% in a syngeneic prostate animal cancer model. In conclusion, the fact that structurally unrelated compounds, identified in independent screens, converge at the level of apoptosome formation or caspase activation suggests that screening for small molecule caspase modulators might be a valid approach.

VI. Inhibitor of Apoptosis Protein-Based Therapeutics: Releasing the Apoptotic Brakes

Inhibitor of apoptosis proteins (IAPs) are acknowledged today as a major control point in the execution of cell death. IAPs comprise a family of caspase-inhibiting proteins characterized by a shared conserved sequence region, termed the BIR domain (Salvesen and Duckett, 2002). Currently, eight endogenous IAPs are known in humans, all of which inhibit apoptosis. Not surprisingly, some of them have been found to be overexpressed in a variety of cancers. So far, the main physiological roles of IAPs seem to be 1) the establishment of a threshold at which caspases are kept inactive, and 2) providing a pool of active caspases which can rapidly execute death after release. Growing evidence also suggests the participation of IAP proteins in other cellular functions apart from inhibiting caspases, including protein degradation, cell cycle control, and signal transduction (Deveraux and Reed, 1999). Survivin, for instance, is an IAP member containing a single BIR domain which is expressed to

high levels in cancer cells, but not in normal cells (Ambrosini et al., 1997). However, it is still unclear whether this is due to an antiapoptotic role of survivin conferring a survival advantage to the tumor cell or due to other reasons. Survivin is proposed to play a role in cell division (Reed and Bischoff, 2000; Uren et al., 2000b) and might therefore be expressed at higher levels in actively dividing cancer cells than in normal differentiated cells.

Antisense approaches targeting survivin and XIAP are currently being tested in preclinical trials by Isis Pharmaceuticals (Carlsbad, CA)/Eli Lilly (Indianapolis, IN) and Aegera (Montreal, Quebec, Canada), respectively (Table 3). Antisense oligonucleotides are designed with a short sequence complementary to the target mRNA. Hybridization of antisense oligonucleotide and mRNA inhibits the translation of the specific mRNA. Furthermore, enzymes like RNase H degrade the mRNA in the antisense/mRNA complex, setting the antisense construct free to target another mRNA strand. Antisense targeting of XIAP has been shown to sensitize a variety of tumor cell lines to radio- or chemotherapy (Holcik et al., 2000; Sasaki et al., 2000). XIAP antisense therapy combined with vinorelbine also proved to be effective in a mouse xenograft model of lung cancer (Hu et al., 2003).

The clinical use of oligonucleotides with natural backbones is limited because they are rapidly degraded by nucleases. In first-generation antisense molecules, one of the oxygens of the phosphate backbone is replaced with sulfur. Those phosphorothioate-modified oligonucleotides have enhanced but yet not optimal stability; they are relatively toxic and not suitable for oral administration. Second-generation oligonucleotides comprise DNA/RNA hybrid backbones with improved pharmacokinetics and reduced toxicity. In collaboration with Hybridon (Cambridge, MA), Aegera developed a second-generation XIAP antisense oligonucleotide AEG35156/GEM640. Knockdown of XIAP by AEG35156 demonstrated significant efficacy in preclinical models of multiple cancer types, Aegera claims. In March 2004, Aegera initiated phase 1 clinical trials evaluating safety and tolerance of the drug in patients with solid tumors.

In contrast to cancer, cerebral ischemia is associated with increased apoptosis that can be greatly reduced by caspase inhibitors. Likewise, adenoviral delivery of XIAP or the neuronal apoptosis inhibitory protein could rescue neurons from death following transient ischemia in the rat forebrain. Even more important, surviving neurons were still functional, as demonstrated by spatial learning tests (Xu et al., 1997; 1999). Overexpression of neuronal apoptosis inhibitory protein also attenuated death of dopamine neurons and dopaminergic fibers in a model of Parkinson's disease (Crocker et al., 2001). Thus, these studies demonstrate that IAPs are promising targets for intervention in several neurodegenerative diseases.

TABLE 3
Drug development targeting the IAP/SMAC pathway

Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
BIR3 antagonists Capped tripeptide XIAP antagonists	Small molecule antagonists of IAPs BIR3 ligands of XIAP	Idun Abbott; Oost et al., 2004	Proapoptotic in tumor cell lines Antitumor activity in cancer cells and breast cancer xenograft models	Preclinical Preclinical
TWX024	Nonpeptidic small molecule inhibitor of BIR2/caspase-3 interaction	Wu et al., 2003	Inhibits XIAP/caspase-3 interaction in vitro, proapoptotic in cell lines, synergistic with anti-CD95 and TRAIL	Preclinical
Polyphenylurea derivatives	BIR2-specific nonpeptidic inhibitors	Schimmer et al., 2004	Direct stimulation of caspase activity, growth suppression of xenotransplanted tumors	Preclinical
Smac-mimetic compounds	XIAP-binding Smac mimetic compounds	Sun et al. 2004	Enhances cisplatin induced apoptosis in prostate cancer cells	Preclinical
Embelin	Herbal cell-permeable XIAP inhibitor	Nikolovska-Coleska et al., 2004	Binds to XIAP BIR3, activates caspase-9, induces apoptosis in XIAP-overexpressing cells	Preclinical
XIAP antisense and RNAi constructs	Inhibition of XIAP expression	Hu et al., 2003; Bilim et al., 2003; McManus et al., 2004	Apoptosis induction in various cancer cells, synergistic with chemotherapy in mouse models	Preclinical
AEG35156/GEM640	XIAP antisense oligonucleotide	Aegera/Hybridon	Exhibits antitumor activity alone or in combination with chemotherapeutics in cancer xenograft models	Phase 1
HIV-Tat- and polyarginine-conjugated SMAC peptides	Cell-permeable peptide inhibitors of caspase/IAP interaction	Fulda et al., 2002; Yang et al., 2003a	TRAIL- and chemosensitization of tumor cells lines, glioma regression in intracranial xenograft models	Preclinical
Nonpeptide small molecule SMAC mimetic	Cell-permeable inhibitor of XIAP, cIAP-1, cIAP-2	Li et al., 2004	Potentiates apoptosis in combination with TRAIL and TNF, lead structure for development of IAP antagonists	Preclinical
LY2181308	Survivin antisense construct	ISIS/Eli Lilly	Preclinical studies show antitumor activity in a broad range of cancers	Phase 1 clinical trials started November 2004
Ad-Survivin T34A	Nonphosphorylatable survivin mutant adenovirus	Mesri et al., 2001	Apoptosis induction in various cancer cells, growth suppression of established breast cancer xenografts	Preclinical

Companies: Abbott Laboratories (www.abott.com), Aegera Therapeutics Inc. (www.aegera.com), Eli Lilly and Company (www.lilly.com), Hybridon (www.hybridon.com), Idun Pharmaceuticals, Inc. (www.idun.com), ISIS Pharmaceuticals (www.isispharm.com).

