Translational Research in Hematologic Malignancies

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Targeting the IAP Family of Caspase Inhibitors as an Emerging Therapeutic Strategy

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The IAPs (inhibitor of apoptosis proteins) are a family of caspase inhibitors that block the execution phase of apoptosis. Overexpression of IAPs confers chemoresistance and, in some groups of patients, is associated with a poor prognosis. Given their role in the development and progression of solid tumors and hematologic malignancies, efforts are underway to develop therapeutic IAP inhibitors, with a focus on X-linked IAP (XIAP) and survivin. Antisense oligonucleotides that target XIAP and survivin have been developed and are currently in phase I clinical trial. Small-molecules that bind and inhibit XIAP have also been identified and are in the process of clinical development. This review focuses on the preclinical data that support the development of IAP-targeted therapies.

At a fundamental level, cancer occurs or progresses because the malignant cells fail to die in response to chemotherapy, gamma radiation, or immune surveillance by endogenous cytotoxic T-cells and NK cells. In part, this failure is due to defects in caspase activation, the execution phase of apoptosis. Caspases are cysteine proteases that are organized in a hierarchical cascade with upstream (initiator) caspases activating downstream (effector) caspases (**Figure 1**; see Color Figures, page 549). There are at least four pathways for caspase activation: (a) the mitochondrial pathway where damage to the mitochondria leads to release of cytochrome c, (b) the death receptor pathway with the tumor necrosis factor (TNF) family of death receptors, (c) a pathway connected to the endoplasmic reticulum, and (d) the direct activation of effector caspases by Granzyme B. Ultimately, all of these pathways converge on the activation of effector caspases such as caspases 3 and 7. The inhibitor of apoptosis proteins (IAPs) are a family of caspase inhibitors that bind and inhibit active caspases 3, 7 and 9. By inhibiting downstream caspases 3 and 7, IAPs block the convergence point of multiple caspase activation pathways and thus inhibit apoptosis from multiple stimuli (reviewed in [1]). IAP overexpression is associated with chemoresistance, and therefore therapies that target IAPs may improve outcomes for patients with solid tumors and hematologic malignancies. This review will highlight how knowledge derived from basic and preclinical studies is being translated into developing inhibitors of IAPs for therapeutic use.

The IAP Family

The IAP family of proteins are grouped together based on the presence of a shared BIR (baculovirus IAP repeat) domain and have been identified in diverse organisms including baculovirus, yeast, Drosophila and humans (reviewed in [2]). In addition to BIR domains, IAPs may also possess CARD (*c*aspase *a*ctivation and *r*ecruitment *d*omain) and RING (*r*eally *i*nteresting *n*ew *g*ene) domains. At least

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eight IAP members have been identified in humans, of which XIAP (X-linked IAP) and survivin have received the most attention as therapeutic targets (**Figure 2**; see Color Figures, page 549). XIAP is of interest as a therapeutic target as it was the first IAP identified and remains the best characterized with respect to mechanism and structure. Survivin has garnered interest as a therapeutic target, in part due to its preferential expression in malignant cells over normal cells and its prognostic importance in a variety of malignancies. IAPs are classically regarded as caspase inhibitors, but they also have functions beyond this role. For example, XIAP is an activator of NF-κB[3,4] and is involved in copper homeostasis.[5] Survivin, in fact, is a relatively weak caspase inhibitor but is a potent regulator of cell cycle progression and mitosis.[6]

Rationale for IAPs as Drug Targets

IAPs and chemoresistance

Preclinical studies have validated IAPs as potential drug targets. In cultured cells, overexpression of IAPs such as XIAP and survivin confers resistance to multi-agent chemotherapy and to stimuli that trigger the mitochondrial and death receptor pathways of caspase activation. Likewise, knocking out survivin or XIAP in cultured cells directly induces apoptosis and sensitizes resistant cells to chemotherapy. In animal models, antisense oligonucleotides directed against XIAP and survivin delay tumor growth in lung[7] and gastric[8] cancer xenografts, respectively.

