

## Mini Review

# Targeting the BIR Domains of Inhibitor of Apoptosis (IAP) Proteins in Cancer Treatment

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## ABSTRACT

Inhibitor of apoptosis (IAP) proteins are characterized by the presence of the conserved baculoviral IAP repeat (BIR) domain that is involved in protein-protein interactions. IAPs were initially thought to be mainly responsible for caspase inhibition, acting as negative regulators of apoptosis, but later works have shown that IAPs also control a plethora of other different cellular pathways. As X-linked IAP (XIAP), and other IAP, levels are often deregulated in cancer cells and have been shown to correlate with patients' prognosis, several approaches have been pursued to inhibit their activity in order to restore apoptosis. Many small molecules have been designed to target the BIR domains, the vast majority being inspired by the N-terminal tetrapeptide of Second Mitochondria-derived Activator of Caspases/Direct IAP Binding with Low pI (Smac/Diablo), which is the natural XIAP antagonist. These compounds are therefore usually referred to as Smac mimetics (SMs). Despite the fact that SMs were intended to specifically target XIAP, it has been shown that they also interact with cellular IAP-1 (cIAP1) and cIAP2, promoting their proteasome-dependent degradation. SMs have been tested in combination with several cytotoxic compounds and are now considered promising immune modulators which can be exploited in cancer therapy, especially in combination with immune checkpoint inhibitors. In this review, we give an overview of the structural hot-spots of BIRs, focusing on their fold and on the peculiar structural patches which characterize the diverse BIRs. These structures are exploited/exploitable for the development of specific and active IAP inhibitors.

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## 1. Introduction

Resistance to cell death is considered a hallmark of cancer [1], and it represents a major issue in therapy by frustrating the efficacy of the cytotoxic compounds employed in cancer treatment. Therefore, there is a great interest in understanding the pathological determinants which protect cancer cells and prevent their capacity to undergo apoptosis or other mechanisms of cell death. To this end, many groups have focused their studies on a family of proteins referred to as inhibitor of apoptosis (IAP; [2–5]) proteins which are often deregulated in cancer cells and whose levels have been shown to affect patients' prognosis [6–13]. The distinctive trait which characterizes all IAPs is constituted by the presence of conserved domains called baculoviral IAP repeats (BIRs; [2,3,5]). In baculovirus, these domains are responsible for the interaction with caspases and their blockage [3,14], preventing cell suicide and allowing viral propagation. Due to the presence of BIR domains, IAPs were at first considered direct regulator of caspases [15–21] and they were thought to be mainly inhibitors of apoptosis (hence their name). Nevertheless, later studies have clearly shown that this view is extremely limited and that IAPs display several other functions [8,22]. Indeed, a direct interaction with executioner caspases, such as caspase-3, has been excluded, for at least 7 (neuronal apoptosis inhibitory protein, NAIP; cellular IAP-1, cIAP1; cIAP2; Survivin; Apollon; melanoma IAP, ML-IAP; and IAP-like protein-2, ILP-2) out of the 8 known IAPs. Of note, this does not imply that IAPs cannot regulate cell viability, though their pro-survival activity derives from the regulation of a plethora of different signaling pathways [8] and not from the direct inhibition of caspases.

In this regard, X-linked IAP (XIAP) represents the “black sheep” of the family [23], being the only member which interacts and inhibits both initiator and effector caspases [24]. XIAP, as NAIP, cIAP1 and cIAP2, sequence contains three BIR domains, each made of about 70 amino acids, whose fold is stabilized by a zinc atom that is coordinated by one histidine and three cysteine residues (belonging to the zinc-finger domain family). Based on the absence/presence of a deep peptide-binding, IAP binding motif (IBM), groove, the BIR domains can be grouped into type I and II. XIAP BIR1, lacking the IBM groove, belongs to type I BIR domain and it is the only XIAP BIR domain not involved in the regulation of caspases. XIAP BIR2 and BIR3 are type II BIR domains. BIR2, together with BIR1-BIR2 linker region, has been shown to interact with effector caspase-3 [21] and 7 [19]. BIR3 is known to inhibit caspase-9 activity [20] by binding its N-terminal tetrapeptide (ATPF), hence blocking the intrinsic apoptotic pathway [18].

XIAP represents a potential target in cancer treatment and several approaches have been pursued in order to inhibit its activity or to reduce its expression levels [25–30]. For example, antisense oligonucleotide AEG35156 was tested both in pre-clinical settings [10,31] and in clinical trials [32–35] to decrease the expression of XIAP and enhance the cytotoxic activity in combinatorial regimens [10]. By employing other strategies, small molecules designed to target the BIR2 domain were shown to promote the activity of caspase-3 [36], but BIR3 is indeed the most extensively studied domain for the development of BIR-targeted compounds. This is the case, for example, of Embelin, a molecule derived from the Japanese *Ardisia* herb, which was shown to inhibit cell growth and induce apoptosis in cancer cells expressing high levels of XIAP [37]. However, the main approach to target the BIR3 domain was stirred by XIAP natural antagonist Second Mitochondria-derived Activator of Caspases/Direct IAP Binding with Low pI (Smac/Diablo), which inspired the design and synthesis of a huge number of compounds [38], named Smac mimetics (SMs).

