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Review

Targeting XIAP for the treatment of malignancy

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Abstract

X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitor of apoptosis proteins family of caspase inhibitors that selectively binds and inhibits caspases-3, -7 and -9, but not caspase-8. As such, XIAP blocks a substantial portion of the apoptosis pathway and is an attractive target for novel therapeutic agents for the treatment of malignancy. Antisense oligonucleotides directed against XIAP are effective *in vitro* and are currently being evaluated in clinical trials. Small molecule XIAP inhibitors that target the baculovirus IAP repeat (BIR) 2 or BIR 3 domain are in preclinical development and are advancing toward the clinic. This review will discuss the progress being made in developing antisense and small-molecule XIAP inhibitors.

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Keywords: XIAP; drug discovery; small molecules; antisense; apoptosis

Abbreviations: XIAP, X-linked inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; RING, really interesting new gene; SMAC, second mitochondrial activator of caspases; AML, acute myeloid leukemia; NMR, nuclear magnetic resonance

Introduction

Inhibitor of apoptosis proteins (IAPs) are a family of caspase inhibitors that selectively bind and inhibit caspases-3, -7, and -9, but not caspase-8. By inhibiting effector caspases-3 and -7, IAPs block the downstream portion of the apoptosis pathway and inhibit cell death in response to multiple stimuli (Figure 1). Currently, there are eight members of the IAP family (reviewed in Schimmer[1]). Of these members, X-linked inhibitor of apoptosis protein (XIAP, also known as hILP, MIHA and BIRC4) and survivin have garnered the most attention as potential therapeutic targets. Survivin has been pursued as a therapeutic target, given its role in chemoresistance and its preferential expression in malignant cells over normal adult cells.[2] Currently, survivin antisense oligonucleotides are being evaluated in a phase I clinical trial in patients with refractory malignancies (www.isispharm.com). Small molecule inhibitors that directly target survivin would be attractive therapeutically, but efforts to develop such agents are limited by a lack of knowledge about the mechanisms by which survivin blocks apoptosis and propagates cell proliferation. More detailed discussion on survivin as a therapeutic target and approaches such as use of antisense oligonucleotides, ribozymes and small interfering RNAs can be found in Zaffaroni *et al*.[3] and Altieri.[4] Strategies to target other IAP members such as cIAP1 with antisense oligonucleotides may be promising and are in very early stages of development.[5]

Currently, two approaches are being used to develop XIAP inhibitors: (a) antisense oligonucleotides that are in clinical trial and (b) small molecule inhibitors that are in preclinical development. This review will focus on the very recent progress being made to develop both antisense and chemical XIAP inhibitors as therapeutic agents for the treatment of malignancy.

XIAP has received interest as a therapeutic target as it is probably the IAP member best characterized with respect to structure and biochemical mechanism.[1,6,7] Furthermore, XIAP is the only member of this family able to directly inhibit both the initiation and execution phase of the caspase cascade crucial to mediate the controlled demise of malignant cells. Through its ability to inhibit caspases, overexpression of XIAP renders cells resistant to multiagent chemotherapy. XIAP is frequently overexpressed in malignant cells and, in certain patients, is associated with poor clinical outcome. From the discovery of XIAP in the second half of the 1990s, research on this unique IAP has been exponential giving us a detailed structural and mechanistic view of its activity in addition to abundant cell biology data. As a result, the field has already progressed in developing potential drugs targeting XIAP as a new way to counteract cancer and overcome drug resistance.

It is noteworthy that for an up-to-date field, such as IAPs in cancer treatment a lag exists between recent science in drug discovery and the time when the results of clinical trials are obtained. Thus, although outcome of these trials for XIAP are not known at present, we review the current state of therapeutic agents being tested and additional considerations, such as pan-IAP inhibitors in an attempt to both present an overview and generate discussion around the area of XIAP inhibitors.

Identifying 'Druggable' Targets Based on XIAP's Structure and Function

XIAP is a 57 kDa protein with three zinc-binding baculovirus IAP repeat (BIR) domains (BIR 1–3) and a really interesting

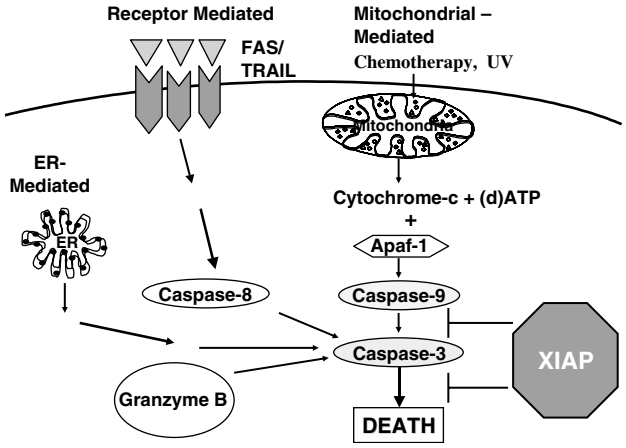


Figure 1 XIAP inhibits apoptosis in response to multiple stimuli. XIAP inhibits active caspases-3, -7 and -9, but not caspase-8. By inhibiting effector caspases, XIAP blocks the downstream portion of the apoptosis pathway and inhibits apoptosis from multiple stimuli including the mitochondrial, death receptor and endoplasmic reticulum-mediated pathways of caspase activation

