Defects in the regulation of apoptosis (programmed cell death) play important roles in many aspects of tumor pathogenesis and progression. For example, apoptosis defects allow neoplastic cells to survive beyond their normally intended life spans.[1] Thus, the need for exogenous survival factors is subverted. Protection is provided against hypoxia and oxidative stress as tumor mass expands; and time is allowed for accumulative genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis, and increase cell motility and invasiveness during tumor progression. In fact, apoptosis defects are recognized as an important complement to proto-oncogene activation, because many deregulated oncoproteins that drive cell division also trigger apoptosis (eg, Myc, E1a, Cyclin-D1).[2] Similarly, defects in DNA repair and chromosome segregation normally trigger cell suicide as a defense mechanism for eradicating genetically unstable cells; thus, apoptosis defects permit survival of the genetically unstable cells, providing opportunities for selection of progressively more aggressive clones.[3] Apoptosis defects also facilitate metastasis by allowing epithelial cells to survive in a suspended state, without attachment to an extracellular matrix.[4] These defects also promote resistance to the immune system because many of the weapons that cytolytic T cells and natural killer cells use for attacking tumors depend on the integrity of the apoptosis machinery.[5] Finally, cancer-associated defects in apoptosis play a role in chemoresistance and radioresistance, increasing the threshold for cell death and thereby requiring higher doses for tumor killing.[6] Thus, defective regulation of apoptosis is a fundamental aspect of the biology of cancer. Because apoptosis defects permit a wide variety of aberrant cellular behaviors, as exhibited in cancer cells, therapeutic strategies that negate the apoptosis advantage for tumors are predicted to selectively kill cancer cells as opposed to normal cells. Fundamentally, cancer cells should be more dependent on apoptosis defense mechanisms than normal cells and thus should be proportionally more sensitive to interventions that target apoptosis proteins and genes. To date, efforts to bring apoptosis-based strategies into animal models or human clinical trials have provided support for this concept of selective vulnerability of neoplastic cells as opposed to normal cells.
A solid knowledge base now exists about the mechanisms of apoptosis regulation, the proteins involved, their 3D structures, and biochemical mechanisms. Over the past 2 decades, a clearer understanding has emerged of the defects in expression or function of apoptosis-regulating genes and proteins relating to cancer. This information can now be exploited for devising strategies for small-molecule drug discovery toward the goal of revolutionary treatments for cancer and leukemia. **Apoptosis Pathways** Apoptosis is caused by proteases known as caspases, which stands for cysteine proteases.
aspartyl-specific proteases. Caspases constitute a family of intracellular cysteine proteases that collaborate in proteolytic cascades, where caspases activate themselves and each other.\[9,10\] Within these proteolytic cascades, caspases can be positioned as either upstream "initiators" or downstream "effectors" of apoptosis.\[11\] Eleven caspases have been identified in the human genome. Several pathways for activating caspases probably exist, though details remain sketchy for some of them (Figure 1). The simplest pathway is exploited by cytolytic T cells and natural killer cells, which inject apoptosis-inducing proteases, particularly granzyme B, into target cells via perforin channels.\[12,13\] Unlike the caspases, granzyme B is a serine protease, but similar to the caspases, granzyme B specifically cleaves substrates at Asp residues. Granzyme B is capable of cleaving and activating multiple caspases and some caspase substrates. Endogenous and viral inhibitors of granzyme B have been identified, accounting for resistance to this apoptotic inducer.\[14-16\] Another caspase-activation pathway is represented by the tumor necrosis factor (TNF)-family receptors. Of the approximately 30 known members of the TNF family in humans, 8 contain a so-called death domain in their cytosolic tails.\[17\] Several of these death domain-containing TNF family receptors use caspase activation as a signaling mechanism, including TNFR1/CD120a, Fas/APO1/CD95, DR3/Apo2/Weasle, DR4/TrailR1, DR5/TrailR2, and DR6. Ligation of these receptors at the cell surface results in the recruitment of several intracellular proteins, including certain procaspases, to the cytosolic domains of these receptors, forming a "death-inducing signaling complex" (DISC) that triggers the activation of caspases and leads to apoptosis.\[18,19\] The specific caspases summoned to the DISC are caspase-8 and, in some cases, caspase-10. These caspases contain so-called death effector domains in their N-terminal prodomains that bind to a corresponding death effector domain in the adapter protein, Fas-associated death domain (FADD), thus linking them to the TNF-family death receptor complexes. Mitochondria also play important roles in apoptosis, releasing cytochrome c into the cytosol, which then causes assembly of a multiprotein caspase-activating complex, referred to as the "apoptosome."\[20,21\] The central component of the apoptosome is Apaf-1, a caspase-activating protein that oligomerizes on binding cytochrome c and that specifically binds procaspase-9. Apaf-1 and procaspase-9 interact via their caspase-associated recruitment domains (CARDs). Such a CARD-CARD interaction plays important roles in many steps in the pathways of apoptosis. The mitochondrial pathway for apoptosis is activated by myriad stimuli, including growth factor deprivation, oxidants, Ca\(^{2+}\) overload, DNA-damaging agents, and others. Mitochondria can also participate in cell death pathways induced via TNF-family death receptors, through crosstalk mechanisms involving proteins such as Bid, BAR, and Bap31.\[22-25\] However, mitochondrial (intrinsic) and death receptor (extrinsic) pathways for the activation of caspases are fully capable of independent operation in most types of cells.\[26\] In addition to cytochrome c, mitochondria also release several other proteins of relevance to apoptosis, including endonuclease G, AIF (an activator of nuclear endonucleases), and inhibitor of apoptosis protein (IAP) antagonists Smac (Diablo) and Omi (HtrA2). Pathways of apoptosis linked to damage in the endoplasmic reticulum and Golgi, as well as a pathway linked to nuclear structures called PODs (PML oncogenic domains) or nuclear bodies, have also been described but are poorly characterized to date.
Suppressors of Apoptosis