In this review, we summarize the structural properties of BIRs, focusing on their fold and on the crucial “hot-spots” exploited for the development of IAP-directed anti-tumor therapies, as SMs. These inhibitors, both peptidic and peptidomimetic, were designed to displace caspase-9 from BIR3 domain and promote the apoptotic machinery, but actually they resulted in unexpected outcomes and acted as pan-IAP inhibitors,

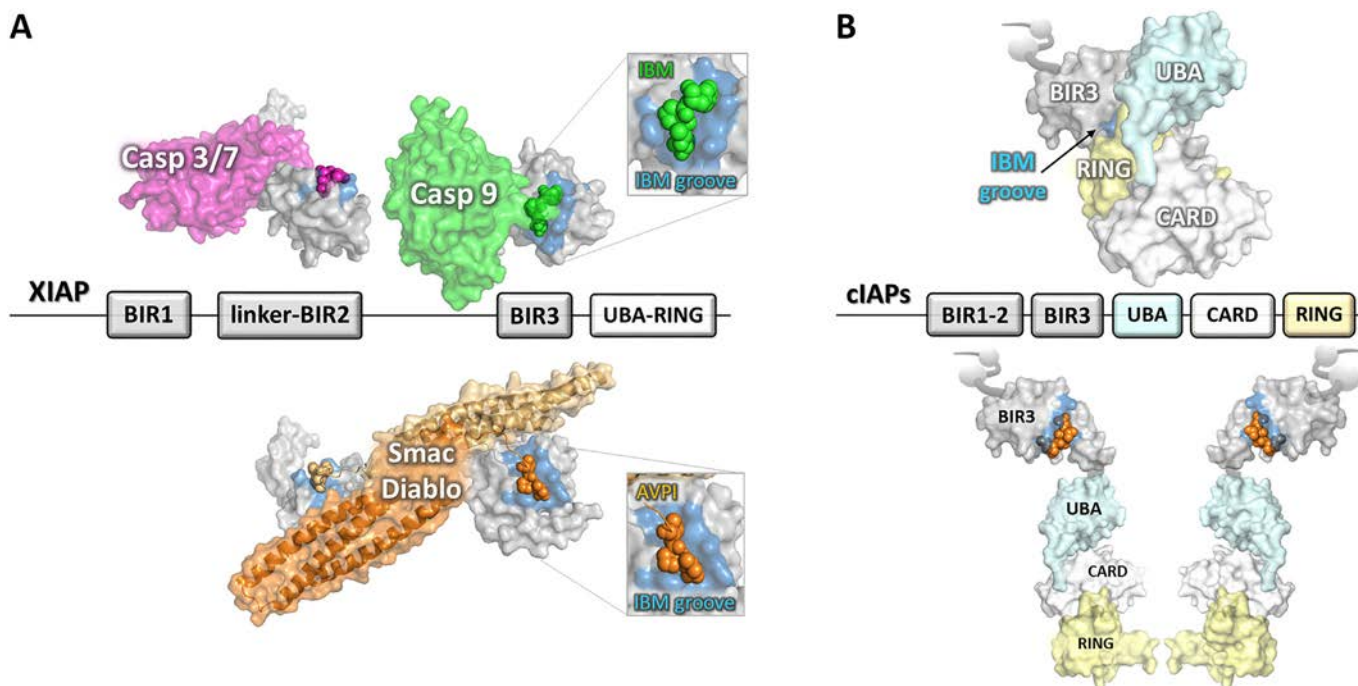
eventually causing the degradation of cIAP1 and cIAP2, while inhibiting XIAP activity.

## 2. Mimicking the IAP Natural Antagonist Smac/Diablo

Smac/Diablo is a pro-apoptotic protein that usually localizes in the mitochondria membranes, being released in response to apoptotic stimuli [39]. Once in the cytosol, Smac/Diablo undergoes maturation with the cleavage of its N-terminal 55 amino acids. The loss of this sequence allows the exposure of the conserved IBM, present also in caspases and responsible for the interaction with the IAP BIRs [40–42] (Fig. 1). Through the IBM, Smac/Diablo prevents the activity of IAPs, causing the release of caspases bound with XIAP and the degradation of some members of the IAP family [43]. It has been shown that small peptides derived from the N-terminal region of mature Smac/Diablo can mimic the activity of the whole protein and display a pro-apoptotic function [44,45]. Later works identified the minimal sequence sufficient to interact with the BIR domains and demonstrated that the first four amino acids, AVPI, are sufficient to mimic the activity of the whole protein [36,46,47]. A number of modifications have been introduced on Smac/Diablo IBM sequence to generate peptidomimetics with increased affinity for the BIR domains and improved pharmacological properties [25,30,48].

