Molecular Pathways: Targeting Inhibitor of Apoptosis Proteins in Cancer—
From Molecular Mechanism to Therapeutic Application

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Abstract
Inhibitor of Apoptosis (IAP) proteins play a critical role in the control of survival and cell death by regulating key signaling events such as caspase activation and NF-κB signaling. Since aberrantly high expression of IAP proteins represents a frequent oncogenic event in human cancers, therapeutic targeting of IAP proteins is considered as a promising approach. Several small-molecule pharmacological inhibitors of IAP proteins that mimick the binding domain of the endogenous IAP antagonist second mitochondrial activator of caspases (Smac) to IAP proteins have been developed over the last years. IAP antagonists have been shown in various preclinical cancer models to either directly initiate cell death or, alternatively, to prime cancer cells for cytotoxic therapies by lowering the threshold for cell death induction. IAP antagonists (i.e. GDC-0917/CUDC-427, LCL161, AT-406, HGS1029 and TL32711) are currently under evaluation in early clinical trials alone or in combination regimens. Thus, the concept to therapeutically target IAP proteins in human cancer has in principle been successfully transferred into a clinical setting and warrants further evaluation as a treatment approach.
Background

Inhibitor of Apoptosis (IAP) proteins are a family of eight human proteins, including neuronal IAP (NAIP), cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), X chromosome-linked IAP (XIAP), survivin, Baculovirus IAP Repeat (BIR)-containing ubiquitin-conjugating enzyme (BRUCE/Apollon), melanoma IAP (ML-IAP), and IAP-like protein 2 (ILP-2) (reviewed in (1)). As reflected by their name, they were initially characterized as endogenous inhibitors of caspases. However, besides the regulation of apoptosis, IAP proteins have also been implicated in the control of non-apoptotic processes, including differentiation, cell motility, migration, invasion and metastasis (2).

All IAP proteins contain at least one of the signature Baculoviral IAP Repeat (BIR) domains, a 70-80 amino acid segment that mediates protein-protein interactions between IAP proteins and caspases, thereby inhibiting caspase activation and activity. IAP proteins such as XIAP, cIAP1, cIAP2, and ML-IAP also contain the Really Interesting New Gene (RING) domain that exhibits E3 ubiquitin ligase activity (3). Depending on the chain type (e.g. K5-, K11-, K48- or K63-linked chains), ubiquitination can lead to proteasomal degradation of substrates or can alter their signaling properties (3).

IAP proteins are key regulators of programmed cell death pathways. Apoptosis represents one of the best characterized forms of programmed cell death and involves two major signaling pathways (4). In the extrinsic (death receptor) pathway, the binding of death receptor ligands, for example Tumor-Necrosis-Factor-related apoptosis-inducing ligand (TRAIL), to death receptors, for example TRAIL receptors, triggers recruitment of adaptor molecules (such as FAS-Associated via Death Domain (FADD)) and caspase-8, which in turn drives activation of caspase-8 and, subsequently, of effector caspases such as caspase-3 (4). Furthermore, caspase-8-mediated cleavage of Bid into tBid provides a link between the extrinsic and intrinsic apoptosis pathways (4). Upon the engagement of the intrinsic (mitochondrial) pathway, mitochondrial proteins such as cytochrome c or Smac are released into the cytosol and trigger apoptosis by promoting caspase activation (4). In the case of Smac, caspases
are indirectly activated via Smac-mediated neutralization of IAP proteins, which in turn results in caspase activation. IAP proteins, in particular XIAP, block caspase activation by binding to caspase-3, -7 and -9 via the BIR domains (1) and negatively regulate both the intrinsic and extrinsic apoptosis pathways. Furthermore, IAP proteins play an important role in the regulation of nuclear factor-κB (NF-κB) signaling, in particular via ubiquitination events (1). NF-κB represents one of the key transcription factors that controls various aspects of tumor biology, including cell death and survival signaling (5). Within the canonical NF-κB pathway, binding of tumor necrosis factor (TNF)α to tumor necrosis factor receptor (TNFR)1 triggers the recruitment of signaling molecules such as TNFR1-Associated Death Domain Protein (TRADD), Receptor-Interacting Protein (RIP)1, TNF Receptor-Associated Factor (TRAF)2, cIAP1 and cIAP2, which results in non-degradative ubiquitination of RIP1 via cIAP proteins and activation of Inhibitor of Nuclear Factor κB Kinase (IKK) (IκB kinase)β via a multiprotein complex containing Transforming Growth Factor (TGF)β-Activated Kinase (TAK)1, TAK1-binding protein (TAB) and NF-κB Essential Modulator (NEMO) (6). This leads to phosphorylation and proteasomal degradation of IκBα followed by nuclear translocation of NF-κB subunits to activate transcription of NF-κB target genes. NF-κB-responsive genes comprise pro-inflammatory genes, for example TNFα and interleukin-8, antiapoptotic genes such as XIAP, Bcl-2 or Bcl-XL, as well as proapoptotic genes such as CD95 and TRAIL receptors (5). cIAP proteins promote canonical NF-κB activation by ubiquitinating RIP1 (3).

