Review

Inhibitor of Apoptosis Proteins: Translating Basic Knowledge into Clinical Practice

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Abstract

The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic proteins that bind and inhibit caspases 3, 7, and/or 9, but not caspase 8. Growing evidence also indicates that IAPs also modulate cell division, cell cycle progression, and signal transduction pathways. As our basic understanding of IAPs has increased, the knowledge is being translated into clinically useful applications in the diagnosis and treatment of malignancy. For example, IAPs such as survivin are being investigated as diagnostic markers for the presence of occult malignancy. In addition, IAP overexpression is a poor prognostic marker in a variety of solid tumors and hematologic malignancies. Finally, IAPs are attractive therapeutic targets, and efforts are under way to develop antisense and chemical IAP inhibitors that may be useful for the treatment of a variety of malignancies. For all of these potential clinical applications, however, the challenge remains to incorporate these findings into actual clinical practice.

Introduction

At a fundamental level, cancer fails to respond to treatment because the malignant cells fail to die in response to chemotherapy, radiation, or the immune surveillance by endogenous cytotoxic T cells and natural killer cells. In part, the failure of cell death is due to failure of the apoptosis and caspase activation pathways. The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic proteins that block cell death, in part, by inhibiting the downstream portion of the caspase activation pathways. This review will describe the mechanism by which these proteins modulate apoptosis and discuss how the basic understanding of IAP function is being translated into the clinical practice of oncology in the form of new diagnostic and prognostic markers as well as novel therapies.

Caspase Activation Pathways

Molecularly, classical apoptosis is caused by the activation of caspases, a family of intracellular cysteine proteases that cleave substrates at aspartic acid residues (1, 2). Caspases lie in a latent (zymogen) state in cells but become activated in response to a wide variety of cell death stimuli. Through a proteolytic cascade, caspases are functionally connected to each other, with upstream (initiator) caspases cleaving and activating downstream (effector) caspases (3).

At present, IAPs inhibit at least two of the major pathways for initiation of caspase activation: (*a*) the mitochondrial pathway with cytochrome *c*, and (*b*) the death receptor pathway with the tumor necrosis factor (TNF) family of death receptors. IAPs also influence a third minor pathway in which granzyme B directly activates caspase 3 (refs. 4 and 5; Fig. 1).

The Mitochondrial Pathway of Caspase Activation. The intrinsic pathway for caspase activation is initiated by the release of cytochrome *c* from the mitochondria. Cytochrome *c* is normally sequestered between the inner and outer membranes of the mitochondria. In response to a variety of proapoptotic stimuli, cytochrome *c* is released into the cytosol (6). Cytochrome *c* then binds and activates Apaf-1. Apaf-1 activates procaspase 9, which in turn cleaves procaspase 3 (7, 8).

The Death Receptor Pathway of Caspase Activation. The death receptor pathway for caspase activation begins with the TNF family of cytokine receptors, which includes Fas (CD95), DR4 (Trail-R1), and TNF-R1 (CD120a). Death receptors are activated by ligand binding to the extracellular domain of the receptor. Once activated, death receptors recruit the death domain protein Fadd/Mort-1. When bound to the death receptor, Fas-associated death domain in turn binds caspase 8 (9, 10), thereby forming the death-inducing signaling complex (DISC). As caspase 8 concentrates at the DISC, it dimerizes and thereby becomes active (11). Cleavage of caspase 8 enhances its stability in the dimerized form (11). Active caspase 8 is released from the DISC into the cytosol, where it cleaves and activates the downstream effector caspases (12, 13).

Convergence Point and Cross-Talk. The intrinsic and extrinsic pathways converge at the activation of downstream effector caspases such as caspase 3. Active caspase 3 then cleaves critical intracellular proteins to induce the final stages of cell death. Although the two caspase pathways are presented as separate entities, cross-talk exists between them. For example, the two pathways cooperate to enhance apoptosis through Bid. Bid, a member of the BH3-only family of proapoptotic proteins, is cleaved and activated by caspase 8. When cleaved, Bid migrates to the mitochondria, where it promotes permeabilization of the mitochondrial membrane, cytochrome *c* release, and initiation of the intrinsic pathway of caspase activation (14–16).

The Inhibitor of Apoptosis Protein Family of Caspase Inhibitors

The IAPs are a family of caspase inhibitors that specifically inhibit caspases 3, 7, and 9 and thereby prevent apoptosis. Crook *et al.* (17) identified the first IAP family member serendipitously while studying vAcAnh-induced apoptosis in SF21 baculovirus cells. During a screen for baculovirus genes that mimicked the actions of p35 and inhibited vAcAnh-induced apoptosis, they identified a novel 1.6-kb gene encoding a 31-kDa antiapoptotic protein with a zinc finger-like motif (17). Subsequent studies identified IAPs in a diverse range of species and discerned that IAPs inhibit apoptosis by blocking caspases (reviewed in ref. 18).