How caspase inhibition by IAPs actually works is best described for XIAP, which inhibits caspase-3, -7, and -9. XIAP contains three separate BIR domains. BIR2 and the linker to the N-terminal BIR1 domain are responsible for binding active caspase-3 and -7. XIAP potently inhibits these caspases by masking their substrate binding site (Huang et al., 2001; Riedl et al., 2001). In contrast, inhibition of caspase-9 solely depends on the BIR3 domain and flanking regions (Deveraux et al., 1999; Sun et al., 2000). Cleavage of caspase-9 results in the exposition of an IAP-binding motif (IBM) that fits into a hydrophobic pocket of the BIR3 domain. Homologous domains have been discovered in SMAC and the serine protease Omi/HtrA2 as well as in the *Drosophila* proteins Reaper, Hid, Grim, Sickie, and Jafrac2. SMAC and Omi/HtrA2 are nuclear-encoded proteins residing in mitochondria. The removal of the mitochondrial targeting sequence then reveals the IBM comprising four amino acids at the new N terminus. After induction of the intrinsic apoptotic pathway, both proteins are released into the cytosol. Most interesting with respect to therapeutic intervention is the fact that the IBMs of SMAC and Omi/HtrA2, termed the “knob”, also fit into the hydrophobic groove of XIAP. Therefore, SMAC is able to replace and release caspase-9 from the XIAP-inhibitory complex.

Currently, several companies are developing cancer therapeutic approaches employing SMAC peptides or SMAC-mimetic drugs to inhibit IAPs and to restore caspase activity (Table 3). So far, approaches involving SMAC-like peptides often failed to induce apoptosis as single agents, but nevertheless sensitized cancer cell lines or xenograft mouse models of human glioma and nonsmall cell lung cancer to TRAIL or chemotherapy (Arnt et al., 2002; Fulda et al., 2002; Guo et al., 2002; Tamm et al., 2003; Yang et al., 2003a). Similarly, a nonpeptidic SMAC-mimetic drug (an oxazoline derivative), which was designed by in silico analysis of the SMAC-IBM conformation, could target XIAP, cIAP1, and cIAP2 and synergized with TNF and TRAIL in the killing of cultured tumor cells (Li et al., 2004). Sun et al. (2004) synthesized structure-based SMAC-mimetic compounds, which bound XIAP and enhanced cisplatin-induced apoptosis in human prostate cancer cells, but did not induce apoptosis on their own.

Based on the NMR structure of the Smac/XIAP-BIR3 complex, capped tripeptides have been developed recently (Oost et al., 2004). These agents bound to the BIR3 domain of XIAP with high affinity, exhibited cytotoxicity for a range of human tumor cell lines, and even slowed down the growth of established breast cancer in a xenograft mouse model. Interestingly, Nikolovska-Coleska et al. (2004) discovered a low-molecular weight, cell-permeable compound of the Japanese Ardisia herb, termed embelin, through a structure-based computational screen of an herbal medicine database. Embelin activated caspase-9 and induced apoptosis in XIAP over-

expressing prostate cancer cells and had only minor effects on normal epithelial cells. Thus, embelin might serve as a lead compound for the development of SMAC agonists.

Idun Pharmaceuticals, in collaboration with Abbott, developed SMAC-mimicking small molecules that are in preclinical testing. However, the treatment of cancer with SMAC agonists still encounters some unresolved questions. For instance, the binding of the SMAC knob in the hydrophobic pocket might be sufficient to derepress caspase-9, but small SMAC peptides often bind to XIAP less efficient compared with the full-length wild-type protein. A second interaction domain of SMAC and BIR3 might play an important role and might be useful if taken into consideration when designing suitable SMAC mimetics (Wu et al., 2000). The second interaction interface might also explain the finding that an alternatively spliced cytosolic form of SMAC (SMAC β) lacking the knob as well as a recombinant deletion mutant of SMAC lacking the first 71 amino acids still induce apoptosis to a similar extent as the wild-type SMAC protein (Roberts et al., 2001). Moreover, IAP-independent functions could account for the antiapoptotic effect of the truncated SMAC proteins.

Targeting the interaction of XIAP with caspase-3/-7 might be even more promising than interfering with the XIAP/caspase-9 complex. Schimmer et al. (2004) employed an elegant enzyme derepression assay to screen for polyphenylurea-based XIAP antagonists. In a fluorogenic substrate assay, a combinatorial library was screened for compounds that could overcome XIAP-mediated repression of caspase-3 activity. The investigators identified several compounds demonstrating a cytotoxic effect predominantly in cancer, but not in nontransformed cells. The observed tumor specificity was suggested to be due to a higher level of already activated, but IAP-bound, caspases in tumor cells compared with normal cells. Furthermore, the identified polyphenylurea derivatives induced apoptosis in cultured leukemia cells as well as growth suppression of xenografted colon cancer in animal models. Even more importantly, overexpression of Bcl-2, Bcl-x_L, or BI-1 (Bax inhibitor-1) did not alter the sensitivity of cells to the phenylurea compounds, whereas the sensitivity to traditional chemotherapeutic drugs was, as expected, reduced (Wang et al., 2004b).

As yet, the targeting of IAPs for clinical use is hampered by the limited information on correlations of IAP expression with certain diseases. High expression levels of XIAP have been correlated with poor prognosis in acute myeloid leukemia patients (Tamm et al., 2004), and overexpression of cIAP1 might be associated with some esophageal squamous cell carcinomas (Imoto et al., 2001). More direct genetic evidence comes from a translocation t(11;18)(q21;q22) found in approximately 50% of *Helicobacter pylori*-induced mucosa-associated lymphoid tissue B-cell lymphomas (Baens et al., 2000). This

translocation causes the expression of a cIAP2-mucosa/associated lymphoid tissue 1 fusion protein, with cIAP2 comprising only the two BIR domains and missing the RING and CARD domain. The fusion protein was demonstrated to mediate constitutive NF- κ B stimulation, thereby activating its own transcription in a positive feedback loop. This mechanism presumably renders the tumor independent of the bacteria-caused NF- κ B activation and might explain why those tumors no longer respond to antibiotics (Uren et al., 2000a). Therefore, this tumor entity might be a suitable target for IAP-based therapy.

Certain tumors show a simultaneous up-regulation of several IAP members, and therefore, drug inhibition of only a single IAP interaction might be insufficient to induce significant *in vivo* effects (Krajewska et al., 2003). In addition, IAP proteins themselves might counteract the therapy; the IAP member ML-IAP, for instance, has only a single BIR domain with a high affinity to SMAC, but with a poor caspase-inhibitory activity. ML-IAP could therefore serve as a sink for applied SMAC agonists, a caveat that might be especially relevant in chemoresistant melanoma, where overexpression of ML-IAP is often observed (Vucic et al., 2000).

In addition to one or three BIR domains, XIAP, cIAP1, cIAP2, IAP-like protein-2, and ML-IAP also contain a RING domain associated with E3 ubiquitin ligase activity. RING proteins can mediate the degradation of other proteins and themselves via the proteasome pathway. The RING domain of XIAP has been shown to target XIAP itself, but also caspase-3 for degradation (Yang et al., 2000). The RING domain might be also involved in caspase-independent antiapoptotic functions of XIAP. Silke et al. (2002) demonstrated that XIAP mutants, which were no longer able to bind to caspase-3 or -9, still retained antiapoptotic activity. Therefore, BIR2- or BIR3-targeting compounds might not achieve full ablation of XIAP's antiapoptotic effects in cancer cells. Interestingly, another XIAP interacting factor XAF1 is down-regulated in various tumor cell lines (Fong et al., 2000). XAF1 is a negative regulator of XIAP like SMAC, but seems to achieve this effect through the complete removal of XIAP from the cytosol by sequestering it in the nucleus.