Within the non-canonical NF-κB pathway, cIAP proteins mediate under resting conditions the constitutive ubiquitination and proteasomal degradation of NF-κB-Inducing Kinase (NIK) together with TRAF2 and TRAF3, thereby negatively regulating non-canonical NF-κB signaling (7, 8). Activation of non-canonical NF-κB signaling, e.g. in response to CD40 stimulation, terminates this cIAP-dependent NIK degradation and allows activation of IKKα via NIK, followed by processing of p100 to p52 and translocation of p52 to the nucleus to activate NF-κB target genes (9).
Moreover, IAP proteins have been implied in the regulation of additional signaling cascades, for example mitogen-activated protein kinase (MAPK) pathway (10), TGFβ signaling (11, 12) as well as innate and adaptive immunity signaling pathways (13, 14). cIAP proteins have been shown to be required for activation of MAPK signaling by members of the TNFR superfamily (10). XIAP has been described to function as a cofactor in TGFβ signaling (11). The immunomodulatory functions of IAP proteins are mediated via their regulation of NF-κB and MAPK signaling pathways or via the control of the activity of the inflammasome, a multiprotein complex that regulates an immune response to microbial triggers (13).

Clinical-translational advances

IAP proteins are highly expressed in multiple human malignancies and have been implicated in promoting tumor progression, treatment failure and poor prognosis, indicating that IAP proteins represent relevant targets for therapeutic exploitation (1). Indeed, IAP proteins have been shown to play a critical role in the regulation of sensitivity versus resistance of cancers to current cytotoxic strategies. Therefore, many efforts have been made over the last decade to develop strategies to neutralize IAP proteins, including antisense oligonucleotides and small-molecule inhibitors (15, 16). Due to space limitations, the current review focuses on small-molecule inhibitors for therapeutic inhibition of IAP proteins.

In principle, IAP antagonists mimick the N-terminal part of the endogenous IAP antagonist Smac that is required for binding to IAP proteins and are composed of non-peptidic elements (1). In addition to monovalent compounds that contain one Smac-mimicking motif, bivalent or dimeric IAP antagonists were developed, which consist of two monovalent Smac-mimicking units that are connected via a chemical linker. Bivalent IAP antagonists were reported to exhibit a several fold higher antitumor activity than monovalent ones, due to their higher binding affinities and their higher potency to promote caspase activation and proteasomal degradation of cIAP proteins (17-20).
Similar to Smac protein, small-molecule IAP antagonists bind to several IAP proteins including XIAP, cIAP1, cIAP2 and ML-IAP. In principle, chemically distinct IAP antagonists can differentially neutralize IAP proteins. The binding of IAP antagonists to XIAP results in activation of caspases (21, 22). The interaction of IAP antagonists with cIAP proteins stimulates their dimerization and increases their E3 ligase activity (17-20, 23). This leads to increased autoubiquitination and degradation of cIAP proteins via the proteasome. Since cIAP proteins are responsible for the constitutive proteasomal degradation of NIK, a critical upstream component of the non-canonical NF-κB pathway (7, 8), IAP antagonist-mediated depletion of cIAP proteins leads to NIK accumulation and non-canonical NF-κB activation (Fig. 1). Subsequent upregulation of NF-κB target genes such as TNFα can then engage TNFR1-mediated caspase-8 activation and apoptosis via a RIP1/FADD/caspase-8 cytosolic complex in an autocrine/paracrine manner (17-20) (Fig. 1). By depleting cIAP proteins, IAP antagonists suppress activation of the canonical NF-κB pathway. Initially, however, they may contribute to canonical NF-κB activation by stimulating the E3 ubiquitin ligase activity of cIAP proteins, thereby promoting RIP1 ubiquitination and NF-κB activation. This initial increase in E3 ligase activity of cIAP proteins is usually only transient, since cIAP proteins are themselves autoubiquitinated and degraded. Five distinct IAP antagonists are currently under clinical development (Tab. 1).