The Inhibitor of Apoptosis Protein Family

To date, eight human IAP family members have been identified (Fig. 2), and IAP homologues have also been described in insects and yeast. IAP proteins are grouped into this family based on the presence of one to three baculovirus IAP repeat (BIR) domains, a zinc-binding region of ~70 amino acids. Although the BIR domain is required for
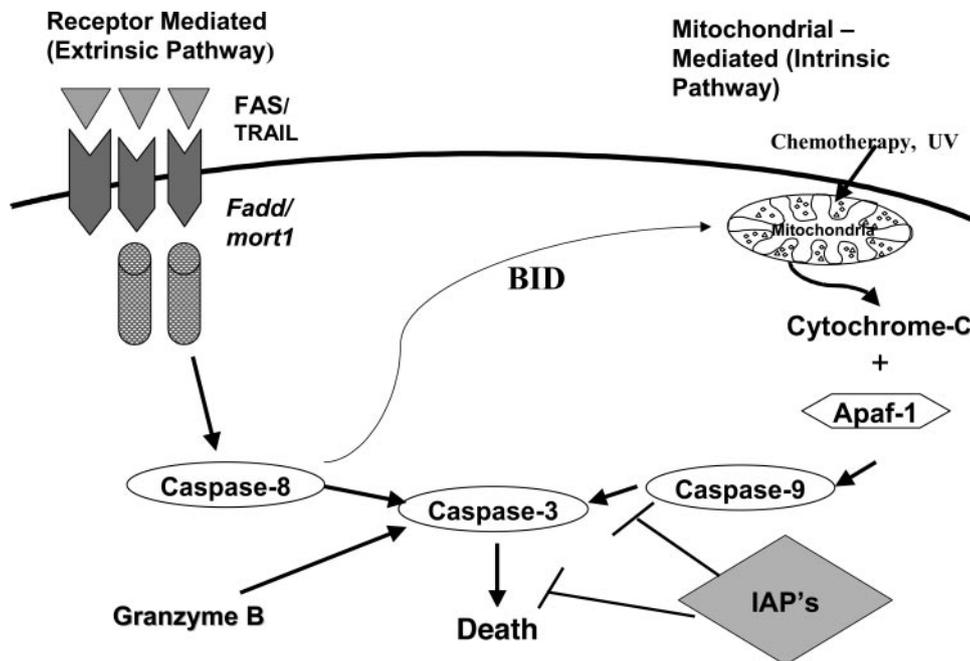
Fig. 1. Caspase activation pathways. Currently, at least two major pathways for initiation of caspase activation have been identified: (*a*) the intrinsic (mitochondrial) pathway with cytochrome *c*, and (*b*) the extrinsic (death receptor) pathway with the TNF family of death receptors. A third minor pathway has been identified in which granzyme B directly activates caspase 3.

membership in the IAP family, not all BIR-containing proteins appear to have antiapoptotic functions (19–22), so the presence of a BIR domain is necessary but not sufficient for inclusion in this protein family. IAP proteins may also contain a RING or caspase activation recruitment (CARD) domain (reviewed in ref. 18). The IAP proteins have been divided into three classes (classes 1, 2, and 3) based on the presence or absence of a RING finger and the homology of their BIR domains (23).

Class 1 Inhibitor of Apoptosis Proteins. Class 1 IAPs contain homologous BIR domains and a RING finger motif. X-linked IAP [XIAP (also known as hILP, MIHA, and BIC4)] has three BIR domains and a RING finger. It was the first IAP in this class to be identified and remains the best characterized. Duckett *et al.* (24) identified XIAP in 1996 in a search for mammalian genes homologous to the baculovirus IAP. It binds and inhibits caspases 3, 7, and 9 with nanomolar affinity, but it does not bind or inhibit caspase 8 (25,

26). cIAP1 (also known as MIHB, hiap2, and BIRC2) and cIAP2 (also known as MIHC, hiap2, and BIRC3) are structurally related to XIAP with three BIR domains and a RING finger. These IAPs were identified through the biochemical purification of proteins associated with the death receptor TNF-R2, but their role at this receptor remains unclear (27). cIAP1 and cIAP2 are expressed in most human tissues, but cIAP1 expression is highest in the thymus, testis, and ovary, and cIAP2 expression is highest in the spleen and thymus (27). cIAP1 and cIAP2 bind and inhibit caspase 3 and 7, albeit less strongly than XIAP (25). They do not inhibit caspases 1, 6, and 8. ML-IAP (also known as livin, KIAP, and BIRC7) and ILP-2 have a RING finger and only one BIR domain, but their BIR domain is most homologous to the BIR3 domain of XIAP, cIAP1, and cIAP2 (hence their inclusion in this class). ML-IAP is expressed in normal fetal liver, kidney, and adult testis and thymus as well as melanoma and lymphoma cell lines. ML-IAP inhibits caspases 3 and 9 with an affinity similar to that of
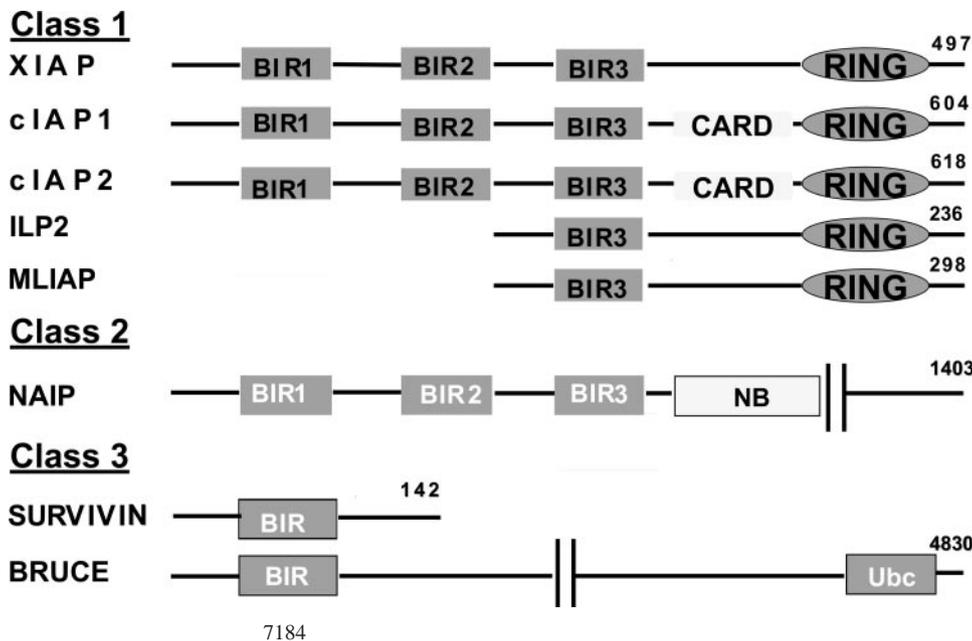
Fig. 2. The IAP family of proteins. To date, eight human IAP family members have been identified, based on shared BIR domains. IAP members may also contain a CARD domain and RING finger motif. BRUCE has an E2 ubiquitination enzyme motif (*Ubc*), and NAIP has a nucleotide binding domain (*NB*). To group the IAP proteins, they have been divided into three classes (classes 1–3), based on the presence or absence of a RING finger and the homology of their BIR domains.

