Review

Livin/melanoma inhibitor of apoptosis protein as a potential therapeutic target for the treatment of malignancy

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Abstract

Livin, also called melanoma inhibitor of apoptosis protein (IAP) or kidney IAP, is a member of the IAP family of caspase inhibitors that selectively binds the endogenous IAP antagonist SMAC and caspase-3, caspase-7, and caspase-9. As such, Livin inhibits apoptosis, and its overexpression renders malignant cells resistant to chemotherapy. Therefore, inhibitors of Livin could be useful adjuncts to chemotherapy in the treatment of malignancies. This review will discuss Livin as a potential therapeutic target and strategies for its inhibition, including antisense oligonucleotides, small-molecule inhibitors, and immune-mediated approaches. [Mol Cancer Ther 2007;6(1):24–30]

Introduction

Caspases are cysteine proteases and are the executioners of apoptosis. These proteases are arranged in a cascade with upstream initiator caspases capable of cleaving and activating downstream effector caspases (Fig. 1). Inhibitor of apoptosis proteins (IAP) are a family of antiapoptotic proteins that bind and inhibit upstream (e.g., caspase-9) and downstream (e.g., caspase-3 and caspase-7) caspases and inhibit apoptosis (1–3). Thus, given their position in the apoptosis pathway, IAPs are important mediators of malignant potential and chemoresistance.

Membership in the IAP family is based on the presence of at least one baculovirus IAP repeat (BIR) domain (1, 3–5). The BIR domain is a zinc-binding region of ~70 amino acids. The BIR domain is required for the antiapoptotic activity of IAPs, but not all proteins with BIR domains have antiapoptotic functions and are part of the IAP family (6). In the family of IAPs, the BIR domains bind caspases and the endogenous IAP antagonist SMAC.

To date, at least eight human IAP members have been identified, including C-IAP1, C-IAP2, NAIP, Survivin, X-linked IAP (XIAP), Bruce, ILP-2, and Livin (ref. 7; Fig. 2). Among them, Livin, also called melanoma IAP (ML-IAP; ref. 8) or kidney IAP (9), is more recently identified (10). Previous reports have indicated that Livin is overexpressed in a variety of malignancies. Moreover, in some studies, levels of Livin are correlated with tumor progression and patient outcome.

Inhibitors of IAPs are currently being developed for clinical use in the treatment of malignancies. Antisense oligonucleotides targeting XIAP (11) and Survivin (12) are in clinical trial, and small-molecule XIAP inhibitors are soon poised to enter clinical trial (13). Given the role of Livin in controlling apoptosis and its selective up-regulation in malignancies, this IAP is also a potential molecular target for anticancer strategies.

This review will discuss Livin as a potential therapeutic target. For a broad review of the IAP family of proteins or a discussion of XIAP and Survivin inhibitors, the reader is referred to previous reviews (1, 13).

Livin, an IAP Family Member that Binds Caspases and Inhibits Apoptosis

In 2000, Vucic et al. searched a sequence database for genes with potential BIR domains (8). From this search, the authors identified a novel IAP, ML-IAP (a synonym for Livin), that had one BIR domain and a RING finger motif. Subsequent studies identified similar proteins by searching in a computer database using the polypeptide consensus sequence of the BIR domain of IAPs in a human fetal kidney cDNA library (9, 10).

In comparing the BIR domains of Livin to the BIR domains of other IAP family members, the BIR domain of Livin is most homologous to the BIR3 domains of NAIP (58.8% identity), c-IAP2 (54.4% identity), and XIAP (50.0%