Inhibiting IAPs is less toxic to normal cells

IAPs are also attractive as therapeutic targets because their inhibition does not appear to be toxic to normal adult cells. In support of this hypothesis, XIAP knockout mice survive until birth and display no significant pathology.[9] Likewise, inhibitors of XIAP and survivin are not toxic when administered to fully developed mice. In contrast to XIAP knockout mice, survivin knockout mice do not develop beyond early embryos in keeping with the role of survivin in fetal tissues and mitosis.

Currently, it is not well understood why IAP inhibitors should be preferentially toxic to malignant cells over normal cells. Perhaps it relates to an intrinsic drive in malignant cells to activate caspases in response to multiple stimuli including oncogenes, hypoxia, and loss of attachment. IAPs may block this drive to apoptosis in normal cells and by inhibiting IAPs the block is removed and apoptosis proceeds. In contrast, normal cells lack these same intrinsic drives and may thus be less sensitive to IAP inhibition. In support of this hypothesis, malignant cells have higher levels of processed caspase 3 than normal cells.[10]

IAPs as prognostic markers

Studies demonstrating the prognostic importance of a potential therapeutic target are important because they help validate the target and help provide a rationale for testing the targeted therapy in that disease site. Multiple studies have demonstrated the importance of survivin in predicting response to chemotherapy, but the prognostic importance of XIAP is more controversial. Overexpression of survivin is an important predictor of clinical outcome in malignancies such as T-cell leukemia[11] and gastric cancer.[12] In contrast the studies with XIAP are less clear. In patients with adult and childhood AML, Tamm et al[13,14] reported that increased levels of XIAP by immunoblotting predicted for decreased overall survival, and levels of XIAP were the best predictors of survival by multivariate analysis. However, not all studies have demonstrated the prognostic importance of XIAP and others even report that overexpression of XIAP is associated with improved outcome.[15,16] While failure to consistently demonstrate the prognostic importance of XIAP does not invalidate this target, it does highlight limitations in our understanding of the biology of XIAP.

Targeting IAPs

Currently, two approaches are being used to develop IAP inhibitors—antisense oligonucleotides and small molecule inhibitors. Antisense oligonucleotides against XIAP and survivin are already in clinical trials, while small molecule XIAP inhibitors are moving towards the clinic.

Antisense oligonucleotides targeting IAPs

XIAP antisense oligonucleotides: Antisense oligonucleotides targeting XIAP have been developed. The antisense molecule currently in clinical trial is a mixed backbone of DNA and RNA oligonucleotides. The antisense molecule inhibits its target by forming a duplex with the native XIAP mRNA. In this double-stranded conformation, intracellular RNAase H cleaves the native XIAP mRNA while leaving the antisense intact. The antisense is released back into the cytoplasm where it is capable of binding additional XIAP mRNA. Thus, antisense oligonucleotides inhibit their targets by promoting the degradation of native mRNA rather than by directly inhibiting translation.

In preclinical studies, the XIAP antisense oligonucleotide directly induced apoptosis and sensitized malignant cells to chemotherapy. In addition it delayed tumor growth of lung cancer xenografts without untoward toxicity to the mice.[7] Currently, a phase I trial of the XIAP antisense is underway in the United Kingdom. The goals of the trial are to assess toxicity of the compound and to determine an optimal dose for the next phase of trials. Future studies are planned to assess the safety and efficacy of the molecule in combination with chemotherapy and to determine the ability of the antisense to knock down its targets in primary patient samples.