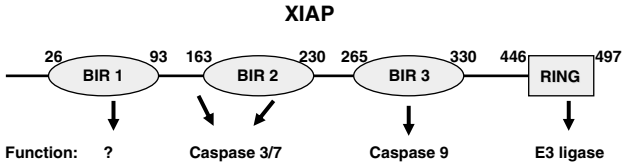


Figure 2 Structure and function of XIAP. XIAP contains threre BIR domains and a RING finger motif. The BIR 3 domain inhibits caspase-9, the BIR 2 domain with its N-terminal linker inhibit caspase-3 and -7, and the function of the BIR 1 domain is unknown. The RING finger contains an E3 ubiquitin ligase

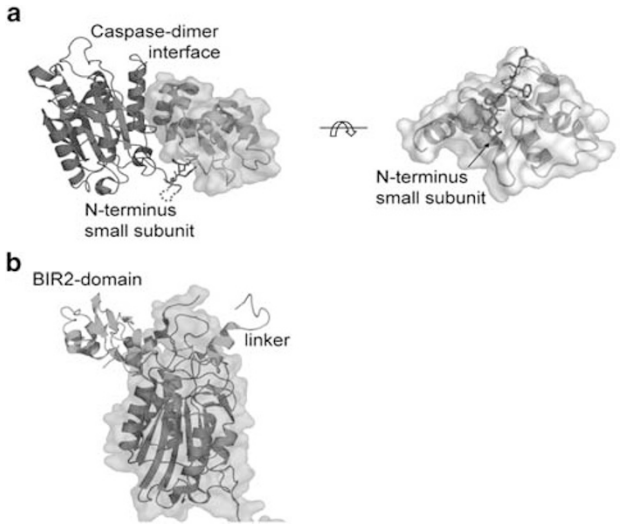


Figure 3 Structural basis of caspase inhibition by XIAP. (**a**) Inhibition of caspase-9 by XIAP/BIR 3. The crystal structure of the capsase-9/BIR 3 complex (left) reveals a two-site mechanism for inhibition. BIR 3 (green) binds caspase-9 (blue) at the interface used for caspase-homodimerization, thereby locking caspase-9 in its monomeric state. In addition, the surface pocket of the BIR 3 domain interacts with the N-terminus of the small subunit of caspase-9 (close up on the right). As a consequence, the active site around the catalytic cysteine (colored red) is rendered inactive. (**b**) Inhibition of caspase-3 by XIAP/BIR2. The linker portion of the BIR 2 region (green) spans across the substrate binding cleft of caspase-3 (grey), thereby sterically blocking the active site of caspase-3 (cysteine in catalytic site is marked red). The actual BIR domain has limited interactions with caspase-3 adjacent to the active site (PDB-accession codes: caspase-9/BIR 3, 1NW9; caspase-3/BIR 2, 1I3O)

new gene (RING)-finger that binds and inhibits caspases-3, -7 and -9 with nanomolar affinity (Figure 2).[8–10] Subsequent structural and functional studies have provided insight into the mechanism by which XIAP inhibits caspases and have demonstrated unique functional properties for the BIR domains and the RING finger. These studies have served as the basis for the recent development of XIAP inhibitors.

The BIR 3 domain as a drug target

The BIR 3 domain of XIAP binds and inhibits caspase-9, an apical caspase in the mitochondrial pathway of caspase activation.[10,11] Consistent with this activity, overexpression of cDNA corresponding to the BIR 3 domain inhibits apoptosis in response to stimuli of the mitochondrial pathway of caspase activation, such as Bax but not stimuli of the death receptor pathway.[12] Likewise, mutations affecting the BIR 3 domain (e.g. W310A) reduce XIAP-mediated inhibition of apoptosis induced by BAX but not by CD95.[12] The crystal structure of BIR 3 bound to caspase-9 reveals a unique two-site mechanism by which the BIR 3 domain inhibits this caspase (Figure 3a). The binding interface of the BIR 3 domain interacts with the interface on caspase-9 that is required for caspase homodimerization. In addition, a groove on the surface of the BIR 3 domain interacts with the N-terminus of the small subunit of caspase-9. As a consequence, BIR 3 locks caspase-9 in its inactive monomeric form with the catalytic site incompetent.[13] This interaction is crucial for the binding and inhibition of caspase-9 by BIR 3 and makes the caspase-9 binding pocket of the BIR 3 domain a potential 'druggable' target as discussed below.