Evaluation of IAP antagonists as single agents revealed that they effectively trigger cell death in a small subset of human malignancies (19), indicating that
IAP antagonist-based combination therapies might be required in the majority of cancers to achieve sufficient antitumor activity. Therefore, a variety of rational targeted combinations have been developed over the years together with different types of cytotoxic stimuli in a large set of cancer entities \textit{in vitro} and \textit{in vivo} in order to exploit additive or synergistic drug interactions (for review see (1)). It is interesting to note that there is some evidence from preclinical studies pointing to a therapeutic window in IAP antagonist-based combination regimens to prime malignant cancer cells for cell death induction, while sparing non-malignant normal cells, e.g. peripheral blood lymphocytes, hematopoietic progenitor cells and neuronal cells (24-27). However, many more studies need to be performed before any definite conclusion can be drawn. IAP antagonist-based combinations range from conventional chemotherapeutic agents and irradiation, the two pillars of many current treatment protocols, to death receptor agonists and various kinds of small-molecule signal transduction inhibitors.

Chemotherapeutic drugs of different pharmacological classes, including e.g. doxorubicin, etoposide, gemcitabine, taxol, cisplatin, vinorelbine, SN38, 5-fluorouracil (5-FU) and cytarabine, were shown to act in concert with IAP antagonists to exert antitumor activity in preclinical models of cancers (26, 28-32). In clinical trials, taxol, daunorubicin, cytarabine or gemcitabine have been selected for combination protocols (Tab. 1). Mechanistically, chemosensitization by IAP antagonists has been linked in some studies to an autocrine/paracrine TNF\(\alpha\)-driven loop that is engaged upon depletion of cIAP proteins (31). However, TNF\(\alpha\)-independent signaling events, i.e. the formation of a cell death complex in the cytosol containing RIP1, FADD and caspase-8, were also shown to be critical for activation of cell death pathways in response to cotreatment with chemotherapeutics and IAP antagonists (32).

In addition to enhancing the antitumor activity of DNA-damaging drugs, IAP antagonists have been reported to increase radiosensitivity in various cancers (27, 33-36). Interestingly, radiosensitization by IAP antagonists was not restricted to the bulk population of the tumor, but was also found in tumor-initiating cancer stem cells that have been described to be particularly radioresistant (27).
Furthermore, the combination of IAP antagonists together with death receptor agonists turned out to be very potent to induce cell death even in otherwise resistant forms of cancer (22, 24, 25, 37-42). Accordingly, simultaneous neutralization of XIAP and cIAP proteins circumvents not only the requirement for mitochondrial amplification of death receptor-mediated apoptosis in many cancers, but also potentiates death receptor-initiated activation of caspase-8 by promoting the aggregation of caspase-8 together with FADD and RIP1 in a multimeric cytosolic complex (complex II) following the release of these signaling molecules from the receptor-bound death-inducing signaling complex (DISC). Just to give two examples, IAP antagonists were found to be able to prime pancreatic carcinoma cells, which are known to be notoriously refractory to most treatment approaches and to cell death induction, when combined with TRAIL receptor agonists such as soluble TRAIL ligand or monoclonal antibodies directed against one of the agonistic TRAIL receptors, resulting in enhanced apoptosis in vitro and reduced tumor growth in vivo (25, 39). Also, CLL cells derived from patients belonging to the poor prognostic subgroups, e.g. those with TP53 mutation, 17p deletion, chemoresistance or unmutated V(H) status, turned out to remain susceptible to cell death induction by IAP antagonists and TRAIL receptor agonists (38).