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Figure 1. Pathways of caspase activation.
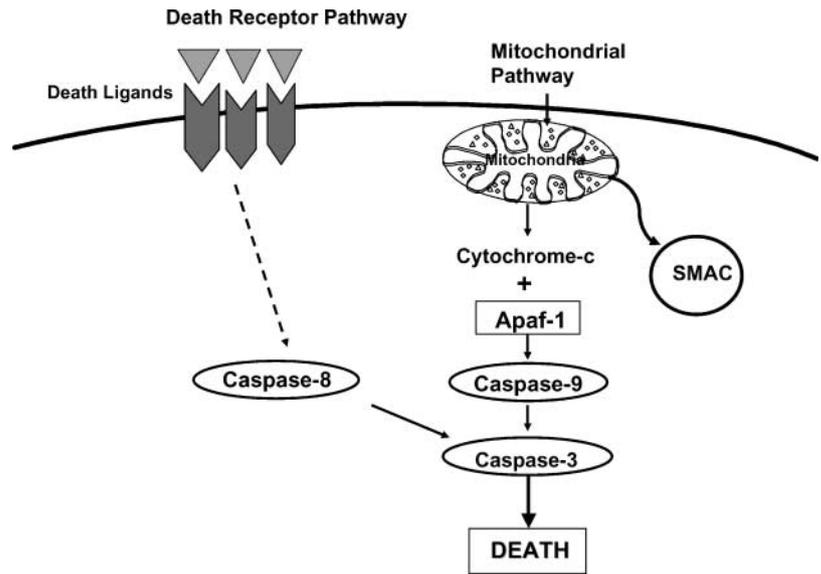
identity). The RING domain of Livin is also homologous to the RING domains of c-IAP2, c-IAP1, and XIAP with 74%, 72%, and 60% identity, respectively (9). Thus, although Livin is similar to Survivin in that it contains only one BIR domain, sequence alignment studies predicted that this IAP would function more like XIAP or c-IAP 1/2.

Using the known nuclear magnetic resonance structures of XIAP and c-IAP-1, Vucic et al. (8) developed a virtual three-dimensional model of the BIR domain of Livin (ML-IAP). According to their model, the Livin BIR has a secondary structure of four α helices and a three-stranded β sheet. The BIR domain was predicted to coordinate a zinc atom with amino acid residues C124, C127, H144, and C151 that stabilized the overall fold. Further studies showed that mutations of these zinc-binding residues reduced the ability of Livin to inhibit apoptosis. The most significant loss in the ability of Livin to inhibit Fas- and tumor necrosis factor–induced apoptosis was found when the structurally important zinc-coordinating amino acid C124 was mutated.

The crystal structure of Livin (ML-IAP) complexed to SMAC was reported by Franklin et al. (14). They confirmed
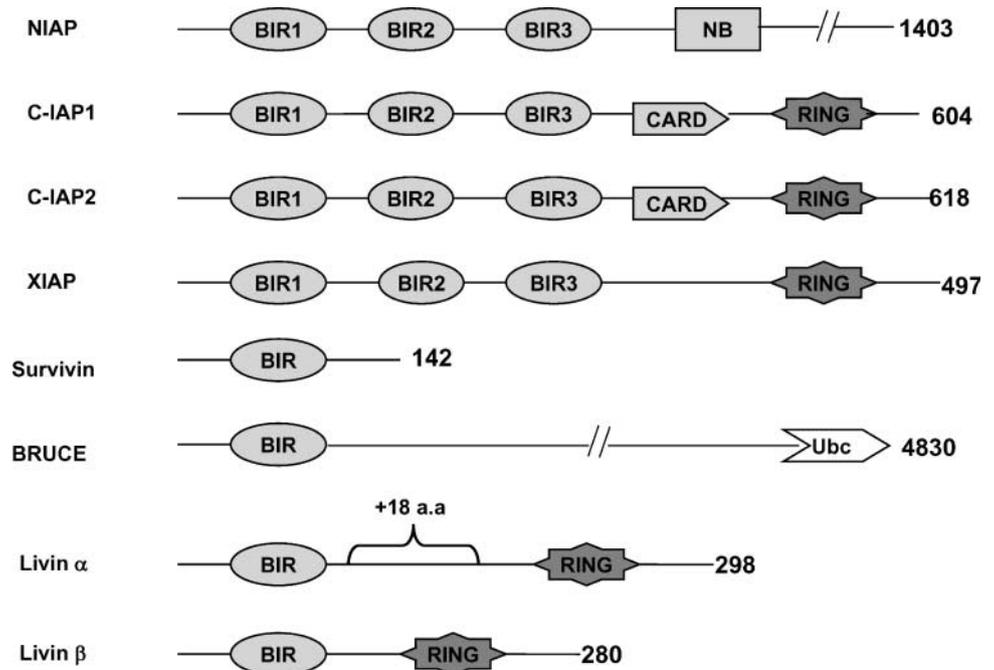
Figure 2. Livin, a member of the IAP family of proteins. To date, eight human IAP family members have been identified based on shared BIR domains, including Livin. IAP members may contain a caspase activation recruitment (*CARD*) domain, RING finger motif, an E2 ubiquitination enzyme motif (*Ubc*), and a nucleotide-binding domain (*NB*). The Livin α isoforms contains an addition 18 amino acids (*aa*) compared with the Livin β isoform.

the α helix and β sheet secondary structure predicted in the virtual model. They also confirmed the chelation of a zinc atom by C124, C127, H144, and C151. As such, the structure of Livin complexed to SMAC was similar to other SMAC/BIR complexes such as XIAP-BIR3/SMAC. Data derived from the virtual model and crystal structure of Livin are important as they facilitate the design of Livin inhibitors.