Several antagonists of the caspase-activation pathways have been discovered, and multiple examples of dysregulation of their expression or function in cancers have been obtained. Because our current knowledge is greatest where the mitochondrial ("intrinsic") and TNF-family death receptor ("extrinsic") pathways for apoptosis are concerned, most available information about antagonists centers on these two apoptotic pathways. In this article, three types of apoptosis-suppressing proteins known to be overexpressed in tumors, including prostate cancers, are considered: IAPs, FLIP, and Bcl-2. **Inhibitor of Apoptosis Proteins** Inhibitor of apoptosis proteins represent an evolutionarily conserved family of suppressors of apoptosis. Members of the IAP family, originally identified in baculoviruses, contain one or more copies of a domain called the baculoviral IAP repeat (BIR). These BIR domains are sometimes accompanied by other domains, including RING domains, ubiquitin-conjugating enzyme folds (E2s), and NACHT-family nucleotide-binding domains. The human genome encodes eight IAP-family members: XIAP, cIAP1, cIAP2, Naip, Apollon (Bruce), ILP2 (Ts-IAP), ML-IAP (K-IAP; Livin), and Survivin. The BIR domains of several IAP-family proteins were originally shown by our laboratory to be responsible for directly binding and specifically inhibiting caspases, thus identifying IAPs as endogenous inhibitors of cell
death proteases.[27-31] Multiple other laboratories have confirmed and extended these findings, providing conclusive evidence that many IAP-family proteins operate as caspase suppressors.[32-41] However, IAPs vary in the specific caspases they inhibit. For example, XIAP suppresses both downstream effector caspases that operate at points of convergence of apoptosis pathways and caspase-9, the apical protease in the mitochondrial pathway for apoptosis.[27,29,30] In contrast, ML-IAP is a potent suppressor of only caspase-9. No examples of IAP-mediated suppression of proteases that operate in the upstream portions of the apoptosis pathway activated by TNF-family receptors have been found (Figure 2). Evidence of overexpression of IAPs in cancer has been obtained, suggesting a role for these suppressors of apoptosis in malignancy.[31,42] For example, the IAP-family member Survivin is overexpressed in most cancers[43] and has become a topic of considerable attention for its dual role as a regulator of cell division (chromosome segregation and cytokinesis) and apoptosis.[44-46] Similarly, the IAP-family member ML-IAP is rarely expressed in normal tissues but is found at elevated levels in melanomas and some renal cancers.[33,40,47] Moreover, XIAP has been reported by our group to be overexpressed in a substantial proportion of acute myelogenous leukemias, with higher levels correlating with shorter remission durations and shorter overall patient survival.[48] Evidence of overexpression of XIAP has also been reported for renal and lung cancers[49,50]; overexpression of cIAP1 has been associated with ovarian cancer. Chromosomal translocations activating cIAP2 are found in some lymphomas.[51] Thus, various IAP-family proteins are overexpressed in specific types of cancer. However, more than one member of the IAP family can be overexpressed simultaneously by some tumors. For example, in prostate cancers, we found evidence that protein levels of XIAP, cIAP1, cIAP2, and Survivin can sometimes become simultaneously increased in tumors.[52] suggesting redundancy in expression of these antiapoptotic proteins. We have also found evidence of apparent simultaneous overexpression of cIAP1, cIAP2, and Survivin in colon cancer (manuscript in preparation). The observation of overexpression of multiple IAP-family members implies that perhaps some aspects of their regulation are shared. Indeed, during a screen of the National Cancer Institute panel of 60 human tumor cell lines, assessing IAP expression at the messenger RNA (mRNA) and protein levels, we obtained evidence that mRNA levels of XIAP, cIAP1, and cIAP2 do not correlate with their protein levels,[48] suggesting that posttranscriptional regulation of these IAP-family proteins is important. Interestingly, all three of these IAP-family proteins contain a RING domain that binds E2s (ubiquitin-conjugating enzymes), implying that alternations in the turnover rate of IAP-family proteins may occur in cancers that overexpress multiple family members simultaneously. The functional importance of overexpressed IAPs for apoptosis suppression in cancers has been supported by antisense experiments.