Moreover, IAP antagonists have successfully been combined with a range of signal transduction modulators depending on the cancer entity, including proteasome inhibitors, various kinds of kinase inhibitors, e.g. FMS-like tyrosine kinase (FLT)3 inhibitors, Platelet-derived growth factor (PDGF) receptor inhibitors, insulin-like growth factor (IGF) receptor inhibitors or Epidermal Growth Factor (EGF) receptor inhibitors, as well as monoclonal antibodies targeting growth factor receptors (43-46). Also, IAP antagonists have been shown to increase the antitumor effects of immunotherapies both by priming cancer cells to immune cell-mediated cytotoxicity and/or by altering immune cell functions (47).

In addition to promoting apoptosis, there is accumulating evidence indicating that IAP antagonists can also potentiate non-apoptotic forms of cell death such as necroptosis, a recently identified programmed form of necrosis (48). Accordingly,
IAP antagonists have been reported to promote the formation of the necrosome, a complex consisting of RIP1 and RIP3, under conditions in which caspase activation is inhibited (49, 50). The IAP antagonist-mediated amplification of necroptosis opens new perspectives to overcome treatment resistance particularly in apoptosis-refractory forms of cancer.

Five distinct IAP antagonists are currently undergoing evaluation in early clinical trials for the treatment of cancer (www.clinicaltrials.gov). Monovalent agents such as GDC-0917/CUDC-427 (Genentech, Inc./Curis), LCL161 (Novartis) and AT-406 (Ascenta Therapeutics) offer the advantage that they can be administered orally. By comparison, bivalent compounds such as HGS1029 (Aegera Therapeutics/Human Genome Sciences), and TL32711 (TetraLogic Pharmaceuticals) require intravenous administration but may turn out to be more efficacious as indicated from preclinical studies. Nevertheless, the question remains open as to whether mono- or bivalent IAP antagonists are the most promising candidates for further clinical development based on their pharmacodynamic and pharmacokinetic properties as well as their toxicity profiles. In addition, the question as to which IAP proteins alone or in combination represent the most critical targets for therapeutic intervention in cancer has not yet been answered. While neutralization of cIAP proteins has been shown to be instrumental for single-agent activity of IAP antagonists by engaging a NF-κB-dependent autocrine/paracrine TNFα loop that triggers cell death upon concomitant depletion of cIAP proteins, the release of the XIAP-imposed block on caspases is considered to be critical for full activation of the effector phase of apoptosis.

Phase I trials examining the safety and pharmacological properties of IAP antagonists trials have been completed for LCL161, HGS1029 and TL32711, demonstrating that IAP antagonists are in principle well tolerated (51-53). Furthermore, several combination protocols with IAP antagonists together with standard-of-care anticancer therapeutics have been initiated, including chemotherapeutic drugs such as taxol, daunorubicin, cytarabine and gemcitabine.
In addition, a trial testing TL32711 in combination with the demethylating agent 5-Azacitidine has recently been launched (Tab. 1). Interestingly, there is also very recent evidence from preclinical studies showing synergistic antileukemic effects when an IAP antagonist was combined together with 5-Azacitidine (54).

Accompanying biomarker studies have demonstrated target inhibition by IAP antagonists by showing depletion of cIAP1 protein levels both in surrogate tissues such as peripheral blood mononuclear cells as well as in tumor tissue (51-53). In addition, increased levels of circulating cytokines and chemokines in plasma specimens were used as pharmacodynamic parameters. However, while the sensitivity of cancer cells to monotherapy with IAP antagonists has been linked to their ability to engage an autocrine/paracrine loop of TNFα production, corresponding in vivo studies largely failed to detect a substantial increase in TNFα levels in the circulation (19). This may be explained by local production of TNFα rather than by its widespread release in the circulation. Also, additional cytokines or chemokines may be relevant for the antitumor activity of IAP antagonists. While these markers may serve as indicators of target antagonism, they will likely not be suitable to predict treatment response.