Certainly, additional complexity is yet to come when interactions with other proteins and post-translational modifications of IAPs are further analyzed. XIAP, for instance, interacts with the type I transforming growth factor- β receptor and consequently activates NF- κ B, c-Jun NH₂-terminal kinase, and SMAD signaling, even though recent studies in XIAP-deficient mice show that this interaction is not required for transforming growth factor- β signaling. XIAP also interacts with type I bone morphogenic protein receptor and TAB1, a cofactor of TAK1 kinase, while cIAP1 and cIAP2 interfere with death receptors. Nevertheless, even though IAP-based drug approaches still encounter unresolved issues, they

have a clear potential for cancer treatment. IAP inhibitors will presumably turn out as effective sensitizers for conventional chemo- or radiation therapy.

VII. Bcl-2 Family Proteins As Targets of Drug Design

Proteins of the Bcl-2 family are crucial checkpoints of the intrinsic mitochondrial death pathway (Cory et al., 2003). Members of this family can either induce or inhibit the release of mitochondrial apoptotic factors and are therefore classified as either pro- or antiapoptotic proteins. Although the precise mechanism of how Bcl-2 family proteins regulate apoptosis is still largely unclear, the stoichiometric balance of pro- and antiapoptotic factors seems to be of central importance. According to this simplistic view, the mitochondrial apoptotic pathway is inhibited by high concentrations of antiapoptotic Bcl-2 family proteins like Bcl-2 itself, Bcl-x_L, A1 or Mcl-1. Overexpression of those proteins is frequently observed in tumors of diverse origin and consequently confers resistance to cancer treatment that mostly targets the mitochondrial pathway. Moreover, inhibition of apoptosis by overexpressed Bcl-2-like proteins also promotes the tumor formation. Therefore, it is not surprising that the *bcl-2* gene, isolated from a B-cell lymphoma, was one of the first oncogenes to be identified. Elevated Bcl-2 levels are frequently found in B-cell malignancies and are often caused by the translocation t(14;18)(q32;q21), which brings the *bcl-2* gene under the transcriptional control of the highly active immunoglobulin heavy chain promoter. Similarly, transgenic mice overexpressing Bcl-2 display a phenotype that is associated with the accumulation of mature B-cells.

Bcl-2 overexpression is not limited to malignancies of B-lymphocytes, but is also frequently found in very different types of human cancers of both hematological and nonhematological origin. In these diseases, Bcl-2 overexpression mostly correlates with poor prognosis. Consequently, it soon became clear that Bcl-2 proteins would be promising targets for both pro- and antiapoptotic therapeutic approaches. Various fields of basic cancer research have contributed to the development of several strategies targeting Bcl-2 proteins (Table 4). Preclinical and clinical trials are now concentrating mainly on 1) antisense techniques to down-regulate protein levels, 2) application of BH3-domain peptides, or 3) synthetic small molecule drugs interfering with Bcl-2-like protein function.

Reed and coworkers were the first to develop antisense constructs to modulate cellular Bcl-2 levels (Reed et al., 1990a,b). A 20-mer oligonucleotide could kill lymphoma and leukemic cells directly, although it was even more effective when combined with standard chemotherapeutic drugs (Campos et al., 1994; Keith et al., 1995). Subsequently, genasense, a synthetic stabilized 18-mer antisense oligonucleotide to Bcl-2 mRNA, was

TABLE 4
Drugs and approaches targeting Bcl-2 proteins

Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
Bcl-2 blocker	Small molecule inhibitors of Bcl-2/Bcl-x _L	Idun/Abbott	Induction of tumor cell apoptosis	Preclinical
GX01 series of compounds	Small molecule inhibitors binding antiapoptotic Bcl-2 proteins	Gemin X		Preclinical
Bcl-2 small-molecule antagonist	Structure-based designed nonpeptidic Bcl-2 antagonists	Structural Bioinformatics		Preclinical
Tetrocarcin-A derivatives	Natural fungal compound inhibitor of Bcl-2	Kyowa Hakko Kogyo Co.	Inhibits mitochondrial functions regulated by Bcl-2, potentiates apoptosis	Preclinical
Chelerythrine	Plant alkaloid inhibiting Bcl-2/Bax interaction	Chan et al., 2003	Inhibitor of Bcl-x _L /Bak-BH3 interaction, induces cell death in Bcl-2 and Bcl-x _L -overexpressing cells	Preclinical
Antimycin A derivatives	Natural and synthetic Bcl-2/Bcl-x _L inhibitors	Tzung et al., 2001	Bind to the BH3 pocket of Bcl-2 and Bcl-x _L and induce apoptosis	Preclinical
HA14-1	Chemical compound binding the Bcl-2 BH3 pocket	Wang et al., 2000b	Induces apoptosis in tumor cells, synergistic with drugs	Preclinical
Synthetic compound	Identified in an HTS, binds to the BH3 of Bcl-2	Enyedy et al., 2001	Induces cell death in Bcl-2-overexpressing cells	Preclinical
Genasense	Bcl-2 18-mer antisense oligonucleotide	Aventis/Genta Inc.	Kills drug-resistant chronic lymphocytic leukemia cells, delays development of fatal lymphoma in mice, increases dacarbazine effectiveness in melanoma models	Phase-3: FDA fast-track status for melanoma, multiple myeloma, chronic lymphocytic leukemia. Phase 3 for non-small cell lung cancer, phase 2 for hormone-refractory prostate cancer
ISIS 22783	Splice-specific Bcl-x antisense oligonucleotide	ISIS; Taylor et al., 1999	Redirects Bcl-x mRNA processing to proapoptotic Bcl-x _s and sensitizes tumor cells for apoptosis	Preclinical
Bispecific Bcl-2/ Bcl-x _L antisense	Antisense construct against both Bcl-2 and Bcl-x _L	Zangemeister-Wittke et al., 2000	Promotion of apoptosis in cell lines	Preclinical
BH3 peptides from Bax, Bak, Bid or Bad	BH3 peptides coupled to protein transduction domains or to fatty acids	Holinger et al., 1999; Wang et al., 2000a	Apoptosis induction in tumor cell lines and mouse xenografts	Preclinical
SAHBs	Peptidomimetic BH3-mimeticum	Walensky et al., 2004	Induces Bax/Bak oligomerization, apoptosis in cell lines and growth suppression of leukemic xenografts	Preclinical
BH3Is	Compounds interfering with Bak-BH3/Bcl-x _L interaction	Degtarev et al., 2001	Induction of apoptosis	Preclinical

Companies: Abbott Laboratories (www.abbott.com), Aventis (www.aventis.com), Gemin X Biotechnologies (www.geminx.com), Idun Pharmaceuticals, Inc. (www.idun.com), ISIS Pharmaceuticals (www.isispharm.com), Kyowa Hakko Kogyo Co., Ltd. (www.kyowa.co.jp), Structural Bioinformatics, Inc. (www.strubix.com).

developed by Genta (Berkeley Heights, NJ). It binds to the first mRNA codons of the Bcl-2 open reading frame and thereby inhibits its expression. Furthermore, upon complex formation, the mRNA is rapidly degraded by RNase H-like endonucleases. Initially, genasense was held as a most promising new anticancer agent. In pre-clinical trials, for instance, it reduced Bcl-2 protein levels in breast cancer cells to 97% and displayed highly synergistic effects combined with doxorubicin in a xenograft breast cancer model. Similarly, genasense could strongly increase the efficacy of dacarbazine in a xenograft melanoma mouse model. Tumors were completely ablated in 10 of 13 animals, whereas dacarbazine alone elicited no effect.