The BIR 2 domain as a drug target

In contrast to the effects of the BIR 3 domain, the BIR 2 region of XIAP binds and inhibits active caspases-3 and -7.[10] Consistent with its ability to inhibit effector caspases, overexpression of cDNA corresponding to the BIR 2 domain inhibits apoptosis from both death receptor and mitochondrial pathway stimuli.[10,12] Likewise, mutations affecting the N-terminal extension of BIR 2 (e.g. D148A) abolish XIAP's protective role in preventing Fas ligand or Bax-induced apoptosis.[12] Structural and biochemical studies have pinpointed the linker region immediately N-terminal to the globular BIR 2 domain as the key mediator of XIAP's inhibition of caspases-3 and -7 (Figure 3b). This linker region binds the catalytic domain of caspase-3 and lies across the active site of the caspase, thereby sterically blocking the caspase's substrate binding pocket.[14–17] Although the linker region alone is a relatively weak inhibitor of these executioner caspases, its fusion to other globular proteins like the BIR 1 domain of XIAP or even GST is sufficient to increase its inhibitory potency into the submicromolar range. Thus, the

BIR 2 domain inhibits caspases-3 and -7 by providing 'bulk' to the linker region and through direct actions on the target. These results are supported by the crystal structure of the caspase-3/BIR 2 complex in which the BIR 2 domain makes limited contact with caspase-3 adjacent to the active site. Full inhibition of caspase-3 and -7, however, requires both the linker and the actual BIR 2 domains, showing that the BIR domain contributes substantially to the inhibition.[18]

It is interesting to note that mutations within the linker region of the BIR 2 domain of XIAP completely abolish the ability of XIAP to inhibit caspase-3, but the same mutation only reduces the ability of XIAP to inhibit caspase-7. In contrast, mutations within the binding groove of BIR 2 equally reduced inhibition of caspase-3 and -7.[18] Thus, the interaction with the linker region of BIR 2 appears more critical for inhibition of caspase-3 activity and the interaction between the BIR 2 binding groove and the caspase appears more important for inhibiting caspase-7 than -3. These structural insights suggest that both the linker region and the binding groove sites in the BIR 2 domain can be exploited to create novel XIAP inhibitors for the treatment of malignancy.

BIR 1 domain

The function of the BIR 1 domain of XIAP is unknown, but studies suggest that it is not an independent caspase inhibitor. For example, the BIR 1 domain with a mutated linker region neither binds nor inhibits caspases-3 or -7.[15,19] However, an intact linker region joined to the BIR 1 domain can bind and inhibit caspase-3 and -7, supporting a role for the BIR 1 domain in providing 'bulk' to the linker region.[19] Potentially, the BIR 1 domain of XIAP has a role beyond caspase inhibition as survivin and BIR-1 in yeast are inefficient caspase inhibitors, but potent regulators of cell cycle and mitosis.[20,21]

RING domain

The RING domain of XIAP has E3 ubiquitin ligase activity and is thus able to promote the degradation of proteins by marking them with ubiquitin molecules.[22] The E3 ligase of XIAP transfers ubiquitin molecules to itself and may thereby promote its own destruction.[22] However, recent reports question the functional importance of XIAP self-ubiquitination and suggest that self-ubiquitination does not promote XIAP degradation.[23] More intriguingly, the E3 ligase of XIAP appears to influence its antiapoptotic activity. Through its RING domain, XIAP promotes the ubiquitination and subsequent degradation of active caspase-9 and -3 but also ubiquitinates the endogenous XIAP inhibitor second mitochondrial activator of caspases (SMAC).[24,25] Mutations of the RING domain reduce but do not abolish the antiapoptotic effects of XIAP.[25] These data suggest that inhibitors of XIAP's E3 ligase could be useful therapeutic adjuncts for the treatment of malignancy.

XIAP – More Than a Caspase Inhibitor

Most studies on XIAP have focused on its role as a caspase inhibitor. However, XIAP also inhibits apoptosis through mechanisms unrelated to its ability to bind caspases. For example, XIAP forms a complex with the TAK1 kinase and its cofactor TAB1 that leads to activation of JNK signal transduction pathways. Activated JNK initiates the MAP kinase phosphorylation cascade leading to activation of NF-κB.[26,27] XIAP also activates NF-κB by promoting the translocation of NF-κB to the nucleus[28] and by promoting the degradation of the NF-κB inhibitor IκB.[29] Activation of NF-κB and JNK by XIAP requires the E3 ubiquitin ligase activity of the RING domain and can be dissociated from the caspase inhibitory effects of XIAP.[30] Thus XIAP inhibitors, targeting the RING domain, could also induce apoptosis by blocking NF-κB activation.

Evidence also implicates XIAP as regulators of the cell cycle. XIAP binds the cell cycle regulators MAGE-D1 and NRAGE, but the significance of this interaction is unclear.[31]

A recent report has identified a role for XIAP in copper homeostasis. The E3 ligase in the RING finger of XIAP ubiquitinates the copper regulating gene MURR1. By promoting the ubiquitination of MURR1, cellular copper levels increase.[32] Thus, some of the antiapoptotic activity of XIAP could be due to affects on copper-dependent enzymes.