Livin and survivin have only one BIR domain, and studies have compared the structure and function of these IAP family members. Structurally, Livin contains a RING domain that is not present in survivin, whereas survivin contains a coiled-coil domain not present in Livin. Functionally, these proteins have different cellular activities. For example, survivin, unlike Livin, has an important role in cell division as it ensures the proper alignment of chromosomes during mitosis (15). Consistent with its role in cell division, survivin localizes to components of the mitotic apparatus (1).

The RING Finger of Livin

The RING domain of IAP family members, such as XIAP, has E3 ubiquitin ligase activity and is responsible for the ubiquitination and degradation of apoptotic regulators such as SMAC, caspase-3, and caspase-9 (16, 17). E3 ubiquitin ligase activity has also been described for the RING finger motif of Livin (18). In Livin, unlike other IAP members, however, the RING domain may also play a role in the subcellular localization of the protein. For example, in HeLa cells, deletion of the RING domain disrupted the subcellular localization of Livin. Wild-type Livin and mutants lacking the BIR domain were localized to the nucleus and in a filamentous pattern in the cytoplasm. In contrast, mutants lacking the RING domain were spread diffusely throughout the cytoplasm (10). These results suggest that the RING finger motif may have a unique role in this IAP family member. However, it is important to note that in MCF7 breast cancer cells, wild-type Livin is normally located diffusely in the cytoplasm (8). As such, the role of the RING finger in subcellular localization is uncertain but may be cell type specific.

Livin Inhibits Apoptosis

Consistent with its membership in the IAP family and its role as an antiapoptotic protein, overexpression of Livin cDNA in MCF7 cells inhibited apoptosis induced by doxorubicin and 4-tertiary butylphenol (ref. 8; Table 1). Surprisingly, in 293 cells, overexpression of Livin increased cell death induced by etoposide. How Livin can assume a proapoptotic role is unclear but may relate to Livin fragments produced after cleavage by caspases (19). In the melanoma cell line MeWo treated with staurosporine, Livin was cleaved into 30- and 28-kDa fragments by caspase-3 and caspase-7, respectively. The resulting Livin fragment (amino acids $DHVD_{52}G$ to $GARD_{238}V$) contained the BIR and RING domains but had proapoptotic activity. Verifying the proapoptotic activity of this Livin fragment, overexpression of this fragment in 293T cells directly induced apoptosis (19).

Although transformation to a proapoptotic protein is unique for an IAP family member, the ability of a traditional antiapoptotic protein to be cleaved into a proapoptotic form has previously been described (20, 21). For example, cleavage of the NH_2-terminal 34 amino acids of Bcl-2 converts this classic antiapoptotic protein into a proapoptotic effector (22). Thus, modalities that increase the cleavage of Livin and its conversion to a proapoptotic protein could be useful therapeutically for the treatment of malignancy.

Livin Binds Caspase-3/7 and Caspase-9

Like other BIR3-containing IAP members, such as XIAP, Livin binds caspase-3, caspase-7, and caspase-9 but not caspase-8. For example, Kasof et al. showed that Livin bound recombinant active caspase-3 and caspase-7 in a cell-free binding assay (10). In HeLa cells, Livin bound pro-caspase-9, but not the 12- and 20-kDa subunits of the active form. Overexpression of Livin also inhibited the activation of this caspase (10). Keeping with its function as an IAP, deletion of the BIR domain abrogated the ability of Livin to bind caspases and inhibit apoptosis. However, this study