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cIAP1 but does not bind or inhibit caspases 1, 2, 6, or 8 (28). ILP-2 expression is normally restricted to the adult testis but has also been documented in a lymphoblastoid cell line. ILP-2 inhibits caspase 9, but not caspases 3, 7, or 8 (29). It remains to be determined whether the variations in affinity for caspases among the different IAPs relate to their endogenous intracellular functions.

Class 2 Inhibitor of Apoptosis Proteins. The Class 2 IAP family member NAIP has three BIR domains but no RING finger motif. Its BIR domains are more distantly related to the BIR domains of the class 1 IAPs. NAIP was identified in 1995 by Roy *et al.* (30), while they were searching for the gene on 5q13 responsible for childhood spinal muscular atrophies. NAIP is expressed in adult liver, placenta, and central nervous system. It inhibits caspases 3 and 7, but not caspases 1, 4, 5, or 8 (31).

Class 3 Inhibitor of Apoptosis Proteins. Class 3 IAP members, such as survivin, contain only a single BIR domain and no RING finger. Survivin is expressed in the fetal liver, kidney, lung, and gastrointestinal tract, but it is not expressed in most normal adult tissue (32). The preferential expression of survivin in fetal tissue suggests that it plays a role in development. Survivin is frequently overexpressed in a variety of malignancies including adenocarcinomas of the lung, pancreas, colon, breast, and prostate (32–36).

The differential expression between normal and malignant cells can be exploited for therapeutic purposes. For example, the survivin promoter could be used as a tumor-specific promoter in which a gene of interest is turned on in malignant cells, but not in normal cells. This transcriptional targeting may be of value in cancer gene therapy (37). Alternatively, survivin expression could be used as a tumor marker for the early identification of malignancy as discussed in detail below.

Inhibitor of Apoptosis Proteins Inhibit Active Caspases

Caspase inhibition is the best understood mechanism by which IAPs prevent apoptosis. In enzymatic reactions, recombinant XIAP inhibits caspase 3, 7, and 9, but not caspase 8. *In vitro*, overexpression of XIAP in 293T cells prevents BAX- and FAS-induced cleavage of procaspase 3 and apoptosis (38). The effect of XIAP on caspase activation has been mapped to its BIR domains, with the BIR2 domain inhibiting caspases 3 and 7, and the BIR3 RING domain inhibiting caspase 9.

Structural Basis of Caspase Inhibition by X-Linked Inhibitor of Apoptosis Protein. Given the cellular and enzymatic data indicating that IAPs inhibit caspases, efforts were undertaken to explore the physical interactions between IAPs and caspases. Most studies have focused on XIAP because it can be produced in recombinant form and crystallized. XIAP inhibits caspases 3 and 7 and caspase 9 through separate domains. Its BIR2 domain (amino acids 163–240) with its NH₂-terminal extension (amino acids 124–162) inhibits caspases 3

and 7, whereas its BIR3 domain (amino acids 241–356) inhibits caspase 9 (39). These studies provided the basis for developing IAP inhibitors that target the caspase-binding pockets of the molecule.

BIR2 Domain Inhibits X-Linked Inhibitor of Apoptosis Protein. A "hook, line, and sinker" model has been proposed to explain how XIAP inhibits caspase 3 (Fig. 3). The "hook" (residues 138–146 of the NH₂ terminus) inhibits capase 3 by lying across the active site of the caspase, thereby blocking the substrate-binding pocket of active caspase 3. The "line" represents two peptide bonds on Val¹⁴⁷ that connect the hook to the sinker. The "sinker" (residues 148–150) stabilizes the interaction between XIAP and caspase 3 (40). In this model, XIAP inhibits caspase 3 through steric hindrance. As such, it inhibits caspases 3 and 7 through a mechanism distinct from peptidyl caspase inhibitors such as benzyloxycarbonyl-VAD-fluoromethyl ketone that compete with the caspase substrate for the binding pocket.