Unfortunately, the hopes raised by those results could not be fulfilled by the outcomes of the actual clinical trials with melanoma patients. Nevertheless, Aventis (Strasbourg, France) and Genta filed a new drug application for genasense in combination with dacarbazine in advanced melanoma that was granted priority review by the FDA last year. However, a major drawback was the decision of the FDA advisory board that recommended against genasense as a treatment in melanoma. Board members were concerned about the unproven effectiveness of the drug because it failed to extend the survival time in the latest phase 3 clinical trial comprising 771 patients, even though it did seem to slow down disease progression. Genta withdrew the application, but further clinical studies involving patients with other tumor diseases are about to be evaluated. Thus, genasense might still turn out as a valuable tool in cancer therapy.

To optimize the impact of an antisense-based therapy, it would be necessary to simultaneously down-modulate at least both Bcl-2 and Bcl-x_L because many human tumors co-overexpress the two proteins. This could be achieved by applying two separate antisense constructs or, as recently shown, by using antisense oligonucleotides against a sequence, which is highly homologous in Bcl-2 and Bcl-x_L, but missing in Bcl-x_S mRNA (Zangemeister-Wittke et al., 2000). Such bispecific antisense oligonucleotides effectively killed various cancer cells.

An innovative antisense approach directed against Bcl-x_L was employed by Taylor et al. (1999). The *Bcl-x* gene encodes several different proteins as a result of alternative splicing. These variants differ in their apoptotic properties; Bcl-x_L blocks apoptosis similar to Bcl-2, whereas Bcl-x_S induces cell death. Taylor et al. (1999) used antisense oligonucleotides coupled to 2'-O-(2-methoxy)ethyl, which does not induce RNase H-like activity, to circumvent the down-regulation of all Bcl-x-proteins. Instead, by choosing an antisense sequence complementary to a region proximal to the splice donor site, which is essential for the production of Bcl-x_L, they triggered the splicing machinery to produce mainly the short-variant protein. Cancer cells treated with these oligonucleotides displayed a decreased Bcl-x_L/Bcl-x_S ra-

tio and were sensitized for UV- and chemotherapy-induced apoptosis.

Compared with the aforementioned down-regulation of Bcl-2 protein levels, the interference with Bcl-2 protein by BH3-domain peptides or small drugs is certainly a more direct and instantaneous approach. All members of the Bcl-2 family contain one to four conserved sequence motifs called the Bcl-2 homology (BH1–4) domains. Among the proapoptotic members of the Bcl-2 family, two groups of molecules are distinguished: the multidomain proapoptotic Bcl-2 proteins (Bax, Bak, and Bok) that contain three BH domains and the so-called BH3-only proteins (Bad, Bik, Bid, Bim, Blk, Hrk/DP5, Noxa, and Puma) that contain only the single BH3 domain (Puthalakath and Strasser, 2002). Some BH3-only proteins selectively interact with antiapoptotic Bcl-2 family members, whereas others also interact with proapoptotic members. BH3-only proteins have been proposed to be allosteric regulators of the Bcl-2 proteins and serve as sensors and effectors of apoptotic signaling. The current thought is that BH3-only proteins require at least one Bax-type partner to induce cell death. Both Bax and Bak undergo a conformational change in response to apoptotic stimuli, which mediates their assembly into homomultimers with channel-forming properties in the mitochondrial membrane, resulting in cytochrome *c* release. This conformational change and multimerization of Bak or Bax is inducible by BH3-only proteins and inhibitable by Bcl-2.

The BH3 domain comprises a 9-amino acid amphipathic α -helix that binds to a hydrophobic pocket of Bcl-2-like antiapoptotic proteins. Therefore, peptides and small molecules mimicking BH3-domain proteins aim at disrupting this complex, thereby sensitizing cells to apoptosis. The disruption of complexes like Bax/Bcl-2 with BH3 peptides and subsequent cytochrome *c* release could be successfully demonstrated in vitro. However, major challenges of using peptides as anticancer agents are the poor solubility and the susceptibility to proteolytic degradation as well as the limited cell permeability.

To enhance cell entry of BH3-domain peptides, several strategies have been employed: 1) coupling of BH3 peptides to protein transduction domains from HIV Tat-protein, HSV VP22, or *Drosophila* Antennapedia (Ant) protein, 2) conjugation to fatty acids, or 3) delivery by cationic lipids. Rapid cell entry and apoptosis induction in HeLa cells was achieved, for instance, by a Bak-BH3 coupled to Ant (Holinger et al., 1999). Ant-Bak-BH3 even sensitized Bcl-x_L overexpressing cells to CD95-mediated cell death. Other investigators coupled a Bad peptide to decanoic acid to enhance cell permeability (Wang et al., 2000b). The peptide induced apoptosis in tumor cells, but left normal human peripheral lymphocytes relatively unharmed. Furthermore, it slowed down the growth of human myeloid leukemia in SCID mice. Shangary and Johnson (2002) introduced BH3 peptides into cells by cationic lipid transfer. Jurkat cells overex-

pressing Bcl-2 or Bcl-x_L were efficiently eradicated by this approach, whereas a Bcl-2 BH3-domain peptide had no effect. Bad-BH3 peptides exhibited by far greater killing in Bcl-x_L than in Bcl-2 overexpressing cells, and Bax peptides were slightly more effective in Bcl-2 overexpressing cells. This observation does not only confirm the higher affinity of Bad for Bcl-x_L that had been found earlier in vitro (Yang et al., 1995), but also points at a possible limitation because the efficacy of BH3 peptides might depend on the affinity of a certain BH3 domain for a limited set of target proteins.

It has been argued that BH3 peptides induce apoptosis by a Bcl-2-independent pathway due to the α -helical structure, which disrupts the mitochondrial membrane on its own and releases proapoptotic factors. Support for this hypothesis came from a study showing apoptosis induction by an α -helical peptide unrelated to BH3 and by a mutant α -helical Bad peptide that was no longer able to bind to Bcl-2 (Schimmer et al., 2001). Recently, Walensky et al. (2004) substituted the Bid BH3 domain with non-natural amino acids on the surface opposite to the interacting region by employing a chemical strategy, called hydrocarbon stapling. This approach resulted in stabilized BH3 peptides termed SAHBs (stabilized α -helix of Bcl-2 domains), with improved pharmacological properties. SAHBs could induce apoptosis in a variety of leukemic cell lines and reduced the growth of leukemia xenografts in mice without adverse side effects. Thus, even though the exact mechanism of action remains unclear, BH3 peptides and probably more so small drugs mimicking BH3 peptides open up new perspectives for cancer treatment.

Several interesting small molecule compounds interfering with Bcl-2/Bcl-x_L function have been recently identified by high throughput screening of chemical libraries. Tetrocarcin A (TC-A), for instance, an antibiotic from *Actinomyces*, was isolated from a library of natural compounds by virtue of its Bcl-2-antagonizing activity. TC-A could sensitize Bcl-2 overexpressing HeLa cells to death receptor- and staurosporine-mediated apoptosis (Nakashima et al., 2000). So far, the cellular target of TC-A is unknown. Cell type-specific susceptibility differences have been reported, since T-ALL cells could be directly killed with TC-A as a single agent, whereas other cells were only sensitized for apoptosis induction (Tinhofer et al., 2002). Thus, TC-A is obviously an interesting candidate for cancer combination therapies.