Validating XIAP as a Potential Therapeutic Target

Three lines of evidence support XIAP as a potential therapeutic target. First, multiple studies in cultured cells have demonstrated that overexpression of XIAP confers resistance to multiagent chemotherapy including stimuli of the mitochondrial and death receptor pathways of caspase activation.[33–35] Second, knocking out XIAP with siRNA or antisense oligonucleotides restores chemosensitivity to a variety of malignant cell lines.[36–39] Finally, knocking out XIAP is not toxic to normal cells, as evidenced by a lack of significant pathology in the XIAP knockout mouse.[40]

Another useful step in validating a drug target is demonstrating that expression levels of the target influence patient outcomes. In the case of XIAP, studies assessing its prognostic importance in patients with malignancy vary among different tumors. For example, in adult[41] and childhood[42] *de novo* acute myeloid leukemia (AML), increased XIAP expression by immunoblotting is correlated with decreased survival compared to patients with lower levels of XIAP. Likewise, increased XIAP expression is associated with more aggressive tumor histology and decreased survival in patients with clear-cell renal carcinoma. In contrast, other studies in leukemia[43] and cervical cancer[44] conclude that XIAP is not prognostic. Interestingly, some reports indicate that increased XIAP expression is associated with a more favourable outcome. For example, in patients with radically resected non-small-cell lung carcinoma, high XIAP expression in the tumor is associated with longer overall survival than patients with low XIAP expression in the tumor.[45] It is not entirely clear why overexpression of XIAP may be associated with a good prognosis, but a few possibilities can be considered. First, XIAP is just one of the eight currently identified IAP family members and a sum or weighted sum of all IAPs may be required to assess the impact IAPs have on

patient outcome. If the clinical outcome is dictated by multiple IAPs, then it would suggest that pan-IAP inhibitors might be more effective therapeutically than inhibitors of a single IAP member. Alternatively, post-translational modifications of XIAP may affect XIAP function without affecting the level of expression. Also, XIAP may be induced in malignant cells as compensation for an increased drive toward of apoptosis. In these cells, XIAP may not be fully protective and the cells may be more susceptible to chemotherapy. Finally, XIAP may have additional roles that have yet to be defined.

Endogenous XIAP Inhibitors – Proofs of Concept for Therapeutic Agents

Endogenous regulatory proteins that bind and antagonize IAPs were first identified in *Drosophila*[46] and subsequently discovered in humans. The endogenous human IAP antagonists, SMAC and HTRA2, are mitochondrial proteins that are released into the cytosol along with cytochrome *c* during mitochondrial disruption. In the cytosol, cleaved and active antagonists are capable of binding and inhibiting IAPs including XIAP.[47–50] Studies using peptides corresponding to the N-terminus of SMAC also provide the proof-of-principle that inhibitors of XIAP could be therapeutically useful for the treatment of malignancy. When internalized into cells, SMAC peptides sensitize malignant cells to chemotherapy, and when combined with cisplatin and tumor necrosis factor-alpha-related apoptosis inducing ligand (TRAIL) delay the growth of lung cancer and glioma xenograft, respectively.[51,52] Therefore, molecules that mimic the actions of endogenous XIAP inhibitors could be therapeutically useful.

Structural studies that map the interaction between XIAP and its endogenous inhibitors provide a basis for the development of small-molecule XIAP inhibitors (Figure 4a). These studies demonstrate that SMAC binds to both the BIR 3 and BIR 2 domains of XIAP. The N-terminal 4–7 amino acids of active SMAC are necessary and sufficient for binding the BIR 3 pocket of XIAP and preventing XIAP from binding and inhibiting caspase-9. Consistent with SMAC and caspase-9 competing for the same site in the BIR 3 domain, mutations in the BIR 3 domain that abolish its binding to caspase-9 also prevent it from binding SMAC.[53–56] These results indicate that small molecules that mimic the actions of SMAC could be identified. They also indicate that a structure-based approach to the design of BIR 3 inhibitors could be successful.

SMAC also inhibits the BIR 2 domain of XIAP. Similar to the BIR 3 domain, biochemical data suggest that SMAC interacts with the analogous surface pocket on the BIR 2 domain. As this pocket is not the primary site by which XIAP inhibits the executioner caspases, however, it is unclear how SMAC binding this pocket inhibits the BIR 2 domain. Potentially, SMAC blocks the supportive role of the BIR 2 pocket as an inhibitor of executioner caspases.[18] Binding of SMAC to the BIR 2 and BIR 3 domains simultaneously may sterically hinder the linker interaction with caspase-3 or -7.[57] Thus, these results offer additional perspectives on the development of therapeutic SMAC-mimics that target the BIR 2 region of XIAP.