BIR3 Domain Inhibits X-Linked Inhibitor of Apoptosis Protein. Early studies determined that the BIR3 domain of XIAP could inhibit caspase 9 (26, 41), but only recently has the mechanism been elucidated. The BIR3 domain of XIAP forms a heterodimer with monomeric caspase 9, thereby preventing caspase 9 dimerization and activation. In addition to trapping caspase 9 in a monomeric form, it also keeps the active site of caspase 9 in an inactive conformation (42). Thus, it is interesting to note that XIAP inhibits caspase 9 without physically touching the active site.

In an extension to the structural studies, the effects of mutations in the BIR domains on the antiapoptotic effects of XIAP have been examined. Mutations affecting the NH₂-terminal extension of BIR2 (*e.g.,* D148A) abolish the protective role of XIAP in preventing Fas ligand (a stimulus of the extrinsic pathway of caspase activation) or Bax-induced apoptosis (a stimulus of the intrinsic pathway of caspase activation). In contrast, mutations affecting the BIR3 domain (*e.g.,* W310A) reduce XIAP-mediated inhibition of apoptosis induced by BAX, but not by CD95 (43). Thus, these mutations support the structural studies demonstrating that the BIR2 domain is required for the inhibition of caspase 3 and that the BIR3 domain inhibits caspase 9.

Survivin. Whereas XIAP inhibits caspases 3, 7, and 9 through direct interactions, the mechanism by which survivin inhibits caspases is less clear. According to some studies (44, 45), survivin binds and inhibits active caspases 3 and 7, but not caspase 8. In contrast, others (46) do not detect an interaction of survivin with caspase 3. A study by Marusawa *et al.* (47) reported that survivin does not inhibit recombinant caspases 3, 7, or 9 in enzymatic reactions or in cytosolic extracts previously stimulated with cytochrome *c* and dATP. However, when survivin is added to cytosolic extracts before the activation of caspase 9 by addition of cytochrome *c* and dATP, it prevents caspase 3/7 activation. Thus, these results suggest that survivin inhib-

Fig. 3. The hook, line, and sinker model for caspase inhibition by XIAP. A hook, line, and sinker model can explain how XIAP inhibits caspase 3 by steric hindrance. The hook in the NH₂-terminal extension of the BIR2 domain of XIAP inhibits caspase 3 by lying across the active site of the caspase, thereby blocking the substrate-binding pocket of active caspase 3. The line is formed by two peptide bonds that connect the hook to the sinker. The sinker stabilizes the interaction between XIAP and caspase 3.

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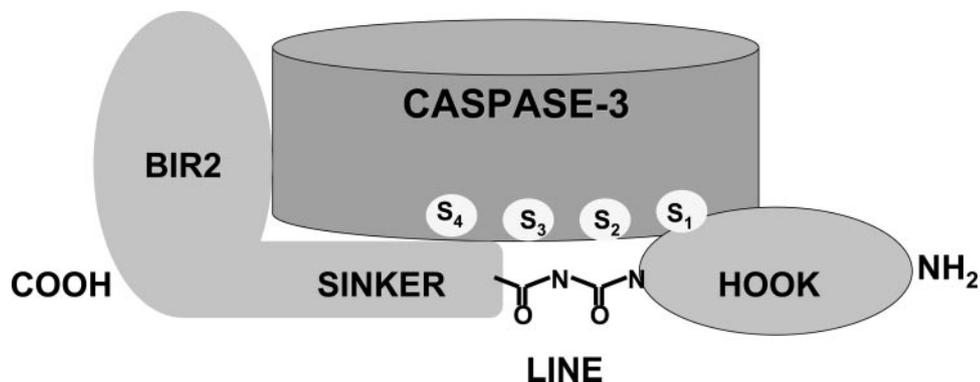
its active caspase 9, but not active caspases 3 and 7, and that the inhibition of caspase 9 requires a cofactor. To identify such a cofactor, Marusawa *et al.* (47) used a two-hybrid screen to identify survivin-binding proteins and identified HBXIP. In enzymatic reactions, survivin and HBXIP in combination (but neither protein alone) inhibited caspase 9 activity. Additional studies will be necessary to resolve these discrepant studies and decipher the mechanism by which survivin inhibits caspases. Furthermore, to generalize the importance of HBXIP as a necessary cofactor for survivin, it will need to be evaluated in other cell systems. Such work will be an important step toward developing chemical survivin inhibitors.

Beyond Caspase Inhibition

Most attention has focused on IAPs as caspase inhibitors, but multiple lines of evidence indicate that IAPs can inhibit apoptosis through effects on cell cycle progression, cell division, and signal transduction.

Inhibitor of Apoptosis Proteins Regulate Cell Division. The IAP proteins likely play a role in cell division. For example, yeast lack caspases but have IAP homologues that contain one BIR domain. Deletion of the yeast IAP leads to inefficient spore formation, indicating that, at least in yeast, IAP plays a role in meiosis (20–22). In mammalian cells, survivin colocalizes with the mitotic apparatus including B tubulin, microtubules, centrosomes, and kinetochores (48–50). Inhibition of survivin with anti-survivin antibody results in delayed metaphase and produces mitotic cells with shorter and less dense mitotic spindles (48, 50).