[53-57] In these experiments, knocking down expression of Survivin, XIAP, or other IAPs has been shown to induce apoptosis of tumor cell lines in culture or to sensitize tumor cell lines to apoptosis induced by anticancer drugs.[53-57] In contrast, gene knockout studies in mice imply that normal cells are possibly less dependent on IAPs than tumor cells because targeted disruption of the genes for xiap, ciap1, and ciap2, both individually and in combination, produces little phenotype.[58] personal communication, T. Mak, 2004] Implications for Treatment
Taken together, these observations imply that drugs that interfere with the action of IAPs could be useful for the treatment of cancer. Recently, a strategy for devising small-molecule inhibitors of IAPs has been suggested by the discovery of natural antagonists of IAPs.[35,38] Proteins such as Smac and Omi (HtrA2) have been shown to bind IAPs and suppress them, releasing caspases to kill cells.[35,38,59] A 7’mer peptide corresponding to the Nterminus of Smac is reported to be sufficient to bind IAPs and block their association with caspases.[60] Moreover, we have confirmed that peptides as short as tetramers can potently reverse caspase inhibition by IAPs, functioning in a stoichiometric manner at micromolar concentrations.[61; unpublished data] By fusing membrane-penetrating peptides onto Smac or Omi peptides, it is possible to induce apoptosis of cancer cell lines in culture as well as to suppress tumor formation in xenograft models in mice.[62-65] Thus, these data provide proof-of-concept evidence that small molecules that mimic the effects of these IAP-binding peptides could potentially be exploited as drugs for cancer treatment. Drug Discovery Strategies
Structural analysis of the interactions of IAPs with caspases and of IAPs with Smac has helped to lay a foundation for such drug-discovery efforts. First, our structure-function studies of IAP-family member XIAP showed that, although this protein contains three tandem BIR domains, a single BIR is sufficient to bind and suppress caspases. These studies demonstrated that the BIR2 domain specifically inhibits caspase-3 and caspase-7, whereas the BIR3 domain of XIAP blocks the activity of caspase-9.[29,30] Thus, discrete domains in IAPs are responsible for binding and inhibiting caspases. Second, the 3D structure of the BIR3 domain complexed with Smac revealed that the
N-terminal 4 amino acids of the mature Smac protein binds in the same crevice normally occupied by the N-terminus of the small subunit of caspase-9, thus suggesting completion for binding.[37,60,66,67] Consequently, small-molecule compounds that mimic the Smac 4?mer peptide should dislodge active caspase-9 from BIR3, thus inducing apoptosis (Figure 3). The structural details regarding the interaction of BIR2 of XIAP with caspases and its relation to Smac are less clear due to poor atomic resolution of the N-terminus of the small subunit of caspases-3 or -7 complexed with BIR2, as determined by x-ray crystallography by scientists at our institution and elsewhere.[32,39] In the crystal structure of the XIAP BIR2-caspase-3 complex, the NH2- terminus of the caspase-3 p10 subunit interacts with the surface of BIR2,[32] which may be an artifact of crystallization. Though, to date, the mechanism of inhibition of XIAP by Smac remains unclear, modeling studies suggest the presence of a similar Smac-binding pocket on BIR2. In addition to chemical inhibitors of IAPs based on mimicking Smac, other strategies can also be envisioned and have begun to be exploited. For example, using an enzyme derepression assay where screens were performed to identify compounds capable of dislodging XIAP from caspase-3 and restoring protease activity, we and other investigators have identified small-molecule antagonists of XIAP.[68,69] These compounds target a non-Smac site on XIAP, which remains to be defined at the structural level. Interestingly, in addition to Smac and Omi (HtrA2), other endogenous antagonists of IAPs have been reported, including XAF1, NRAGE, and ARTS, which operate through an alternative mechanism.[70-72] Thus, it is conceivable that the aforementioned small-molecule antagonists of IAPs mimic one or more of these endogenous antagonists of IAPs, a concept awaiting experimental testing.