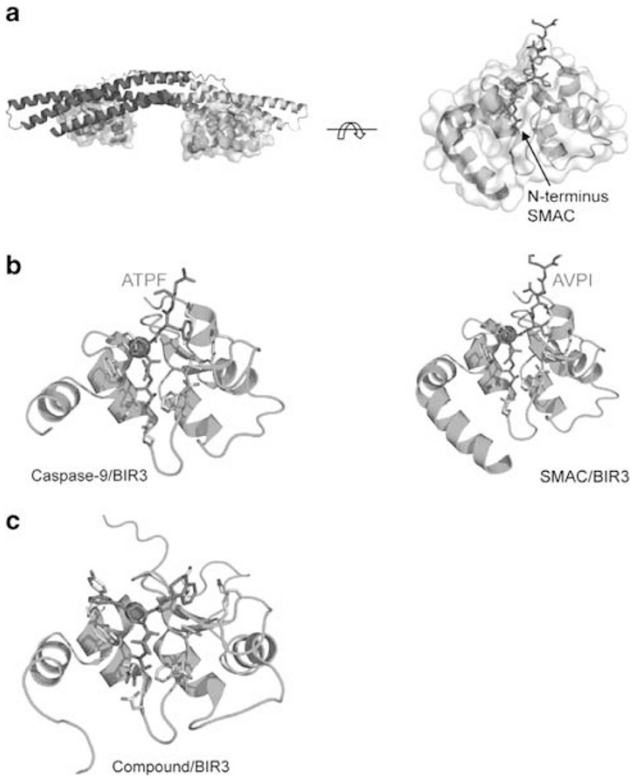


Figure 4 SMAC antagonizes XIAP. (**a**) Structure of the SMAC/BIR 3 complex. The N-termini of SMAC interact with the BIR 3 domains of XIAP (green). (**b**) SMAC and SMAC-like molecules compete with caspase-9 for the surface pocket on the BIR 3 domain. The N-terminus of the small subunit of caspase-9 (ATPF; blue; left) binds the same pocket on BIR 3 as the N-terminus of SMAC (AVPI; blue; right). (**c**) Molecules that mimic SMAC can bind the BIR 3 domain and antagonize the caspase-9–BIR 3 interaction. The binding of SMAC-like tripeptides identified by Oost *et al*.[60] is shown (PDB-accession codes: SMAC/BIR 3, 1G73; caspase-9/BIR 3, 1NW9; antagonist/BIR 3, 1TFT)

Unanswered Questions Regarding XIAP as a Therapeutic Target

A number of outstanding questions that can influence the effectiveness of XIAP inhibitors still have to be addressed. For example, XIAP is just one of eight human IAPs and the roles of the different IAP family members such as cIAP1 and cIAP2 are not yet fully defined. If the other IAP members are more important inhibitors of apoptosis than XIAP, then molecules that specifically inhibit XIAP may be less effective than pan-IAP inhibitors as these would overcome the redundancy in the pathway. Studies that help to better define the roles of the other IAP members will assist in the development of the optimal IAP inhibition strategy. In addition, structural models for a potential SMAC pocket on the BIR domains of other IAPs, such as cIAP1, cIAP2 or survivin, can be generated with current crystallographic data. This could be useful in generating agents to target these IAPs.

Furthermore, another possible concern is whether XIAP inhibitors will induce upregulation of the other IAP family members and thereby diminish the efficacy of the compounds. Of note, cIAP1 and cIAP2 are upregulated in the XIAP knockout mouse and may protect it from pathology.[40] In

addition, the importance of XIAP's role beyond caspase inhibition is unresolved. Depending on the importance of these functions for tumor growth and progression, therapeutic agents that fail to target these actions may not be optimal.

Development of XIAP Inhibitors

Two broad approaches have been taken to develop clinical inhibitors of XIAP – antisense oligonucleotides and small molecule inhibitors (Table 1). Antisense oligonucleotides, aside from disadvantages inherent to the method, are advantageous as they target the entire protein, while small molecules bind a single domain. However, small molecule inhibitors offer the potential of more rapid inhibition of their target *in vivo* and more predictable duration of action.

XIAP Antisense Oligonucleotides

Antisense oligonucleotides directed against XIAP are being developed for the treatment of solid tumors and hematologic malignancies by Aegera pharmaceuticals (Montreal, Canada). The antisense molecule currently in clinical trial (AEG 35156) is a second-generation 19-mer antisense oligonucleotide that targets XIAP. It contains a mixed backbone of chemically modified DNA/RNA nucleotides. Antisense oligonucleotides inhibit their target by forming duplexes with intracellular native mRNA. The duplexes recruit RNAase 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. In cultured cells, uptake of antisense oligonucleotides requires transfection, infection or electroporation protocols. In xenografts and patients, intracellular uptake of antisense molecules is achieved after intravenous or subcutaneous administration, but the mechanism facilitating the uptake is not clear.

In cultured cells, knockdown of XIAP mRNA with the XIAP antisense oligonucleotide occurs 6–9 h after transfection of the antisense molecule, and knockdown of the protein is observed 12–18 h after transfection. In cultured cells, a $\sim$70% knockdown of XIAP mRNA target is achieved. Antisense oligonucleotides against XIAP can directly induce apoptosis and sensitize cells to chemotherapy. For example, antisense XIAP oligonucleotides, but not control sequences, directly induce apoptosis in H460 lung carcinoma cells and sensitize these cells to Taxol, vinblastine and etoposide chemotherapy. In H460 lung cancer xenograft models, XIAP antisense knocks down its protein target and delays tumor growth. When combined with vinblastine, a synergistic delay in tumor growth is achieved.[58] Encouraged by these results, Aegera has moved its antisense molecule into phase I clinical trials in the UK. In addition to standard safety and efficacy end points, trials will also measure pharmacodynamic end points and test whether the antisense molecule can knock down its target in tumor biopsies after administration to patients.[59]