Inhibitor of Apoptosis Proteins Regulate Cell Cycle Progression. Evidence also implicates IAPs as regulators of the cell cycle. For example, overexpression of XIAP arrests cells in the G_0-G_1 phase of the cell cycle, and this growth arrest is associated with down-regulation of cyclin A and D1 and induction of the cyclin-dependent kinase inhibitors p21 and p27 (51). In addition, XIAP binds the cell cycle regulators MAGE-D1 and NRAGE, but the significance of this interaction is unclear (52). Survivin has also been implicated in the regulation of the cell cycle. In HeLa cells, survivin is virtually undetectable in G_1 cells and rises ~5- and 40-fold in S-phase and G_2-M cells, respectively (50). Changes in survivin mRNA levels also correlate with increases in survivin protein and promoter activity.

Inhibitor of Apoptosis Proteins Regulate Cell Signaling. The IAP family of proteins also plays a role in cell signaling by activating nuclear factor (NF)-κB. XIAP and NAIP, for example, form a complex with the TAK1 kinase and its cofactor, TAB1, that leads to activation of c-Jun-NH_2-terminal kinase 1 (53). Activated c-Jun-NH_2-terminal kinase 1 subsequently activates NF-κB through the mitogen-activated protein kinase phosphorylation cascade (54). In addition, XIAP promotes the translocation of the NF-κB p65 subunit to the nucleus, which is a prerequisite for NF-κB activity (55). Finally, XIAP promotes the degradation of the NF-κB inhibitor I$\kappa\beta$ (51).

As the role of IAPs in regulating cell division, cell cycle, and signal transduction is clarified, it will be important to identify the IAP domains responsible for these activities. If it becomes possible to identify separate domains of the proteins that inhibit caspases, cell cycle, and signal transduction, then mutant IAPs could be made that dissociate these functions. This work could lead to the development of IAP inhibitors that specifically block the inhibition of caspases by IAPs while IAPs retain their role as cell cycle and signal transduction regulators.

Regulation of Inhibitor of Apoptosis Protein Function by Endogenous Inhibitory Proteins

IAP family members are regulated at the level of the gene, message, and protein, but the details of this regulation are beyond the scope of this review. Rather, this review will focus on the regulation of IAPs by endogenous inhibitory proteins because they serve as prototypes for therapeutic chemical IAP inhibitors.

Regulatory IAP-binding proteins were first identified in *Drosophila*. The proteins Reaper, Hid, Grim (56), and Sickle (57) were shown to bind and inhibit the *Drosophila* IAP, DIAP1. Later, human versions of Reaper (Rpr), Hid, Grim (Grm), and Sickle (Skl) were identified and named SMAC/DIABLO and HTRA2. These IAP inhibitors share a homologous sequence in their NH_2 terminus that is responsible for binding and inhibiting IAPs (Fig. 4).

SMAC and HTRA2. Human SMAC and HTRA2 are mitochondrial proteins that are released along with cytochrome *c* during the disruption of the mitochondria. On release, they are cleaved to an active form. In their active state, SMAC and HTRA2 bind IAPs, thereby preventing their association with caspases (58–61). The IAP-inhibitory functions of the SMAC family of proteins are encoded in their NH_2 terminus. Peptides corresponding to the seven NH_2-terminal amino acids are capable of binding XIAP (62). Mutation of the NH_2-terminal alanine to glycine abolishes the ability of the SMAC peptide to bind IAPs and exert its proapoptotic function (63). When internalized into cells, peptides corresponding to the seven NH_2-terminal amino acids of SMAC are capable of sensitizing H460 lung cancer cells to cisplatin and Taxol (64) and neuroblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand [TRAIL (65)]. Similar results have been observed with HTRA2 and the IAP inhibitors in *Drosophila*. The antitumor properties of SMAC peptides have been extended into xenografts, where cell-permeable versions of these peptides shrink tumors when combined with cisplatin (64) or TRAIL (65) in lung carcinoma and glioma xenografts, respectively. Thus, these SMAC peptides serve as prototypes for small molecules that mimic the actions of SMAC and inhibit IAPs and would be useful therapeutically for a variety of malignancies.

Structural Studies. Given the potential clinical utility of SMAC-like molecules, efforts have been made to understand the physical interactions between SMAC and IAPs. Structural studies have demonstrated that SMAC binds XIAP at two distinct sites. The NH_2 terminus of active SMAC (residues 56–59) binds the BIR3 pocket of XIAP and competitively inhibits the BIR3 domain from binding caspase 9. Mutations in the BIR3 domain that prevent the binding to caspase 9 (*e.g.,* W310) also prevent the BIR3 domain from binding SMAC, suggesting that the binding sites of SMAC and caspase 9 overlap. However, the binding sites are not identical because some mutations in BIR3 (*e.g.,* H343A) abolish the binding of BIR3 to caspase 9 but not SMAC (63, 66).

SMAC/DIABLO		A	V	P	I	A	Q	K	S	E	P	H
OMI/HTRA2		A	V	P	S	P	P	P	A	S	P	R
Rpr	M	A	V	A	F	Y	I	P	D	Q	A	T
Grim	M	A	I	A	Y	F	I	P	D	Q	A	Q
Hid	M	A	V	P	F	Y	L	P	E	G	G	A
Skl	M	A	I	P	F	F	E	E	E	H	A	P

Fig. 4. The SMAC family of IAP inhibitors. Members of the SMAC family of IAP inhibitors share a homologous NH_2-terminal region. The NH_2 terminus is sufficient for binding the BIR3 domain of XIAP.