Table 1. Effects of Livin overexpression and knockdown

Method	Isoform	Cell line	Effect	Ref.
Overexpression				
	Full length	MCF7	Inhibited doxorubicin-induced cell death	Vucic et al. (8)
	Full length	293	Increased etoposide-induced cell death	Lin et al. (9)
	Full length	293	Inhibited menadione-induced cell apoptosis	Lin et al. (9)
	Fragment 52-238	293T	Induced apoptosis	Nachmias et al. (19)
	Livin α	Jurkat	Inhibited staurosporine, TNF, and CD95-induced cell death	Ashhab et al. (28)
	Livin β	Jurkat	Inhibited etoposide, TNF, and CD95-induced cell death	Ashhab et al. (28)
Knockdown				
Antisense	Full length	HeLa	Decreased viability	Kasof et al. (10)
siRNA	Full length	HeLa	Sensitized to doxorubicin, UV, and TNF	Crnkovic-Mertens et al. (38)
Antisense	Full length	G361	Decreased viability	Kasof et al. (10)
siRNA	Full length	MeVo	Decreased colony formation	Crnkovic-Metens et al. (38)
siRNA	Livin β	HeLa	Decreased clonogenic growth, sensitized to etoposide and TNF	Crnkovic-Metens et al. (29)
siRNA	Livin α	HeLa	No effect	Crnkovic-Metens et al. (29)

Abbreviations: siRNA, small interfering RNA; TNF, tumor necrosis factor.

did not directly test the ability of Livin to inhibit the enzymatic activity of caspases.

Although capable of binding and inhibiting apoptosis, the ability of Livin to directly inhibit the enzymatic activity of caspases has been brought into question by a recent report by Eckelman et al. (23). In this study, the authors showed that the IAP family members cIAP1 and cIAP2 bound caspase-3, caspase-7, and caspase-9 but were very weak inhibitors of their enzymatic activity compared with XIAP. The weak inhibitory activity was related to critical differences in the amino acid sequence of the caspase-binding region of the BIR domains of XIAP and cIAP1/2. Livin also lacks the critical amino acid residues that render XIAP a potent inhibitor of the enzymatic activity of caspases. Thus, the mechanism by which Livin, as well as cIAP1/2, inhibit apoptosis is unclear. Potentially, Livin acts as a sink to bind and sequester the endogenous IAP antagonist SMAC.

Livin as a Sink for SMAC

SMAC is an endogenous mitochondrial protein that is released from the mitochondrial during apoptosis (24, 25). In the cytoplasm, SMAC binds and inhibits IAPs and thereby acts as a proapoptotic protein (26). SMAC can also bind Livin. Interestingly, Livin binds SMAC with an affinity similar to XIAP. However, Livin binds and inhibits caspase-9 with an affinity $\sim$300-fold weaker than XIAP. Thus, Vucic et al. speculated that Livin might predominantly function as a sink for SMAC (27). In this scenario, Livin would bind and sequester SMAC, thereby permitting other IAPs, such as XIAP, to function uninhibited. This observation would explain the antiapoptotic activity of Livin as well as the findings of Eckelman et al. (23) that suggest that Livin does not directly inhibit the enzymatic activity of caspases. Livin acting as a sink for SMAC also has important therapeutic implications. If binding SMAC is the predominant function of Livin, then Livin inhibitors should be molecules that are designed to target the Livin-SMAC interaction rather than the Livin-caspase interactions.

Splice Variants of Livin

Two splice variants of Livin (Livin α and Livin β) have been recognized to date. The amino acid sequences of Livin α and β are essentially identical save for an extra 18 amino acids in the BIR-RING linker region of Livin α. The increased 18 amino acids in Livin α are predicted to form an addition α helix in this isoform (28). Thus, Livin α may have additional binding partners compared with Livin β. The functional differences between the two isoforms are uncertain as studies are contradictory (Table 1). In HeLa cells, selective small interfering RNA silencing of Livin β but not Livin α blocked growth of these cells in clonogenic survival assays. In addition, silencing of Livin β but not Livin α sensitized the cells to tumor necrosis factor-α– and etoposide-induced cell death (29). In contrast, in Jurkat cells, overexpression of Livin α but not Livin β protected cells from staurosporine, whereas overexpression of Livin β protected cells from etoposide-induced cell death. Both isoforms protected cells from tumor necrosis factor and

anti-CD95–induced apoptosis (28). Thus, the effects of the different isoforms seem to vary based on cell type and apoptotic stimulus. However, the explanations for these differences are unclear at present.