Unanswered Questions Pertaining to Antisense Therapies in General

Although the xenograft data have been encouraging, the efficacy of antisense therapies in previous clinical trials has not matched the initial expectations. To date, most antisense trials have indicated that the drug is safe, but efficacy is modest. For example, in a phase III trial in melanoma, antisense Bcl-2 prolonged disease-free survival, but not overall survival (www.genta.com). As a result, the FDA did not approve Bcl-2 antisense for the treatment of metastatic melanoma. Recently, patients with refractory chronic lymphocytic leukemia who were randomized to receive chemotherapy and Bcl-2 antisense had a higher response rate than patients assigned to chemotherapy alone (17 *versus* 7%) and the responses in the antisense group appeared durable (www.genta.com). In part, the limited efficacy of antisense in these early trials may reflect the inclusion of patients with chemoresistant disease. Responses to the Bcl-2 antisense may be better in less resistant groups and these trials are currently underway (www.nih.gov). Alternatively, the poor response to Bcl-2 antisense may indicate that the selected target is not optimal for an antisense approach due to redundancy among related family members. Given a similar redundancy in the IAP family, the selective targeting of XIAP may not produce an optimal effect. In this case, a different approach using pan-IAP inhibitors may be needed. Lack of

Table 1 Summary of lead compounds targeting XIAP

Lead compound	Developing organization	Target	Stage
Antisense			
AEG35156	Aegera Therapeutics	XIAP mRNA	Phase I
Peptidometic			
Tripeptide	Abbott Laboratories	BIR 3	Preclinical
Tripeptide	University of Michigan	BIR 3	Preclinical
Natural product			
Embeline	University of Michigan	BIR3	Preclinical
Synthetic			
Tetrazoyl thioether C_2-symmetric diyne	University of Texas Southwest Medical Center	BIR 3	Preclinical
Aryl sulfonamide	Novartis	BIR 2-linker	Preclinical
Polyphenylurea	Burnham Institute/TPIMS	BIR 2	Preclinical

significant response to antisense may also reflect a failure to achieve adequate target knockdown, as it is unknown how much knockdown is required to inhibit the function of the target. Finally, it remains unclear whether the effects of antisense therapies are related to knockdown of their target or nonspecific effects from altering gene regulation. Given these concerns, XIAP antisense therapy may also be hampered in clinical trial. Therefore, there is great interest in developing small-molecule inhibitors of XIAP to overcome limitations with antisense oligonucleotides.

Small-Molecule Approaches to XIAP Inhibition

Small molecules that inhibit the BIR domains of XIAP are another promising approach for developing therapeutic XIAP inhibitors (Table 2). Owing to the differences in the mechanism of caspase inhibition by the BIR 2 and BIR 3 domain and the fact, that they inhibit different caspases, molecules have been developed to specifically target the BIR 2 or BIR 3 region of XIAP.

BIR 3 inhibitors

The structural data surrounding the interaction between the BIR 3 domain of XIAP and caspase-9 suggests that small molecules that bind the BIR 3 pocket of XIAP could mimic the action of SMAC and inhibit the interaction between XIAP and caspase-9. These structural studies have facilitated a variety of chemical biology approaches including fluorescent polarization, nuclear magnetic resonance (NMR), 'in silico' virtual screening and computer modelling to identify BIR 3 inhibitors. For example, Oost *et al*.[60] designed a fluorescent polarization-based assay in which fluorescently labelled peptides based on the SMAC mimic HID were incubated with recombinant BIR 3 protein. With this assay, they screened a peptide library and identified pentapeptides that bind the BIR 3 domain of XIAP (Figure 4c). Based on these peptide leads, new tripeptides with unnatural amino-acid residues were synthesized that reduced the peptide character of the compounds. The most potent tripeptide BIR 3 inhibitors contained an unnatural (*4S*)-4-phenoxy proline residue and directly induced apoptosis in a variety of malignant cell lines with LD_{50}s in the low to submicromolar range.

Tripeptide BIR 3 inhibitors with unnatural amino acids have also been identified by Sun *et al*.[61,62] who modified a series of SMAC-pseudomimics and tested their binding to the BIR 3 domain of XIAP by NMR. Binding of peptides to BIR 3 was confirmed by a fluorescent polarization assay. Through this approach, they identified compounds that bound the BIR 3 domain with nanomolar affinity and were more avid for the BIR 3 pocket than the natural SMAC peptides. Furthermore, they determined that aromatic groups in the fourth residue were necessary for binding the BIR 3 pocket.[63] Unlike the BIR 3 inhibitors described by Oost *et al*.,[60] these molecules were not directly toxic, but sensitized breast and prostate cancer cell lines to chemotherapy.