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SMAC full-length protein and NH_2-terminal peptides also bind the BIR2 domain of XIAP, but with an affinity ~5- to 10-fold lower than that for BIR3. The mechanism by which SMAC disrupts the association of BIR2 from caspase 3 is unclear, but it may be related more to steric hindrance than competitive binding (63).

HTRA2 binds to the BIR3 domain of XIAP, but with weaker affinity than SMAC (67). In its active state, HTRA2 exists as a trimer, and mutations that prevent trimer formation render HTRA2 inactive. In addition to inhibiting IAPs through binding the BIR3 pocket, HTRA2 can also cleave and inactivate multiple IAPs including XIAP, cIAP1, and cIAP2, but not survivin (68).

SMAC β. SMAC and HTRA2 may also exert their proapoptotic activity through effects independent of IAP binding. A study by Roberts *et al.* (69) described a naturally occurring alternate splice form of SMAC, termed SMAC β, that lacked the mitochondrial-targeting sequence. SMAC β did not interact with XIAP, cIAP1, or cIAP2, likely due to loss of its NH_2 terminus. Although unable to bind IAPs, SMAC β enhanced TRAIL- and VP-16-mediated apoptosis in 293 cells. Likewise, mutant versions of HTRA2 (67) that are unable to bind IAPs are still able to induce apoptosis when overexpressed in MCF7 breast cancer cells. It is unclear from this study how these alternate splice forms can still induce apoptosis. Perhaps they maintain their ability to ubiquitinate IAPs, thereby promoting IAP destruction. Alternatively, SMAC and HTRA2 may have additional binding partners unrelated to the IAPs through which they exert a proapoptotic influence. In the case of HTRA2, it has protease activity independent of its role in binding IAPs, and this protease activity may regulate apoptosis in some systems (68).

XAF1. XAF1 is another IAP inhibitor. XAF1 is a nuclear protein that binds and sequesters XIAP in the nucleus. In biochemical reactions, XAF1 binds and inhibits XIAP. In cells, overexpression of XAF1 blocks XIAP-mediated inhibition of apoptosis. It remains unclear, however, whether the sequestration of XIAP in the nucleus simply separates the XIAP from cytosolic caspases or whether there are additional effects from XIAP located in the nucleus (70). Molecules that mimic XAF1 would likely function differently than SMAC and would be an alternative strategy to the development of an XIAP inhibitor.

Translational Applications of Inhibitor of Apoptosis Proteins

Given the importance of IAPs as downstream inhibitors of apoptosis and contributors to chemoresistance *in vitro*, efforts are being made to translate this information into clinical practice. Currently, IAPs are being evaluated as diagnostic markers of malignancy, prognostic markers, and therapeutic targets.

Inhibitor of Apoptosis Proteins as a Diagnostic Marker for the Presence of Occult Malignancy

Survivin shows significant differential expression between malignant and normal adult cells, with very low to absent levels in normal adult tissue but increased levels in a wide variety of solid tumors and hematologic malignancies. Therefore, the detection of survivin in body fluids could serve as a diagnostic marker and allow the early detection of malignancy. Such a study was performed by Smith *et al.* (71), who measured survivin protein in urine samples from patients with bladder cancer and controls. Using a novel detection method for survivin, urine samples were filtered onto nitrocellulose membranes and probed with an anti-survivin antibody. In their study, 100% of patients with bladder cancer and none of the control patients had detectable levels of survivin in their urine. Similar results were obtained by measuring survivin by reverse transcription-polymerase chain reaction in cells isolated from urine samples. In a study of oral

cancers, Lo Muzio *et al.* (72) reported increased survivin expression by immunohistochemistry in 94% of precancerous oral lesions that progressed to squamous cell carcinoma but in only 33% of lesions that did not progress. Taken together, these results suggest that survivin can be measured in samples easily obtained from patients and can be used to screen for the presence of malignancy. Furthermore, the results suggest that survivin expression can identify the lesions at highest risk for malignant transformation and invasion. The use of survivin as a diagnostic might also find a role in other clinical settings. For example, survivin in bronchial washings could be used to support a diagnosis of malignancy in the work-up of patients with a lung nodule, but this application has not been tested in clinical trial.

Inhibitor of Apoptosis Protein Expression as a Prognostic Marker

The importance of IAPs as prognostic markers in malignancy has also been investigated; here too, most attention has focused on survivin. The prognostic importance of survivin expression at both the level of the message and protein has been demonstrated in multiple studies. For example, in a study of 43 patients with astrocytic brain tumors, survivin mRNA expression in the tumor was associated with increased malignant grade of the tumor and decreased overall survival (73). Survivin mRNA levels were also measured in a series of 144 patients with colon cancer treated with curative resection. In this study, increased survivin mRNA was associated with decreased overall survival (53% *versus* 77.5% estimated 5-year survival for patients with low *versus* high survivin mRNA; ref. 34). Likewise, increased survivin mRNA levels have been reported as a poor prognostic factor in malignancies including gastric (74), urothelial cell (75), and hepatocellular carcinoma (76). In addition to its level of expression, the localization of survivin may also be prognostically important. In a study of 84 patients with esophageal carcinoma, increased nuclear survivin expression correlated with reduced overall survival (estimated mean survival of 28 months for patients with nuclear survivin *versus* 108 months for patients with no nuclear survivin), but cytoplasmic levels of survivin were not predictive of outcome (33). This discrepancy between nuclear and cytoplasmic survivin highlights the different antiapoptotic functions of survivin and suggests that the role of survivin as a cell cycle regulator may be a more important determinant of patient outcome than its role as a caspase inhibitor.