Screening of a chemical library for compounds able to bind to the BH3 pocket of Bcl-2 proteins also led to the identification of HA14-1. This compound was shown to compete with Bak for the binding to Bcl-2 and to induce apoptosis in HL60 cells (Wang et al., 2000a). Furthermore, it acted synergistically with mitogen-activated protein kinase inhibitors on leukemic cell lines and might therefore be a valuable therapeutic tool (Milella et al., 2002). In a similar way, antimycin A₃, an inhibitor of the mitochondrial electron transport chain,

has been demonstrated to bind to the hydrophobic BH3 pocket and to induce apoptosis in Bcl-2 and Bcl-x_L overexpressing cells. This effect was independent of antimycin's effect on cellular respiration because a structural derivative of antimycin A₃ without respiration-inhibitory properties still promoted cell death (Kim et al., 2001; Tzung et al., 2001).

A library of 16,320 preselected compounds was screened in an HTS assay by Degterev et al. (2001) for the ability to displace a fluorescent Bak BH3 peptide from Bcl-x_L in a fluorescent polarization assay. This led to the identification of two classes of agents called BH3 inhibitors (BH3Is), which disrupted Bak-Bcl-x_L complexes at low micromolar concentrations. BH3Is could also disrupt the Bcl-x_L complex with Bax and Bad in intact cells. The BH3I-2 compound directly decreased the mitochondrial transmembrane potential prior to cytochrome *c* efflux, arguing for a more direct influence of BH3Is on mitochondria (Feng et al., 2003). Although the affinities to their targets are as yet too low to be used as drugs, BH3Is might provide promising lead structures for the devise of similar high-affinity compounds.

Other approaches used computational screens based on the NMR structure of Bcl-x_L. From more than 200,000 organic compounds from the NCI 3D database, several hit molecules were identified and proposed to inhibit antiapoptotic Bcl-2 proteins by binding to the BH3 pocket (Enyedy et al., 2001). The most potent compound induced apoptosis in Bcl-2 overexpressing cancer cell lines, but was less effective in cells with low Bcl-2 expression. Chan et al. (2003) derived chelerythrine, a natural benzophenanthridine alkaloid, in an HTS assay as an inhibitor of Bcl-x_L/Bak-BH3 interaction (IC₅₀ 1.5 μ M) from a library of natural compounds. Chelerythrine induced apoptosis effectively in Bcl-2 or Bcl-x_L overexpressing cells and triggered cytochrome *c* release from isolated mitochondria. Taken together, a variety of strategies have been successfully employed to attack Bcl-2 family proteins and the checkpoints they confer on apoptosis regulation. Although our understanding of the precise mechanism of action of Bcl-2 proteins is still incomplete, Bcl-2 inhibitors and compounds that mimic BH3 domains are assumed to have a great potential in future anticancer therapy.

VIII. Targeting Mitochondria Directly

An important apoptotic alteration is an event characterized as mitochondrial membrane permeabilization (MMP). Several cytotoxic drugs induce MMP by a direct action on mitochondria and might therefore trigger death in cells in which upstream apoptotic signals have been disabled (Debatin et al., 2002). Components acting directly on mitochondria can be either proteins of the permeability transition pore complex, including the peripheral benzodiazepine receptor (PBR) and the adenine nucleotide translocase (ANT), or can be small molecules

that exert a cytotoxic effect by virtue of their physico-chemical properties (Table 5). Interestingly, certain compounds acting directly on mitochondria can overcome the antiapoptotic effect of Bcl-2 proteins, suggesting that their action is independent of the Bcl-2 pathway. Some of these agents are already used in anticancer chemotherapy, and new drugs specifically designed to target mitochondria are being developed.

Synthetic ligands of the PBR, such as the isoquinoline carboxamide PK11195 or the diazepam Ro5-4864, induce apoptosis or sensitize cells by overcoming the cytoprotective effects of Bcl-2 or Bcl-x_L. This sensitizing effect has been demonstrated in several tumor cell lines in vitro and also in SCID mice transplanted with human tumor cells (Hirsch et al., 1998; Maaser et al., 2001; Decaudin et al., 2002). In these models, PBR ligands could not induce cell death on their own. When used in combination with other proapoptotic agents, however, these agents were highly cytotoxic and neutralized the antiapoptotic potential of Bcl-2. A number of agents, such as for instance lonidamine and 2-chloro-2'-deoxyadenosine, have been reported to create a conformational change of ANT to induce mitochondrial channel formation. Lonidamine has been shown to revert the resistance to cisplatin and to potentiate its cytotoxicity in experimental models. Lonidamine kills a wide range of tumor cells in vitro and in animal models (Ravagnan et al., 1999). The drug is currently tested in phase 2/3 trials for metastatic breast and nonsmall cell lung cancer with encouraging results so far.

The lipid composition of the inner mitochondrial membrane is very different from that of other intracellular membranes. Therefore and due to the mitochondrial membrane potential, cationic ampholytes may enrich and insert in the inner mitochondrial membrane to induce MMP. This mode of action probably applies to MKT-077, a cationic rhodacyanine dye that is selectively toxic to cancer cells and is now undergoing clinical trials (Britten et al., 2000). Another cationic ampholyte is the α -helical peptide D(KLAKLAK)₂ that directly permeabilizes mitochondria. In elegant studies, D(KLAKLAK)₂ has been fused to targeting peptides that interact with surface receptors expressed on angiogenic endothelial cells in tumors (Ellerby et al., 1999; Arap et al., 2002). These fusion peptides are translocated into the cytoplasm and then induce apoptosis via mitochondrial permeabilization in the targeted tumor cells. Treatment of mice with this peptide delayed the development of cancer in a mouse model of prostate carcinoma and human breast cancer xenografts.

Several agents cause thiol oxidation of mitochondrial membranes and proteins, which might then result in MMP. For instance, arsenite, which is used to treat acute promyelocytic leukemia and multiple myeloma, causes glutathione depletion and permeabilizes purified mitochondria in vitro. A number of other agents that act on mitochondria possess a steroid-like structure. This

TABLE 5
Drugs acting on mitochondrial permeability transition

Reagent	Principle	Reference	Experimental Effects	Clinical Trial/Status
Peripheral benzodiazepine receptor ligands	PK11195, Ro5-4864, FGIN-1-27	Hirsch et al., 1998; Maaser et al., 2001	Potentiate chemosensitivity, induce tumor xenograft regression	Preclinical
Lonidamine	Dichlorinated indazole-3-carboxylic acid derivative	Ravagnan et al., 1999	Permeabilizes isolated mitochondria, potentiates chemotherapeutic effects	Phase 2/3 in metastatic breast cancer and non-small cell lung carcinoma
CD437	Synthetic retinoid	Marchetti et al., 1999	Apoptosis induction in several tumor cells, MMP transition in vitro (several other actions known)	Preclinical
Cladribine	2-Chloro-2'-deoxyadenosine	Genini et al., 2000	Direct disruption of mitochondrial membrane potential	Approved for chronic lymphocytic and hairy cell leukemia
Arsenite	Arsenic trioxide	Larochette et al., 1999	Oxidative disruption of mitochondrial membrane and proteins	Approved for acute promyelocytic leukemia
D(KLAKLAK) ₂	Positively charged α -helical peptide	Ellerby et al., 1999	Disrupts mitochondrial membranes and shows anticancer activity in mice	Preclinical
Verteporfin	Porphyrine photosensitizer	Belzacq et al., 2001	Direct triggering of cytochrome c release	Used in photodynamic therapy
Betulinic acid	Pentacyclic triterpenoid from Birch	Pisha et al., 1995; Fulda et al., 1998	Proapoptotic activity in isolated mitochondrial cell lines and xenograft models	Preclinical
MKT-077	Cationic rhodacyanine	Modica-Napolitano et al., 1996	Selective cytotoxicity for tumor cells, also mitochondria-independent actions known	Phase 1