**Interleukin-1beta-Converting Enzyme Inhibiting Protein (FLIP)** The pathway of apoptosis triggered by TNF-family death receptors is fundamental to the mechanisms by which cytolytic T cells attack and kill tumor cells.[73-78] Cytolytic T cells, natural killer cells, macrophages, and dendritic cells have been demonstrated to produce one or more of the TNF-family death ligands, such as FasL, TNF, or TRAIL. On binding their specific receptors on susceptible target cells, these receptors recruit procaspase-8 and/or -10 to the receptor complex, forming a DISC that results in the activation of caspases.[11,79] Perhaps not surprisingly, many tumors develop resistance to this extrinsic pathway for apoptosis at some point in their pathogenesis or progression, reducing or ablating their sensitivity to immune cell attack.[5] Multiple antagonists of the extrinsic pathway have been identified, including several death effector domain-containing proteins that compete for binding to the adapter proteins or procaspases that participate in TNF-family death receptor signaling, including FLIP, BAR, and possibly Bap31.[80,81] Among them, FLIP has received the most attention for its role in producing Fas-resistant states in tumor cells.[82,83] The FLIP protein is highly similar in its overall sequence to procaspases-8 and -10, containing tandem copies of the death effector domain, followed by a pseudocaspase domain that lacks enzymatic activity. FLIP can promote apoptosis in some circumstances.[84] However, for the most part, this protein is antiapoptotic, forming complexes with procaspase-8 and -10, and preventing their effective activation, as well as competing for binding to adapter proteins required for the recruitment of caspases to receptors of death-receptor complexes.[5,83] Overexpression of FLIP occurs commonly in cancers. Our laboratory has determined by antisense and gene transfer studies that FLIP is an important determinant of resistance of some tumor cell lines to the induction of apoptosis by TNF, Fas, and TRAIL.[85,86] Moreover, in a collaborative effort with other investigators, we have identified a class of compounds called synthetic triterpenoids that cause reductions in FLIP in multiple human tumor cell lines, correlating with the restoration of sensitivity to TRAIL-induced apoptosis.[85] Thus, small-molecule drugs that ablate expression or function of FLIP represent an attractive approach to sensitizing tumor cells to TNF-family death ligands. The prototype triterpenoid shown to reduce the expression of FLIP is CDDO (2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid). At submicromolar levels, this compound, as well as selected analogs, reduces the levels of FLIP protein by a mechanism involving ubiquitination and proteasome-dependent degradation of the FLIP protein without affecting FLIP mRNA levels.[85,87,88] CDDO has no effect on the levels of many other apoptosis proteins at submicromolar levels sufficient to reduce FLIP, including FADD, caspase-8, DAP3, XIAP, Bcl-2, Bcl-XL, Bax, Mcl-1, and Bak. However, the mechanism of this compound is presently undefined, and, indeed, it is likely that other proteins besides FLIP are involved in its proapoptotic mechanism. Indeed, when employed at higher doses, CDDO and related compounds are reported to have effects on a number of cancer-relevant targets.[89] When tested on solid-tumor cell lines at submicromolar concentrations, CDDO generally sensitizes only to TRAIL and Fas, but alone CDDO does not induce significant apoptosis. Applied to primary leukemia cells, however, CDDO and related compounds demonstrate single-agent activity, inducing robust apoptosis via a caspase-8-dependent mechanism, including chemorefractory chronic lymphocytic leukemias and acute myelogenous
leukemias.[87,90,91] Thus, by activating the extrinsic pathway, CDDO and related triterpenoids may provide a pharmacologic route to bypass roadblocks to intrinsic pathway (mitochondrial) apoptosis, thereby achieving apoptotic destruction of chemorefractory leukemias.