Survivin: Second generation survivin antisense oligonucleotides with a mixture of RNA and DNA-like oligonucleotides on a modified 2′-O-methoxyethyl phosphorothioate backbone have been developed. In preclinical studies in cultured cells, the antisense knocked down its

protein and mRNA targets as measured by immunoblotting and QRT-PCR, respectively. In cultured cells, survivin antisense, but not the control compound, induced apoptosis, activated caspase-3, sensitized cells to chemotherapy, and arrested cells in the G_2/M phase. Like XIAP antisense oligonucleotides, survivin antisense oligonucleotides, but not control sequences, delayed tumor growth in xenograft models.[17] Currently, survivin antisense is being evaluated in a phase I clinical trial in the United Kingdom as a single agent in patients with refractory malignancies. Biopsies of tumor tissue are being obtained pre- and post-treatment to assess the effects of the antisense on its target.

Small molecule IAP inhibitors

An alternate approach to inhibiting IAPs involves small molecules that block active sites on the IAP protein. Small molecule inhibitors that target survivin would be attractive therapeutically, but efforts to develop such agents are limited by a lack of knowledge about the structure of survivin and the mechanisms by which survivin inhibits caspases and blocks mitosis. In contrast to survivin, the mechanism by which XIAP inhibits caspase is better understood and structural models on which to base drug design are available. Currently, first generation small-molecule XIAP inhibitors have been identified and efforts are underway to develop these agents for clinical use.

Druggable Sites in XIAP

Structural and functional studies have demonstrated a groove in the BIR 3 domain of XIAP that binds and inhibits caspase 9, and two surfaces on the BIR 2 domain that bind and inhibit active caspases 3 and 7.[18-20] Thus, small molecules that bind these sites could be used to inhibit the interaction between XIAP and caspases and thereby repress XIAP-mediated inhibition of caspases.

Endogenous XIAP inhibitors as proof-of-concept molecules

Endogenous XIAP inhibitors, such as SMAC, have provided the proof-of-concept that the development of small-molecule XIAP inhibitors is feasible and may be an effective therapeutic strategy. SMAC is a mitochondrial protein that is released into the cytosol upon disruption of the mitochondria. Upon release, SMAC is cleaved into an active form that binds and inhibits the BIR 3 and BIR 2 domains of XIAP. Peptides corresponding to the 4-7 N-terminal amino acids of SMAC are necessary and sufficient for binding and inhibiting XIAP. When internalized into cells, SMAC peptides or full-length proteins induce apoptosis and sensitize cells to chemotherapy. In addition, cell-permeable SMAC peptides delay tumor growth of lung cancer and glioma tumors in xenograft models.[21,22] Thus, these biochemical, structural, and functional studies provide the basis for designing small-molecule SMAC mimics and provide the proof-of-concept that small-molecule SMAC mimics could be effective therapeutically.

Small-molecule BIR 3 inhibitors

The N-terminus of SMAC and the active site of caspase 9 bind the same pocket in the BIR 3 domain of XIAP,[18,23] and this interaction has been exploited to develop small molecule BIR 3 inhibitors. Using a high throughput fluorescent polarization competitive binding assay, pentapeptides that compete with the SMAC homologue HID for the BIR 3 domain were identified.[24] Based on these leads, tripeptides with unnatural amino acids were synthesized that bound and inhibited XIAP more potently than HID peptides. Using a similar strategy, another group[25] also identified tripeptide inhibitors of the BIR 3 domain.

Inhibitors of BIR 3 domain have also been identified by virtual screening.[26] A library of Chinese herbal remedies was docked into the BIR 3 domain "in silico" and from this screen, the structurally simple natural benzoquinone embelin was identified. Subsequent studies with embelin confirmed that it that bound and inhibited the BIR 3 domain and induced apoptosis.