applies to betulinic acid and the retinoid analog CD437, which perturb the lipid composition of mitochondrial membranes and induce the opening of the permeability transition pore complex (Fulda et al., 1998; Marchetti et al., 1999). Betulinic acid, a pentacyclic triterpenoid isolated from Birch, has the interesting ability to selectively kill neuroectodermal tumor cells including neuroblastoma, medulloblastoma, glioblastoma, and Ewing sarcoma cells (Pisha et al., 1995; Fulda et al., 1999). Isolated mitochondria from different cell types are permeabilized by betulinic acid, and this effect is prevented by the ANT ligand bongkrekic acid as well as by Bcl-2 overexpression (Fulda et al., 1998). It is unclear, however, how betulinic acid acts on mitochondria and how its relative cell type specificity can be explained. Nonetheless, it appears that derivatives of betulinic acid with improved pharmacological properties might be useful in treating neuroectodermal tumors.

Whether the apoptogenic or chemosensitizing effects of the above-mentioned agents are truly due to a direct effect on mitochondria is at least in some cases a matter of debate. For instance, the doses of PBR ligands required to obtain cytotoxic effects are several orders of magnitude higher than the K_d of the high-affinity PBR. Furthermore, for betulinic acid, arsenite, or adenine deoxynucleotides, several other cellular effects have been described that contribute to the cytotoxic effects as well. Nevertheless, the fact that Bcl-2 does not confer resistance against some of these agents underscores that direct targeting of mitochondria might be useful for the eradication of tumor cells.

IX. Tumor Suppressor p53

The tumor suppressor protein p53 is an important integrator of cellular stresses that can either trigger cell cycle arrest or apoptosis. The importance of p53 for the prevention of tumorigenesis becomes evident by the fact that p53 is mutated and thereby inactivated in more than 50% of human cancers. Due to the importance of p53 in apoptosis induction following DNA damage, different strategies have been undertaken to target the tumor suppressor (Table 6). A promising approach is the restoration of normal functions of mutant p53. For instance, the radioprotector amifostine can restore the transcriptional activity of some p53 mutants in yeast functional assays, provided that they are conformationally flexible (Maurici et al., 2001). Similarly, introduction of small synthetic molecules, which stabilize p53 in a transcriptionally active state by allosteric modulation of its conformation, is thought to especially target tumor cells with a high accumulation of mutant p53 (Foster et al., 1999). Some reports demonstrated that the transcriptional activity of mutant p53 can be rescued by application of synthetic peptides derived from the C terminus of p53. These peptides interact with the core domain of mutant p53 and restore growth-suppressing

functions of p53 (Selivanova et al., 1997, 1999; Kim et al., 1999).

Another possibility to stabilize and to restore the tumor suppressor function of p53 is by interfering with the binding of p53 to Mdm2, a negative regulator that targets p53 for proteasomal degradation. This has been evaluated by employing peptides or synthetic drugs (Chene et al., 2000; Stoll et al., 2001). Chalcone derivatives have been identified to exert such an activity, but so far lack sufficient specificity. Last year Roche (Basel, Switzerland) developed so-called "nutlins", which comprise imidazoline compounds that bind into the p53 pocket of Mdm2 and thereby increase p53 activity (Vassilev et al., 2004). Nutlins were shown to inhibit tumor growth in mice to 90% without accompanying harmful side effects. The latest drug discovery in the area of Mdm2 antagonists is RITA. This furan derivative [2,5-bis(5-hydroxymethyl-2-thienyl)furan] was selected from the NCI compound library after comparing the antiproliferative effect of drugs in isogenic tumor cell lines differing in their p53 status. RITA interfered with p53/Mdm2 binding both in vitro and in vivo and suppressed the growth of wild-type p53 colon carcinoma xenografts in mice (Issaeva et al., 2004). In addition to compounds disrupting p53/Mdm2 binding, drugs inhibiting the E3 ligase activity of Mdm2, which is responsible for ubiquitin-mediated degradation of p53, are currently being evaluated for drug discovery (Buolamwini et al., 2005).

Genetic reintroduction of wt-p53 into p53-deficient cancer cells leads to suppression of tumor growth, and synergistic effects with conventional chemotherapy have been demonstrated in several studies. However, the low efficiency of gene therapy vectors limits the success of this therapeutic approach. Hepatotoxicity associated with systemic application of adenoviral vectors is another drawback. Nevertheless, several clinical trials currently evaluate the delivery of wt-p53-expressing adenovirus (Ad-p53) by intratumoral injection. INGN201, an Ad-p53 supported by Introgen Therapeutics (Austin, TX), is now being tested in a phase 3 trial for the treatment of head and neck cancer. Similarly, Schering Plough is investigating the use of the Ad-p53 virus SCH58500 in advanced ovarian cancer. Ad-p53 has also been proposed for the elimination of cancer cells ex vivo out of bone marrow from breast cancer patients receiving high-dose chemo- or radiotherapy before autologous bone marrow transplantation. As contamination of bone marrow by cancer cells is a main cause for tumor relapse, purging the transplant with Ad-p53, which specifically targets tumor epithelial and not bone marrow cells, might provide an effective strategy to reduce the risk of tumor relapse (Seth et al., 1996). Furthermore, another intriguing adenoviral strategy was devised to specifically target p53-deficient tumor cells. The adenovirus mutant ONYX-015 is deficient in E1B protein expression, which normally targets and inactivates p53 to

TABLE 6
Reagents targeting the tumor suppressor p53

Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
INGN201	p53-Expressing adenovirus	Introgen Therapeutics	Apoptosis induction in tumor cell lines and xenograft models	Phase 3 for head and neck cancer, clinical trials for other advanced solid tumors
SCH58500	p53-Expressing adenovirus	Schering-Plough	Apoptosis induction in tumor cell lines and xenograft models	Phase 3 for advanced ovarian cancer
ONYX-015	p53 delivery with mutant adenovirus	Onyx; Khuri et al., 2000	Virus demonstrates significantly greater antitumor activity against mutant p53 tumors in vivo	Phase 2/3 for combination therapy of advanced squamous cell cancer; phase 1/2 trials for other cancers
C-Terminal p53 peptides	Stabilization of wild-type and reactivation of mutant p53	Selivanova et al., 1997; Kim et al., 1999	Restores transactivation and growth-suppressing function of mutant p53	Preclinical
CDB3	Rationally designed 9-mer peptide	Friedler et al., 2002	Binds and stabilizes the p53 core domain, restores activity of mutant p53	Preclinical
Amifostine	Restoration of p53; prodrug is converted to the phosphorothiol WR 1065	Maurici et al., 2001	Restoration of the transcriptional activity of mutant p53 shown in vitro	Established as a chemo- and radioprotector with complex mechanisms of actions
CP31398	Styrylquinazoline that stabilizes wild-type p53 and restores a normal conformation of mutant p53	Pfizer; Foster et al., 1999	Inhibition of p53 ubiquitination, increases p53 activity in cells and inhibits growth of xenografted tumors	Preclinical, might serve as a small-molecule lead structure
Prima-1	Synthetic small molecule restoring activity of mutant p53	Bykov et al., 2002	Drug restores DNA binding and active conformation of mutant p53	Preclinical, might serve as a lead compound
HPV E6-binding peptide aptamers	Inhibition of E6 protein-mediated p53 degradation	Butz et al., 2000	Apoptosis induction in papillomavirus-positive cancer cells	Preclinical
Nutlins	Imidazoline derivatives that antagonize p53/Mdm2 interaction	Roche; Vassilev et al., 2004	Drugs bind to the p53 pocket of Mdm2 and inhibit protein interaction in vitro and in vivo	Preclinical lead compounds
Chalcones	Small molecule antagonist of p53/Mdm2 interaction	Stoll et al., 2001	Compounds with presumably insufficient specificity	Preclinical
RITA	Small molecule antagonist of p53/Mdm2 binding	Issaeva et al., 2004	Inhibits the binding of p53/Mdm2 in vitro and in vivo, reduces tumor growth in xenograft animal models	Preclinical
Small peptide compounds	Small molecule antagonist of p53/Mdm2 interaction	Garcia-Echeverria et al., 2000	High-affinity peptide that stimulates the p53 pathway	Preclinical
Pifithrin- α	Synthetic p53 inhibitor	Komarov et al., 1999	Inhibits p53-dependent transcription, protects mice from lethal genotoxic stress associated with cancer treatment	Preclinical