The prognostic importance of the other IAP members has also been examined, For example, IAP expression was studied in a series of 48 men with early $(T_2N_0M_0)$ prostate cancer treated with external beam radiation. cIAP1, cIAP2, XIAP, and survivin expression was measured by immunohistochemistry in needle biopsy samples that contained both normal and malignant prostate tissue. In this small cohort, increased levels of cIAP2 in the malignant cells were associated with a shorter relapse-free survival (77).

XIAP levels were studied in a series of 78 patients with newly diagnosed acute myelogenous leukemia. In this study, patients with the lowest levels of XIAP had significantly longer median survival (133 weeks) than patients with higher levels (52.5 weeks; ref. 78). This study has recently been expanded to include 172 patients with newly diagnosed acute myelogenous leukemia. In this follow-up analysis, XIAP no longer correlated with remission or survival (79).

Paradoxically, some studies have demonstrated that increased levels of XIAP are associated with an improved prognosis. For example, XIAP expression was studied in a series of patients ($n = 144$) with stage I–IIIA non–small-cell lung cancer treated with surgical resection. The number of cells within a sample staining positive for XIAP varied from 0% to 100% (median, 20%). Patients with XIAP expression above the median had a longer overall survival (median, 60 months) than patients with lower XIAP expression (median, 24

months). Patients with high levels of XIAP were also more likely to have earlier stage disease (80).

It is not entirely clear why some studies have demonstrated that increased levels of XIAP are associated with a good prognosis. XIAP is just one of eight family members, so perhaps a sum or a weighted average of all eight IAP members is needed to accurately predict patient outcome. Likewise, incorporating levels of IAP inhibitors such as SMAC, HTRA2, and XAF1 may be required to demonstrate prognostic importance.

The prognostic importance of IAPs such as survivin has been demonstrated repeatedly, but the challenge remains how to incorporate these molecular markers into clinical algorithms. Perhaps patients with elevated IAPs such as survivin could be offered more intensive or novel therapies at diagnosis. However, such investigational approaches should only be done as part of a properly controlled trial.

Inhibitor of Apoptosis Proteins as Therapeutic Targets

Because IAPs are preferentially expressed in malignant cells and are prognostically important, they are attractive therapeutic targets, and efforts are under way to develop IAP inhibitors for clinical use using antisense oligonucleotides or small molecule chemical inhibitors. The interest in developing therapeutic IAP inhibitors has been piqued by the observation that temporary inhibition of IAPs may not be toxic to normal cells. In support of this hypothesis, XIAP knockout mice have a normal survival with no histologic or gross abnormalities. *In vitro*, cells from XIAP knockout mice undergo chemotherapy and ultraviolet-induced apoptosis like wild-type control cells. However, deleting XIAP in these mice results in a compensatory increase in cIAP1 and cIAP2 protein levels that could mask the effects of IAP inhibition in normal cells (81).

Like the XIAP knockout mice, NAIP knockout mice are also normal, without any gross or histologic defects or any evidence of cognitive dysfunction. However, the survival of pyramidal neurons in the hippocampus after kainic acid-induced seizures is decreased in NAIP knockout mice compared with wild-type controls, suggesting that NAIP is required for normal neuronal survival under certain stresses (82).

In contrast to the effects of the IAP knockout studies in mice, deletion of the *Drosophila* IAP, DIAP1, results in embryonic lethality due to widespread apoptosis (83). Whereas the mammalian knockout models are reassuring, this study must be considered as one begins testing therapeutic IAP inhibitors.

Antisense Oligonucleotides as Inhibitor of Apoptosis Protein Inhibitors. One therapeutic strategy to inhibit IAPs uses antisense oligonucleotides to decrease the target IAP mRNA and subsequently decrease the protein. Antisense oligonucleotides inhibit IAPs by forming duplexes with intracellular native mRNA. The duplexes disrupt ribosome assembly and inhibit protein translation. More importantly, the mRNA-antisense oligonucleotides complex recruits RNase H enzymes that cleave the native mRNA strand while leaving the antisense oligonucleotide intact. The antisense oligonucleotide is then released back into the cytosol, where it is capable of inhibiting additional native mRNA (reviewed in ref. 84). Antisense oligonucleotides are being actively developed for both XIAP and survivin.

Survivin Antisense Oligonucleotides. The efficacy of survivin antisense oligonucleotides has been demonstrated both *in vitro* and *in vivo*. *In vitro*, survivin antisense molecules directly induce apoptosis in lung cancer and mesothelioma cell lines overexpressing this IAP. However, cell lines and normal cells such as peripheral blood mononuclear cells that do not express survivin are unaffected by the antisense oligonucleotides (85, 86). *In vivo*, antisense survivin oligonucleotides administered by transfecting the tumor cells with plasmids

encoding survivin antisense before tumor implantation reduced tumor growth in xenograft models of gastric carcinoma (87) and thymic lymphoma (88). Although not strictly an antisense strategy, adenoviral delivery of a nonphosphoryable dominant negative of survivin suppressed *de novo* tumor formation in a breast cancer xenograft and shrunk existing tumors by almost half without significant toxicity to the mouse (89). Clinical grade antisense survivin oligonucleotides are currently under development by Isis Pharmaceuticals and Lily Pharmaceuticals and should enter phase I clinical trials shortly.