Using computer-based rational drug design, Li et al[27] synthesized a tetrazoyl thioether dimeric SMAC-mimic that bound the BIR 3 domain with nanomolar affinity. This molecule also blocked the BIR 2 domain of XIAP and crossreacted with cIAP1 and cIAP2. These molecules sensitized cells to death receptor ligands and promoted the activation of caspase 8. XIAP inhibitors that promote the activation of caspase 8 are unique as XIAP does not inhibit this caspase. Rather, this activity may relate to a reported role of cIAP1 and cIAP2 as inhibitors of a JNK signaling pathway that promotes caspase 8 activation.[28]

Small-molecule BIR 2 inhibitors

The BIR 2 domain of XIAP binds and inhibits caspase 3 and 7 with two interaction sites. The linker region immediately to the N-terminus of the BIR 2 domain binds the catalytic domain of caspase 3 and blocks the active site of the enzyme by steric hindrance. This interaction is relatively weak, but is stabilized by a stronger interaction between the binding groove of BIR 2 and a site on the small subunit of caspase 3.[20,29] Using a high throughput enzymatic derepression assay, Wu et al[30] identified a series of aryl sulphonamides that inhibited XIAP and increased the enzymatic activity of caspase 3. These molecules bound the BIR 2-linker region and sensitized resistant cells to death receptors.

Using a similar enzymatic assay, we screened a combinatorial library of approximately one million peptidyl and nonpeptidyl small molecules for XIAP inhibitors.[31] After library deconvolution, several active compounds with different pharmacophores were identified, including the polyphenylurea series. These active polyphenylurea XIAP inhibitors derepressed XIAP- and BIR 2-mediated derepression of caspases 3 and 7 in an enzymatic assay, but did not derepress BIR 3-mediated inhibition of caspase 9.[31,32] In cell-free binding studies, the active XIAP inhibitors bound the BIR 2 but not the BIR 3 domain of XIAP.[32] The active

compounds, but not inactive controls, were directly toxic to both hematologic and solid tumor cell lines. Of note, these molecules were predominantly toxic as single agents, but sensitized select cell lines to stimuli of death receptor ligands.[31]

In a study of primary samples from patients with acute myeloid leukemia (AML), active polyphenylurea XIAP inhibitors including 1396-12 preferentially induced apoptosis in primary leukemia patient samples over normal hematopoietic cells with an $LD_{50} < 10$ μM in 60% of samples. In contrast, the molecules were not toxic to normal hematopoietic cells with an $LD_{50} > 40$ μM in short term cytotoxicity assays. However, the compounds inhibited growth of primary hematopoietic cells in long-term clonogenic assays, suggesting that the molecules might be toxic to normal hematopoietic progenitor/stem cells. The response to the XIAP inhibitors correlated with XIAP protein levels by immunoblotting, with the polyphenylurea XIAP inhibitors inactive in AML patient samples with low to absent levels of XIAP, and toxic to samples with higher levels of XIAP.[33] These findings and related experiments suggest that the polyphenylurea-based XIAP antagonists induce apoptosis through their intended mechanism. These results also suggest that there may be two groups of patients with AML. In the first group, XIAP levels are low and do not contribute to disease pathogenesis. In these patients, XIAP inhibitors are ineffective. In the other group, XIAP levels are elevated and may be important in blocking caspase function. It is these patients where XIAP inhibitors seem to be most effective.

Polyphenylurea XIAP inhibitors have also demonstrated efficacy and safety in xenograft models of prostate and colon cancer. Treatment of mice with brief courses of active XIAP inhibitors delayed the growth of prostate and colon cancer xenografts and suppressed prostate tumor metastasis without untoward toxicity.[31,34]

Second generation polyphenylurea XIAP inhibitors have now been synthesized and pharmacokinetic studies with these molecules are planned. Furthermore, studies to assess the affinity for other IAP members are also underway.

Conclusions

Over the last ten years, significant progress has been made in developing IAP inhibitors for therapeutic use. Antisense oligonucleotides against XIAP and survivin are currently in clinical trial and small molecule IAP inhibitors are not far behind. Initially, these molecules will be tested as single agents in heavily pretreated patients with refractory malignancies. However, it is anticipated that their greatest utility will be in combination with chemotherapy in less advanced disease.

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