Companies: Introgen Therapeutics (www.introgen.com), Onyx Pharmaceuticals (www.onyx-pharm.com), Pfizer (www.pfizer.com), Roche (www.roche.com), Schering-Plough (www.sch-plough.com).

promote viral replication. Therefore, the virus is thought to replicate only in cells with deficient wt-p53 expression, although this selectivity has been questioned recently (Rothmann et al., 1998). The potential therapeutic use of ONYX-015 in combination with cisplatin and 5-fluorouracil is under investigation in phase 2/3 clinical trials for patients with recurrent squamous cell cancers (Khuri et al., 2000).

Similar to Mdm2, the E6 protein of human papillomavirus 16 and 18 is another protein inactivating p53. With the help of a cellular ubiquitin E3 ligase, E6 targets p53 for proteasomal degradation, thereby rendering the phenotype of the cells p53-null (Scheffner et al., 1993). Papillomavirus infection is thought to be causative for about 90% of human cervical cancers and 50% of other anogenital cancers. In earlier studies, it has been shown that degradation of p53 in these cancers can be overcome by a combined treatment with transcriptional inhibitors, which induce p53 protein accumulation and inhibit E6 synthesis, together with inhibitors of the proteasome to prolong p53 half-life time. Synthetic peptide aptamers competing intracellularly for E6 binding with p53 are another promising pharmacological approach that has been shown to induce apoptosis in HPV16-positive cancer cells (Butz et al., 2000).

Mutant p53 accumulates to high levels in cancer cells. As mutant p53 is not simply dysfunctional but can antagonize other p53 family members, such as p63 and p73 and thereby gain tumor-promoting functions, the selective depletion of mutant p53 might also exert a beneficial effect. A strategy to promote the degradation of mutant p53 by the proteasome might be the targeting of the heat shock factor (Hsp)-90 by geldanamycin. Hsp90 is a protein chaperone that binds to different "client proteins", including a list of cancer relevant targets such as mutant p53, Raf-1, Akt, and others. When geldanamycin binds to Hsp90, it disrupts the Hsp90-client protein complex, leading to degradation of the client proteins. Accordingly, geldanamycin has been reported to deplete mutant p53 in various tumor cell lines (Blagosklonny et al., 1995; An et al., 1997). Kosan Biosciences (Hayward, CA) has initiated a first phase 2 monotherapy trial in patients with metastatic melanoma using 17-AAG, a 17-allylamino-17-demethoxy derivative of geldanamycin. 17-AAG is also being evaluated in clinical trials for the treatment of multiple other cancer indications.

A common problem of traditional chemo- and radiotherapy are genotoxic side effects on normal tissues, which limits the applicable dosage and the antitumor effect. An ingenious approach to tackle this problem was developed by the group of Gudkov. Most cancers harbor mutated inactive p53, whereas surrounding normal tissues express functional wild-type p53. Those patients could benefit from a treatment with the small molecule drug pifithrin- α , a p53 inhibitor. In normal tissues, pifithrin- α would block p53-mediated transcription and

apoptosis, relieving the genotoxic stress of the cells. Indeed pifithrin- α completely rescued mice from doses of radiation that usually kill 60% of the mice, and none of the mice developed tumors in 8 months (Komarov et al., 1999). It must be mentioned, however, that the specificity of pifithrin- α for p53 has been recently questioned, as it was also found to inhibit heat shock and glucocorticoid receptor signaling (Komarova et al., 2003).

X. Viral Death Proteins Apoptin and E4orf4

Promising candidates for tumor therapy are the viral proteins apoptin (derived from the chicken anemia virus) and the adenoviral protein E4orf4 (Table 7). Sharing the same preference as TRAIL, they specifically kill tumor cells while leaving normal cells unharmed (Kleinberger, 2000; Rohn and Noteborn, 2004). The reason for this tumor selectivity is still unclear. In the case of apoptin, it seems to depend on its subcellular localization, as the protein is localized in the cytoplasm of normal cells, but migrates into the nucleus of transformed cells. This is at least in part a consequence of apoptin phosphorylation at Thr-108, which seems to take place only in transformed but not in normal cells. An interesting feature of apoptin is that it triggers cell death very efficiently in tumor cells that are resistant to chemotherapy due to Bcl-2 overexpression, p53 mutations, or expression of the BCR-ABL fusion oncoprotein. Both viral proteins are still in preclinical studies. Delivery with gene therapy vectors or fusion with the protein transduction domain from HIV-Tat, which enable fused proteins to transfer cell membranes, are currently being investigated (Lavoie et al., 1998; van der Eb et al., 2002; Guelen et al., 2004). Preclinical studies showed no adverse effects of apoptin delivered by an adenoviral vector, and an antitumor effect was observed in nude mice challenged with human hepatoma (Pietersen and Noteborn, 2000).

XI. Interfering with Protein Degradation: Targeting the Proteasome

The 26S proteasome is the core protein degradation machinery of eukaryotic cells. Intracellular levels of a vast number of different proteins are regulated by polyubiquitination and subsequent turnover mediated by the proteasome. Interference with proteasome function therefore leads to disturbances in a variety of cellular tasks. Interestingly, tumor cells are obviously more sensitive to inhibition of the proteasome than nontransformed cells, which makes it an attractive target for cancer treatment.