Regulation of Livin Expression

Livin can be regulated through a few different mechanisms, and understanding Livin regulation highlights different strategies for developing Livin inhibitors. Like other IAP family members, SMAC binds and inhibits Livin (30). In addition, SMAC potentiates the ubiquitination of Livin. Although SMAC can ubiquitinate Livin, this modification of Livin did not increase Livin degradation (31). Thus, the functional importance of Livin ubiquitination by SMAC is unclear.

IAPs, such as Survivin and XIAP, can also be regulated at the level of transcription. Although not yet reported for Livin, one might speculate that factors that modulate the Livin promoter could increase or decrease Livin expression. The selective expression of Livin in normal tissues and its increased expression in malignancy also supports this level of regulation of Livin levels.

Livin as a Therapeutic Target

Overexpression of Livin as a Prognostic Marker

Studies showing the prognostic value of Livin are important because they help validate the protein as a potential therapeutic target and help provide a rationale for testing the targeted therapy in that disease site. The prognostic importance of Livin has been shown in neuroblastoma and bladder cancer. For example, in patients with neuroblastoma, overexpression of Livin when coupled with amplified Myc oncogene expression predicted shorter median survival in these patients compared with patients without increased expression of both of these markers (median overall survival = 32 versus 135 months; ref. 32).

Likewise, Livin expression also predicted patient outcome in patients with bladder cancer (33). In 30 patients with bladder cancer, Livin α and β expression were measured by reverse transcription-PCR. In this study, patients with increased Livin α had a shorter time to relapse compared with patients without increased Livin α expression (3.5 versus 25.8 months). Although Livin α expression correlated with prognosis, it did not relate to other clinical variables such as stage and grade of the malignancy. In this study, Livin β expression did not correlate with outcome.

Although Livin may have a prognostic role in neuroblastoma and bladder cancer, studies in patients with lung cancer (34), colon cancer (35), nasopharyngeal cancer (36), and metastatic melanoma (37) did not show an association with clinical outcome despite the expression of Livin in these malignant cells. For example, in a study of 80 of patients with nasopharyngeal cancer, Livin expression was detected in 48% by immunohistochemistry (36). Livin expression was dichotomized into high and low levels based on the percentage of the tumor cells with positive

staining. Using this dichotomization, Livin expression did not predict for overall or disease-free survival. However, the sample sizes of these studies were small. Thus, these studies are likely underpowered to conclude that Livin expression is not prognostic. Potentially, small differences in outcomes could be shown with larger sample sizes.

Coexpression of other IAP family members with Livin has only been assessed in a few studies. For example, in studies of Livin expression in patients with lung cancer (34) or nasopharyngeal cancer (36), coexpression of survivin was measured but did not correlate with Livin expression. The levels of other IAPs were not measured in these studies. In patients with bladder cancer, Livin but not survivin levels correlated with rate of relapse, but levels of other IAP members were not measured (33). In the studies of patients with neuroblastoma (32), where increased Livin predicted a worse outcome, expression of other IAP members was not reported. Therefore, in the studies of patients with neuroblastoma and bladder cancer, one cannot exclude the possibility that there were alterations in the expression of other IAP members that may have explained the poor prognosis of the patients with high levels of Livin.

The failure to consistently show the prognostic importance of Livin does not invalidate this target, but it does highlight limitations in our understanding of the biology of Livin. For example, there may be mutations or posttranslational modifications of Livin that influence its biological activity. These alterations would not have been measured in the prognostic studies highlighted above. Furthermore, Livin may have to be considered in the context of levels of other IAP members or other apoptotic proteins to show its prognostic importance.

Targeting Livin: Proof-of-Concept Studies

Given its role as a downstream inhibitor of apoptosis, attention has turned to Livin as a potential therapeutic target. A number of proof-of-concept studies have been conducted that suggest that targeting Livin could be therapeutically useful. For instance, as discussed above, inhibiting Livin β with small interfering RNA decreased HeLa cell survival in clonogenic assays and sensitized cells to etoposide chemotherapy (29, 38). Likewise, knocking out both isoforms with Livin antisense in HeLa and G361 cells reduced the levels of Livin protein, thereby inducing apoptosis and decreasing viability (10). Thus, these studies show that antisense oligonucleotides targeting Livin could be useful for the treatment of malignancy. Small interfering RNA strategies might also be useful, but the therapeutic use of small interfering RNA is still in its infancy.