X-Linked Inhibitor of Apoptosis Protein Antisense Oligonucleotides. Like survivin, XIAP antisense oligonucleotides can reduce XIAP mRNA and protein. XIAP antisense molecules can directly induce apoptosis as well as sensitize cells to chemotherapy and γ-irradiation (90–92). In a lung carcinoma xenograft, XIAP antisense therapy combined with vinorelbine reduced tumor establishment in mice (93). Clinically, antisense XIAP oligonucleotides are being developed as therapeutic agents by Aegera Therapeutics Inc. Their antisense XIAP molecule AEG35156 is currently in phase I clinical trials.

Small Molecule Inhibitor of Apoptosis Protein Inhibitors. Another strategy to block IAPs involves identifying small molecules that reverse IAP inhibition of caspases. A prototype for such a compound is the endogenous IAP inhibitor SMAC. Peptides that correspond to the NH_2 terminus of SMAC are necessary and sufficient for binding and inhibiting XIAP. Given these encouraging results, efforts have been made to identify nonpeptidyl small molecule IAP inhibitors to serve as the lead compounds for therapeutic agents. Currently, most efforts are focusing on developing small molecules that inhibit XIAP because its crystal structure is known and the mechanism by which it inhibits caspases is well understood.

To date, three separate screening strategies have been used to identify XIAP inhibitors. The first strategy was based on the ability of SMAC peptides to bind to the BIR3 pocket of XIAP. Using fluorescence polarization assays or similar fluorescence-based assays, peptides and small molecules were identified that compete with SMAC peptides for binding to the BIR3 pocket (94, 95). Further work, however, is required to determine whether these peptides can induce apoptosis or sensitize cells to chemotherapy. In the second strategy, phage display was used to identify XIAP-binding peptides. This approach identified peptides unrelated to SMAC that bound selectively to the BIR2 domain but not to the BIR1, BIR3, or RING finger domains. These XIAP inhibitors and directly induced cell death in leukemia cells (96). In the third screening strategy, biochemical-based assays have been used to identify small molecules and peptides that inhibit XIAP (97). Here, libraries were screened for compounds that reversed XIAP-mediated repression of recombinant caspase 3, as evidenced by increases in the cleavage of a fluorogenic substrate. Through these screens, both small molecule peptidyl and nonpeptidyl XIAP inhibitors were identified. The small molecules identified by Wu *et al.* (97) appear to inhibit XIAP by binding to the linker region between the BIR1 and BIR3 domains of XIAP. These molecules are capable of sensitizing TRAIL-resistant cells to TRAIL-induced apoptosis. In a separate screen based on a similar strategy, peptidyl and nonpeptidyl XIAP inhibitors were identified that reversed XIAP-mediated inhibition of caspase 3. In a recently reported study (98), XIAP inhibitors based on the polyphenylurea pharmacophore were shown to derepress inhibition of caspase 3 by the BIR2 domain of XIAP but not to derepress the inhibition of caspase 9 by the BIR3 domain of XIAP. These XIAP inhibitors directly induced apoptosis in cell lines and primary leukemia blasts and sensitized cells to chemotherapy-induced apoptosis. When extended into mouse models, these compounds directly reduced tumor size in a prostate xenograft model, even when used as single agents.

Given the current developmental status of the IAP inhibitors, antisense oligonucleotides against survivin or XIAP will likely be used in clinical practice before chemical IAP inhibitors. Over the next 10 years, however, I speculate that chemical IAP inhibitors will also move forward into clinical evaluation. An algorithm for the clinical use of antisense and chemical IAP inhibitors can be derived from the experience with the BCR-ABL tyrosine kinase inhibitor, STI571. STI571 was first used in patients with refractory chronic myelogenous leukemia and acute lymphocytic leukemia, but it is now used as first-line therapy. As more experience with the compound is gained, it is being studied in combination with standard chemotherapy, and it is in combination with standard treatment that it is likely to be most effective. Likewise, molecules that target IAPs such as XIAP or survivin will initially be used as single agents or in combination with low-dose chemotherapy in patients with relapsed or refractory disease. As more experience is gained, these targeted therapies will be used up-front in the treatment of *de novo* disease in combination with standard chemotherapy. In the future, when more small molecules that modulate the apoptosis cascade are developed, they will be used together to simultaneously target different molecular defects.

Summary

In summary, the IAPs are potent caspase inhibitors, but they also inhibit cell death by modulating cell cycle progression, cell division, and signal transduction pathways. Our basic understanding of the structure and function of IAPs is now being translated into clinical practice as IAPs are being investigated as diagnostic markers for early-stage malignancy and novel prognostic markers. In addition, these molecules have been validated as therapeutic targets, and efforts are under way to develop IAP inhibitors. These IAP antagonists will be important biological tools to further our understanding of the role of IAPs in normal and malignant cells; hopefully, they will also find a place in the clinical management of patients with malignancy.

Acknowledgments

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