Proteasome inhibitors comprise five important classes of substances: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones, and β -lactones. Several drugs targeting the proteasome are currently in preclinical and clinical testing (Table 7). The first compounds used as proteasome inhibitors were pep-

TABLE 7
Viral proapoptotic strategies and proteasome inhibitors

Molecular Target	Reagent	Principle	Company/Reference	Experimental Effects	Clinical Trial/Status
Viral proteins	Apoptin	Proapoptotic VP3 protein from chicken anemia virus	Zhuang et al., 1995	Induces p53-independent cytotoxicity in tumor but not nontransformed cells in vitro and in animal models	Preclinical gene therapeutic approaches
	E4orf4	Proapoptotic adenoviral protein	Gemin X; Lavoie et al., 1998	Induces p53-independent, transformed cell-specific apoptosis presumably by binding to protein phosphatase 2A	Preclinical gene therapeutic approaches
Proteasome	Bortezomib (Velcade, PS-341)	Dipeptide boronic acid	Millennium	Stabilization of cell-cycle and proapoptotic proteins, inhibition of antiapoptotic proteins, effects on tumor microenvironment	FDA approved for relapsed and refractory multiple myeloma, ongoing trials for several tumors
	MLN519 (PS-519)	Lactacystin derivative	Millennium	Potent anti-inflammatory and neuroprotective effects	Phase 1, intended for acute stroke and myocardial infarction
	Epoxomicin, Eponemycin	Streptomycetes epoxyketones	Meng et al., 1999a,b	Cytotoxic effects in various tumor cells	Preclinical
	Nitrophenyl trileucine vinyl sulfone	Trileucine vinyl sulfone	Bogyo et al., 1997	Irreversible inhibition of trypsin- and chymotrypsin-like proteasome activities	Preclinical
	Ritonavir	Peptidomimetic protease inhibitor	Abbott	HIV protease inhibitor, also inhibits chymotrypsin-like activity of proteasome	Approved for AIDS, phase 2 studies in tumor patients to be started

Companies: Abbott Laboratories (www.abott.com), Gemin X Biotechnologies (www.geminx.com), Millennium Pharmaceuticals, Inc. (www.milmm.com).

aldehyde moieties that, however, are rather unspecific and also target other proteases. Substitution of the aldehyde moiety with boronic acid resulted in compounds with superior specificity and potency forming covalent, reversible complexes with proteasome subunits (Adams et al., 1998). Bortezomib (Velcade, PS-341), a dipeptidyl boronic acid proteasome blocker developed by Millennium Pharmaceuticals (Cambridge, MA) in cooperation with Johnson and Johnson (New Brunswick, NJ), was tested against a panel of 60 tumor cell lines and displayed promising anticancer properties. Furthermore, bortezomib reduced tumor growth in xenograft mouse models of human prostate and several other cancer types (Adams et al., 1999). Bortezomib is the first proteasome inhibitor to enter clinical trials and is already FDA-approved for the treatment of recurrent multiple myeloma. Phase 2 studies demonstrated a response to bortezomib in 27% of patients irrespective of prior treatments (Richardson et al., 2003). This is consistent with preclinical findings that the response of myeloma cells to the drug is independent of their sensitivity to other chemotherapeutics. Nontoxic doses of bortezomib also sensitize several other cancer cells to chemotherapy in vitro and in animal models.

It was suggested that the proapoptotic effect of proteasome inhibitors might be due to the observed stabilization of I κ B resulting in NF- κ B inhibition and the down-regulation of antiapoptotic NF- κ B target genes (Berenson et al., 2001). Another observation was the down-regulation of Bcl-2 in pancreatic cancer cells treated with bortezomib, which would in itself probably be sufficient to induce apoptosis by misbalancing pro- and antiapoptotic proteins (Bold et al., 2001). Accumulation of cell cycle inhibitors such as p21 and concomitant cell cycle arrest and apoptosis might also play a role (Adams et al., 1999). Bortezomib like other proteasome inhibitors, e.g., lactacystin, also induce p53 accumulation, even though p53 does not seem to be crucial for sensitization of tumor cells (An et al., 2000). Microarray-based transcriptional profiling of the human multiple myeloma cell line MM.1S demonstrated that bortezomib induces a down-regulation of several gene transcripts important for cell cycle and survival pathways as well as a simultaneous up-regulation of proapoptotic transcripts (Mitsiades et al., 2002). Bortezomib induced the mRNA expression of several caspases, death receptors, and death ligands. It also decreased the antiapoptotic proteins Bcl-2 and BIRC3 as well as Toso and c-FLIP that are negative regulators of CD95-mediated apoptosis. It is important to note that bortezomib has presumably multiple mechanisms of action that involve not only the tumor cell itself, and that might contribute to the clinical success of the drug. An important target is also the tumor microenvironment, in which proteasome inhibitors abrogate the expression of adhesion molecules and soluble growth factors and thereby might block angiogenesis and tumor metastasis. Thus, bortezomib,

which is already a proven and validated drug, will certainly enter the clinic for the treatment of several tumor entities.

XII. Prospects of Apoptosis-Targeted Therapies

The importance of apoptosis as a pathogenic mechanism with a causative or contributing role for many diseases has become increasingly evident. The identification of the genes and gene products that regulate apoptosis, together with an increased knowledge about their mechanisms of action, has laid the foundation for the discovery of new drugs targeting apoptosis. The process of apoptosis is controlled at multiple molecular levels, each of which is influenced by different pro- and antiapoptotic proteins. The various decision points of life and death do not only provide an exciting multitude of molecular targets, but also offer a variety of therapeutic options.

Depending on the molecular target, different strategies are being employed. Recombinant biologicals including death ligands or agonistic and antagonistic antibodies that inhibit or trigger death receptor signaling have proven efficacy in various animal models. It will be exciting to see whether TRAIL agonists that have now reached clinical trials will be useful in combating cancer or other diseases, either as a single agent or more likely in combination with classical therapeutic regimens. Among all the apoptosis-based drug targets, strategies that target caspases are at the forefront for blocking apoptosis in numerous diseases. Proof-of-concept data have been obtained in several experimental models revealing that blockade of apoptosis by means of caspase inhibitors is a validated therapeutic strategy.

Despite very encouraging data both in vitro and in vivo, several open key issues which are critical for the success of apoptosis-based therapies still remain to be resolved. It is rather unknown whether caspases have physiological roles beyond that of apoptosis and if so, whether this would bring the risk for toxicity in the long-term use of caspase inhibitors. Another open question is whether preservation of cell survival by caspase inhibitors truly results in preservation of cell function. Although small molecule drugs have been slow to progress to the clinic, there are now two unrelated caspase inhibitors that have shown promising results in early clinical trials. At present, many compounds developed to modulate apoptosis are only micromolar inhibitors. Small molecule drugs or peptides like caspase inhibitors have to be optimized to readily penetrate the cells. There is still a great discrepancy between their in vitro and in vivo efficacy, which decisively limits their current use as therapeutics.

Despite these obstacles, many compounds bring the hope that, with sufficient modification by tools of structural biology and combinatorial chemistry, it might be possible to derive sufficiently potent drugs to inhibit

apoptosis. This caveat also holds true for endogenous inhibitors of apoptosis. The IAPs are of particular interest because they are the most powerful intrinsic inhibitors of cell death. Several studies have demonstrated that IAP antagonists are promising candidates for anti-cancer therapies by either directly killing cancer cells or by augmenting the established chemotherapy. Finally, setting the threshold of apoptosis susceptibility by modulating the Bcl-2/Bax rheostat is another appealing approach for therapy of cancer and other diseases. Nowadays, small molecules that mimic BH3 domains represent prototype therapeutics, and the structure-activity relation profile of these compounds remains to be improved. Moreover, it is still unclear whether Bcl-2 antagonists and BH3 mimetics should exert a selective or a broad-spectrum activity against the various members of the Bcl-2 family.

Many new drugs are currently being designed; however, of the hundreds or so molecules in development, most of them will remain in the preclinical state. The relatively low rate of clinical entry associated with these molecules is related to the lack of specificity, low efficacy, or development to drug resistance. These issues are being addressed as our understanding of the field evolves. Exploiting the various mechanisms at which apoptosis can be targeted offers hope that apoptosis-based therapies and improved clinical outcome for a wide range of illnesses may be not far from realization.

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