Pharmacodynamic assays to determine treatment response include for example the detection of apoptotic markers in tumor biopsies such as cleavage products of caspase-3, -8 and poly (ADP-ribose) polymerase (PARP). However, the determination of markers of apoptotic cell death may not be sufficient to properly assess treatment response, since under certain conditions IAP antagonists can also trigger alternative forms of cell death besides apoptosis, for example necroptosis (49, 50).

In summary, IAP proteins represent promising targets for the development of small-molecule cancer therapeutics, since they are expressed at aberrantly high levels in multiple human malignancies and since they block cell death pathways while supporting cancer cell survival. The therapeutic potential of IAP antagonists may particularly reside in rational combinations together with other cytotoxic
strategies to take advantage of additive or synergistic drug interactions. This has convincingly been demonstrated in various preclinical cancer models. In addition, clinical studies have recently been launched to test this concept. As many of these clinical trials are currently ongoing, it is too early to draw conclusions on the clinical efficacy of IAP antagonists.

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Figure Legend

Figure 1. Regulation of apoptosis and NF-κB signaling by IAP proteins and their antagonists.

XIAP negatively regulates both the extrinsic and intrinsic apoptosis pathways by inhibiting caspase-3 and -9. The extrinsic (death receptor) pathway is triggered upon ligation of death receptors such as TRAIL receptors (TRAIL-R) by their ligands such as TRAIL, leading to activation of caspase-8 and -3. The intrinsic (mitochondrial) pathway is engaged by the release of mitochondrial proteins such as cytochrome c (CytC) or Smac into the cytosol, which promotes caspase-9 and -3 activation. Neutralization of XIAP-mediated caspase inhibition by IAP antagonists promotes caspase-dependent apoptosis. cIAP proteins (cIAPs) control activation of canonical and non-canonical NF-κB pathways. cIAP proteins promote canonical NF-κB activation via non-degradative ubiquitination of RIP1, while they inhibit non-canonical NF-κB signalling via ubiquitination of NIK and its degradation via the proteasome.

IAP antagonists stimulate the E3 ubiquitin ligase activity of cIAP proteins, thereby promoting their autoubiquitination and proteasomal degradation. In turn, NIK is stabilized and activates non-canonical NF-κB signaling. Induction of NF-κB target genes such as TNFα can then in an autocrine/paracrine manner trigger TNFR1-mediated caspase-8 activation and apoptosis via a RIP1/FADD/caspase-8 cytosolic complex. IAP antagonists suppress activation of the canonical NF-κB pathway by depleting cIAP proteins. Initially however, they may stimulate canonical NF-κB signaling by increasing the E3 ubiquitin ligase activity of cIAP...
proteins, which leads to RIP1 ubiquitination and NF-κB activation. Stars marks IAP antagonists and their targets. Please see text for more details.
References


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Table 1. Clinical trials with IAP antagonists

<table>
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<th>Compound</th>
<th>Combination</th>
<th>Cancer type</th>
<th>Status</th>
<th>Phase I/II</th>
<th>Reference</th>
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Abbreviations: AML, acute myelogenous leukemia; GEM, gemcitabine; MDS, myelodysplastic syndrome; 5-Aza, 5-Azacitidine.
Figure 1:

![Diagram of TNFα and TRAIL signaling pathways.](image-url)

- TNFα activates TNF-R1 which leads to the formation of TRADD, TRAF2, TRAF3, and NIK.
- cIAPs are involved in the regulation of NF-κB.
- TRAIL activates TRAIL-R, leading to the formation of FADD and RIP1.
- Caspase-8 and Caspase-9 are involved in the caspase cascade, with Smac and XIAP playing roles in inhibition.
- Bid is cleaved by Caspase-8, leading to the release of CytC.
- TNFα can also activate TNF-R1, leading to the formation of TRADD and TRAF2.
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