Figure 3: Structure of XIAP BIR3 Domains Bound to Smac Peptides—A model of the putative Smac-binding pocket on BIR3 of XIAP is presented, showing the super position of the 4’mer SMAC peptide (AVPI) and N-terminal peptide from the small subunit of caspase-9 (blue).
agents that trigger the extrinsic pathway are also being explored for their utility in cancer treatment. For example, an agonistic antibody that activates TRAIL receptor-1 (DR4) has been tested in phase I trials of patients with malignancy. Recombinant trimeric TRAIL protein has produced impressive preclinical results in mouse models, either alone or in combination with chemotherapy, and also may soon enter clinical trials.[92] 

**Bcl-2-Family Proteins**

Bcl-2 protein is the founding member of a large family of apoptosis-regulating proteins that govern the intrinsic pathway of apoptosis. Overexpression of the antiapoptotic protein Bcl-2 occurs in roughly half of human cancers, contributing to resistance to anticancer drugs, hormone ablative therapy, and radiotherapy.[1] Several homologs of Bcl-2 protein have been identified and characterized, with some functioning as blockers (n = 5 in humans) and others as promoters of cell death (n = 19 in humans),[93-95] comprising a gene family of 25 members.[96; unpublished data]. Alterations in the expression of several members of the Bcl-2-family protein have been documented in cancers, including overexpression of antiapoptotic members and loss of expression of proapoptotic members.[97] Simultaneous overexpression of more than one of the six antiapoptotic members of the Bcl-2-family proteins can occur in some cancers, creating challenges with respect to overcoming roadblocks to apoptosis. Our prior analysis of prostate tumors, for example, revealed that levels of antiapoptotic proteins Bcl-2, Bcl-XL, and Mcl-1 are generally elevated in advanced prostate cancers, whereas proapoptotic proteins Bax and Bak generally remain present at high levels during progression of these tumors to a hormone-independent, metastatic phenotype.[98] Similar findings have been made for melanomas, which commonly overexpress proteins Bcl-2, Bcl-XL, and Mcl-1.[99] Bcl-2-family proteins operate as regulators of the mitochondriadependent pathway for apoptosis (intrinsic pathway). These proteins govern the permeability of the mitochondrial membrane, dictating whether apoptogenic proteins such as cytochrome c are released into the cytosol.[21,95,100] One of the prominent mechanisms by which the mitochondrial (intrinsic) pathway for apoptosis cross-talks with the death receptor (extrinsic) pathway involves caspase-8-mediated cleavage and activation of the proapoptotic Bcl-2 homolog Bid. Normally, Bid resides in the cytosol in a latent (inactive) state; however, on cleavage by caspase-8, this protein translocates to the outer membrane of the mitochondria, where it dimerizes with other Bcl-2--family proteins, inducing the release of cytochrome c and

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**Figure 4: BH3 Peptide Bound to Bcl-2 Protein**—The 3D structure of Bcl-2 protein is depicted in space-filling mode, with a BH3 peptide (in ribbon mode) bound to a hydrophobic crevice.
Apoptosis Mechanisms: Implications for Cancer Drug Discovery

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Apoptosis, a process crucial in tissue development and maintenance, involves a cascade of events that ultimately result in cell death. Understanding the mechanisms of apoptosis is vital in cancer research, as it can provide new avenues for drug discovery.