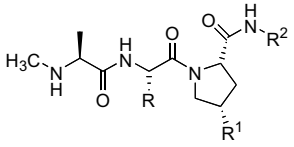
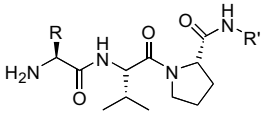
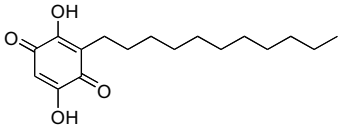
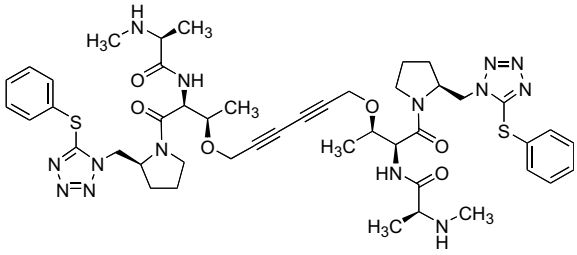
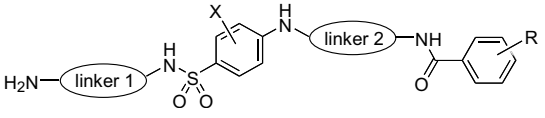
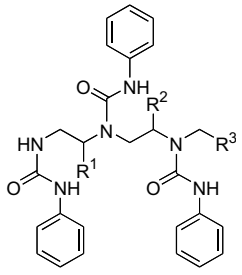
The detailed structural data on the BIR 3–caspase-9 interaction also permit virtual screening of molecules 'in silico' (with approaches reviewed in Toledo-Sherman and Chen[64]). Using computer modelling of the BIR 3–caspase-9 interaction site and docking software, a database of virtual molecules was screened for entities that fit into the desired region. With this strategy, Nikolovska-Coleska *et al*.,[65] screened a virtual library of 8221 molecules isolated from Chinese herbal remedies and identified putative binding partners. The putative binding partners were screened in a fluorescent-polarization binding assay with the BIR 3 domain of XIAP and a fluorogenic SMAC peptide. With this secondary binding assay, five compounds were identified that compete for the SMAC-binding groove in the BIR 3 domain. Among these five compounds was embelin, a natural product isolated from the Japanese Ardisia herb and oils, which is considered an herbal anticancer agent and male contraceptive. Embelin is a remarkably simple benzoquinone with an 11-carbon alkyl side chain that binds the surface groove in the BIR 3 domain of XIAP with an affinity similar to the SMAC peptide and induces apoptosis of malignant prostate cancer cells at low micromolar concentrations. Prior studies of embelin and related analogues have demonstrated its antitumor effects in xenograft models.[66] In studies in mice, rats and dogs embelin was safe, but produced a reversible decrease in testosterone and infertility.[67,68] Thus, given the prior pharmacokinetic and toxicity data, embelin could be rapidly advanced into clinical trials for the treatment of solid tumors and hematologic malignancies.

Computer-simulated drug design was performed by Li *et al*.[69] who modelled the interaction of the peptidyl SMAC-mimic AVPF with the BIR 3 domain of XIAP. The SMAC peptide was modified 'in silico' with nonpeptidyl moieties to create chemical SMAC mimics. Identified compounds included a tetrazoyl thioether that was modified via Glaser coupling to form a C_2-symmetric diyne. This lead bound the BIR 3 domain of XIAP with an affinity similar to the SMAC peptides and also bound cIAP1 and cIAP2. When added to HeLa cell lysates, it increased caspase-3 activity more than a synthetic SMAC peptide. The increased derepression of XIAP compared to the SMAC peptide is likely attributable to its dimeric and bivalent nature that may permit the compound to interact simultaneously with the BIR 2 and BIR 3 domains of XIAP, better resembling wild-type SMAC. Of note, SMAC is also a dimer and interacts simultaneously with the BIR 2 and BIR 3 domains to inhibit XIAP.[57]

These molecules were not directly toxic, but sensitized cells to TRAIL and TNF-α-mediated apoptosis. In the presence of TNF-α, the compound induced activation of caspase-8 before activation of caspase-3. An XIAP inhibitor that activates caspase-8 before caspase-3 is unique given that XIAP does not bind or inhibit caspase-8. The activation of caspase-8 may relate to the molecule's role as a cIAP1 or cIAP2 inhibitor and supports a previously described role for cIAP1 and cIAP2 as inhibitors of a JNK signalling pathway leading to activation of caspase-8.[70] Alternatively, the molecules may have targets beyond IAPs resulting in caspase-8 activation.

Studies with the different BIR 3 inhibitors are contradictory with respect to whether inhibition of the BIR 3 domain directly induces apoptosis or whether it only sensitizes cells to chemotherapy. A number of explanations may account for

Table 2 Small-molecule XIAP inhibitors

Author	Target	Pharmacophore	Structure
Oost *et al*.[56]	BIR 3	Tripeptides (unnatural amino acids)	
Sun *et al*.[58,59]	BIR 3	Tripeptides (unnatural amino acids)	
Nikolovska-Coleska *et al*.[61]	BIR 3	Benzoquinone	
Li *et al*.[65]	BIR 3	Tetrazoyl thioether C_2-symmetric diyne	
Wu *et al*.[68]	BIR 2-linker	Aryl Sulfonamide	
Schimmer *et al*.[69]	BIR 2	Polyphenylurea	

the differences between these studies. First, the BIR 3 inhibitors reported by Oost, Sun, Nikolovska-Coleska and Li were tested in different cell lines. Thus, it is quite possible that inhibitors of BIR 3 directly induce apoptosis in some cell lines, but are only chemosensitizers in other contexts. In support of this hypothesis, antisense oligonucleotides against XIAP are also directly toxic to some cell lines but act as chemosensitizers in other systems.[37,39,71] Alternatively, the molecules' affinities for the other BIR domains of XIAP and the BIR 3 domain of other IAPs has not been fully reported. For example, the inhibitor by Li appears to also target the BIR 2 domain of XIAP, but additional studies are needed. Potentially, differences in crossreactivity for the other BIR domains of XIAP and other IAP members could explain differences in cell-based activity. Finally, one cannot entirely dismiss the possibility that these molecules have targets beyond XIAP and induce apoptosis through mechanisms unrelated to XIAP.