As noted above, Livin may function as a sink for the endogenous IAP antagonist SMAC and thereby inhibit apoptosis. SMAC peptides corresponding to the NH_2 terminus of SMAC are necessary and sufficient for binding Livin. Therefore, Franklin et al. examined the effects of SMAC peptides in MCF7 cells overexpressing Livin (14). In this study, SMAC peptides with amino acid sequences corresponding to the nine residues at the NH_2 terminus of active SMAC bound and inhibited Livin. Moreover, the peptide (AVPIAQKSE) could be modified to increase their binding affinity and selectivity for Livin by substituting the Pro3′ with (2*S*,3*S*)-3-methylpyrrolidine-2-carboxylic acid. Internalization of SMAC peptides abrogated the ability of Livin to inhibit apoptosis and sensitized Livin-expressing cells to chemotherapy. Thus, small molecules that inhibit the SMAC-Livin interaction by mimicking the interaction with SMAC could be useful therapeutic agents for the treatment of malignancy. Furthermore, these studies show the Livin inhibitors could be developed through rational structure modification of binding peptides.

Given the differences in sequence and structure between Livin and other IAPs, such as XIAP, it is possible to use virtual drug design or high-throughput screening methods to identify molecules that preferentially bind to one IAP member over the others. For example, phage display was used to select peptides that bound the BIR domain of Livin with higher affinity than XIAP and vice versa (14). Thus, molecules could be created with preferential affinity for selected IAP members. Alternatively, molecules that bind and inhibit the BIR domains of multiple IAP members could also be developed. As discussed in more detail below, it is currently unknown whether a pan-IAP inhibitor or a specific IAP inhibitor would be preferential for therapeutic use.

An interesting approach to developing anti-Livin therapies is based on immunotherapy (3, 39, 40). Supporting an immunotherapy-based approach to developing anti–Livin/ML-IAP therapies, patients with gastrointestinal cancer (41), breast cancer (42), and lung cancer (43) have anti-Livin antibodies in their serum, indicating Livin may act as a major tumor-associated antigen that can be presented by MHC class I molecules. Furthermore, Livin-derived peptide can induce CTL reactivity against Livin-expressing cells in an HLA-restricted manner, leading to CTL-induced death of both autologous Livin-expressing cells and an HLA-matched Livin-expressing melanoma cell line. Thus, there could be a role for Livin immunotherapy after reduction of the bulk of the tumor burden with chemotherapy or surgical resection (44–46).

Livin: Unanswered Questions

The ability of Livin to inhibit apoptosis and its overexpression in malignant cells make this molecule a potential therapeutic target. However, a number of questions about Livin remain unanswered, and clarification of these issues will be important in determining whether Livin inhibitors should move forward for clinical development.

First, the prognostic value of Livin has not been fully clarified. Although the lack of correlation with patient outcome may reflect our lack of knowledge about this protein, it may also indicate that Livin is not an optimal therapeutic target. Second, the effects of Livin inhibition on normal cells are unknown. Studies in Livin knockout mice and silencing Livin in primary normal cells will thus be important in judging the potential toxicity and therapeutic index of potential Livin inhibitors.

Finally, it is currently unknown whether pan-IAP inhibitors or a specific Livin inhibitor would be most useful therapeutically for the treatment of malignancy. If a pan-IAP inhibitor would be most useful, then developing small molecules that inhibit Livin and other IAPs would be a better therapeutic strategy as opposed to antisense oligonucleotides that only target Livin. If an inhibitor of a specific IAP seems optimal, it will be important to determine whether a compensatory up-regulation of other IAP members might occur and thereby negate the effects of inhibiting a single IAP.

Given the overall role of Livin and its relation to other IAPs, it would seem that specific Livin inhibitors that do not block other IAPs would have a very narrow therapeutic indication and be useful for a very selected subgroup of patients with cancer. However, IAP inhibitors that fail to inhibit Livin might be suboptimal as the uninhibited activity of Livin could negate the beneficial effects of inhibiting other IAP members. Thus, we propose that a pan-IAP inhibitor that also blocks Livin will be the most appropriate choice for therapeutic development. However, this statement is merely conjecture and needs to be proven experimentally.

Through future research, we predict that the above questions will be clarified. In the process, we will further our knowledge about the role of Livin in malignant and normal cells. This information will also improve the ability to target Livin with greater proficiency.

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