**Apoptosis Mechanisms**

Apoptosis occurs in multiple steps, including binding of a death receptor (e.g., Fas, TNF, or TRAIL) to its receptor on the cell surface, which triggers the activation of caspases, a family of proteases.

**Overexpression of Antiapoptotic Proteins**

Many cancer cells exhibit a wide variety of abnormal behaviors and molecular processes that normally would trigger an apoptosis response, including apoptosis sensitivity compared with normal cells. Cancer cells exhibit a wide variety of abnormal behaviors and molecular processes that normally would trigger an apoptosis response, including apoptosis sensitivity compared with normal cells.

**Overexpression of Bcl-2**

Overexpression of Bcl-2, a protein that inhibits apoptosis, is a common occurrence in cancer cells. This overexpression can confer a survival advantage to cancer cells, making them resistant to apoptosis induction.

**Bcl-2 Antagonists**

Bcl-2 antagonists are molecules that work to reverse the effects of Bcl-2 overexpression. This can be achieved through various mechanisms, including the delivery of BH3 peptides that mimic the antiapoptotic properties of Bcl-2, thereby promoting apoptosis.

**Clinical Testing**

Bcl-2 antagonists have been tested in clinical trials. One such compound is oblimersen sodium (Genasense), which inhibits Bcl-2 by hybridizing with the first 18 nucleotides within the coding region of Bcl-2 mRNAs, reducing the expression of Bcl-2 protein and thereby promoting apoptosis.

**Conclusions**

Knowledge of the pathways of apoptosis and of the mechanisms of the proteins that govern them is beginning to reveal a variety of targets for the discovery of cancer drugs. Detailed structural analysis of apoptotic proteins and studies of their biochemical mechanisms have suggested strategies for lead generation, resulting in numerous novel chemical entities with mechanism-based activity. Encouraging proof-of-principle data have been provided that help to validate several targets of apoptosis. Much work lies ahead, however, in terms of optimizing the spectrum of activity of compounds that interact with multiple members of apoptosis protein families, improving the stability and pharmacologic properties of these compounds, establishing their optimal formulations for stability and delivery, and elucidating attendant rate-limiting toxicities. Many of the most logical targets for promoting apoptosis of cancer and leukemia cells are technically challenging and often involve either disrupting protein interactions or altering gene expression, as opposed to traditional pharmaceuticals that typically target the active sites of enzymes. Modern techniques of structure-based drug optimization render this task feasible, but still challenging. Such targets require long-term commitments, often outstripping the usual drug discovery and development cycle incorporated into the practices of pharmaceutical companies. Long-term commitments to research may create a new era in cancer therapy, where the intrinsic or acquired resistance of malignant cells to apoptosis can be pharmacologically reversed, reinstating natural pathways for cell suicide. There is good reason to suspect that malignant cells will be preferentially susceptible to restoration of apoptosis sensitivity compared with normal cells. Cancer cells exhibit a wide variety of abnormal behaviors and molecular processes that normally would trigger an apoptosis response, including...
cell-cycle checkpoint dysregulation, oncogene activation, chromosome segregation defects, cell detachment from substratum, and outgrowth of blood supply (hypoxia). These defects render tumor cells more dependent on apoptosis-suppressing genes and proteins, and thus withdrawing this support from malignant cells may promote self-destruction of transformed cells while sparing normal cells. The full validity of this hypothesis awaits verification in human clinical trials. However, present insights from animal studies and current forays into the clinic are encouraging. Apoptosis-based strategies for the discovery of cancer drugs promise to yield effective therapies against cancer and merit further research support.

Disclosures: Dr. Reed is a shareholder in Genta Incorporated.

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