BIR 2 inhibitors

The first small-molecule XIAP inhibitors were reported by Wu *et al*.,[72] in 2003. These inhibitors were identified using a high throughput enzymatic derepression assay in which recombinant XIAP was combined with active caspase-3 to inhibit caspase-mediated cleavage of the fluorogenic peptide substrate. With this assay, they screened $\sim$160 000 compounds in 1536-well format and identified potent XIAP inhibitors including the compounds TWX006 and TWX024, aryl sulfonamides with flexible acyclic diamines in the first and third fragments. These compounds derepressed XIAP-mediated inhibition of caspase-3 more potently than SMAC. In addition, these molecules bound the BIR 2-linker region of XIAP, and, in enzymatic assays, relieved the repression of caspase-3 more potently than SMAC peptides.

Using a similar enzymatic assay, a combinatorial library of approximately 1 million peptidyl and non-peptidyl compounds was screened to identify molecules that inhibit XIAP and thereby increase caspase-3-mediated cleavage of a fluorogenic substrate.[73] After library deconvolution, XIAP inhibitors based on different pharmacophores were identified including the polyphenylurea series. These polyphenylurea XIAP inhibitors bound and inhibited the BIR 2 but not the BIR 3 domain of XIAP,[73,74] and were directly toxic to a wide spectrum of malignant cell lines. The polyphenylurea BIR 2 inhibitors also demonstrated preferential toxicity to primary malignant cells over normal cells. For example, in a series of primary samples from patients with AML, the BIR 2 inhibitors induced apoptosis with an $LD_{50} < 10\,\mu$M in 60% of samples in short-term toxicity assays, but were not toxic to primary bone marrow mononuclear cells. In clonogenic assays, however, the compounds inhibited growth of primary hematopoietic cells, suggesting that the molecules could be toxic to normal progenitor/stem cells. The compounds were also active in xenograft models where they delayed the growth of tumors of the prostate, breast and colon carcinoma cells without obvious untoward toxicity to the mice.[34,73] Currently, second-generation polyphenylurea inhibitors have been synthesized and are under evaluation. Like the BIR 3 inhibitors, the affinity of these compounds for the BIR 2 domains of other IAP members has not been reported.

E3 ligase inhibitors

E3 ligases are attractive therapeutic targets and molecules such as the nutlins that target MDM2 are being developed for therapeutic use.[75] Thus, molecules that selectively inhibit the E3 ligase in XIAP's RING domain could be useful therapeutic adjuncts for the treatment of malignancy. Such molecules could prevent XIAP from ubiquitinating caspases-3 and -9 and block XIAP's role in NF-κB activation and copper homeostasis. In cell lines, however, selective mutation of the E3 ligase only partially reversed XIAP's inhibition of apoptosis. Therefore, therapeutic inhibitors of the E3 ligase of XIAP might be more effective when combined with small-molecules that target other domains of XIAP or when used in combination with standard chemotherapy.

Unanswered Questions Pertaining to Small-Molecule XIAP Inhibitors

A number of questions are to be considered that might influence the efficiency of small-molecule XIAP inhibitors in the clinic. As discussed above, there is redundancy among the IAP family members. If the other IAP members are equally or more important inhibitors of apoptosis than XIAP, then small molecules that specifically inhibit XIAP may not be optimal for therapeutic use and pan-IAP inhibitors may be needed. To date, the spectrum of activity of the small molecule XIAP inhibitors has not been fully characterized. Furthermore, in some studies, XIAP inhibitors are directly toxic, while other XIAP inhibitors only sensitize malignant cells to chemotherapy. Understanding the differences between these studies and determining whether the toxicity is XIAP-related will also help optimize the best molecules for clinical trials.

Finally, as with every compound, the presence or development of resistance may ultimately limit the use of small-molecule XIAP inhibitors. It is unknown whether malignant cells acquire mutations in the BIR domains of XIAP and whether these mutations affect XIAP's function or its response to inhibitors. It is also unknown whether mutations in XIAP can develop after treatment with small-molecule XIAP inhibitors akin to the development of mutations in ABL kinase after treatment with the inhibitor Gleevec.[76]

Conclusion

Within a time span of less than 10 years, the XIAP gene has been identified, a targeted therapy generated and a clinical trial with this antisense therapy launched. In addition, small molecules that inhibit XIAP have been identified and are moving through the pipeline of clinical development. Hopefully, these early trials of XIAP inhibitors will show promise and improve the outcome for patients with malignancy, but the rapid transition from target to therapy is already a victory for translational research.

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