## 3. Finding the Right Combination for IAP-Targeting Compounds

IAP-directed compounds were initially imagined as broad activators of apoptosis [36]. Accordingly, they have been tested in combination with a plethora of cytotoxic compounds [38,49–51]. Nonetheless, due to the crucial role of XIAP in protecting from the tumor necrosis factor (TNF) superfamily ligands, and in particular from the cytotoxic activity of TRAIL [52–56], SMs have been tested in combination with the latter molecule. The genetic and pharmacologic inhibition of XIAP has indeed been proved to sensitize cancer cells to TRAIL in pre-clinical models [57,58]. Later works have proved that XIAP-directed compounds have a dramatic effect also on other members of the IAP family, and in particular on cIAP1 and cIAP2 [59–61]. Upon stimulation with SMs, cIAPs undergo a conformational change which allows self-ubiquitination and rapid proteasome-dependent degradation [62,63]. The depletion of cIAP1 and cIAP2 causes the stabilization of NIK which is responsible for the activation of the non-canonical NF- $\kappa$ B pathway [62,64,65]. Of note, in about 10–15% of cancer cell lines, this event is sufficient to induce cell death due to NF- $\kappa$ B-dependent secretion of TNF which kills cells in an autocrine/paracrine fashion [59]. Moreover, since cIAP1 and cIAP2 have been described in a number of receptor complexes [4,66,67], it is not difficult to imagine that their depletion results in the activation and/or inhibition of different pathways. In particular, IAPs have been shown to modulate immunity-related receptors such as TLRs and NOD receptors [8,68–70] and protect from inflammation [71,72]. Accordingly, the treatment with SMs has been shown to display an immune modulatory activity [73,74], resulting in the massive secretion of pro-inflammatory cytokines with anti-tumor activity [75,76], but also inducing systemic toxicity [77]. Therefore, SMs have recently been proposed in combination with immune checkpoint inhibitors (ICIs; [73]), which are currently attracting a huge interest in cancer treatment [78], both in preclinical experiments [73] and in clinical trials [79]. Although the treatment with SMs in monotherapy resulted at most in partial responses in multiple myeloma [74] and glioblastoma [75] mouse models, notably, the combination with ICIs produced durable effects and was even curative in treated mice. These findings support the idea that SMs, even if not effective in cancer treatment as standalones, could be employed successfully in immune-based therapies.



**Fig. 1.** Smac/Diablo and SMs target homologous IAPs involved in different cellular pathways. IAPs are composed of three BIR domains (BIR1 to BIR3), an ubiquitin-associated (UBA) domain and a C-terminal RING domain. cIAPs additionally contain a CARD domain. (A) XIAP inhibits initiator (Caspase-9, green surface) and effector caspases (Caspase-3 and -7, magenta surface), through the interaction of its type II BIRs (BIR3 and BIR2, respectively), represented with grey boxes and surfaces. In particular, type II BIRs display a conserved IBM groove (blue region in the zoomed view), which hosts the N-terminal IAP-binding motif (IBM) reported in spheres. Smac/Diablo released from mitochondria upon stress stimuli, displaces caspases by exposing the N-terminal tetrapeptide AVPI (orange spheres), the base for SM design. (B) cIAPs are present in the cytosol as inactive monomers (panel B, on top). SM treatment induces dimerization and rapid auto-ubiquitination of cIAPs, leading to their degradation.

#### 4. Cancer Cell Mechanisms of Resistance to SM Treatment

As already mentioned, only a small percentage of cancer cell lines are sensitive to SMs in monotherapy [59] due to autocrine/paracrine expression of high levels of TNF [60,61] which results in apoptotic and necroptotic death [80]. A number of works focused on the mechanisms that could predict the cellular response to this class of compounds and explored strategies able to overcome cancer cell resistance. In this regard, the combination of TNF with SMs appears the most obvious way to increase their cytotoxic activity [47], but it is effective only in a small portion of cancer cell lines [81] and it is not practicable in the clinic.

Cancer cells have generally been shown to express higher levels of IAPs compared to normal cells [82], but this up-regulation is not necessarily responsible for reduced sensitivity to SMs. Accordingly, other mechanisms have been proposed. For example, CLL cells have been shown to be resistant to SMs due to their inability to form a ripoptosome [83], while other works suggest that, in the absence of cIAP1, the levels of cIAP2 massively increase [84] and this IAP is ultimately responsible for the resistance [81]. In these settings, inhibitors of phosphoinositide 3-kinase (PI3K) have been shown to prevent cIAP2 up-regulation and could therefore represent a strategy to enhance the cytotoxic activity of SMs. Biomarkers predictive of response have been proposed, especially in head and neck squamous cell carcinoma (HNSCC) [85,86], finding that the mediators of the extrinsic apoptotic pathway are essential for the efficacy of SMs, as shown also in a previous loss-of-function screening [87]. Unfortunately, the activity of SMs appears to be completely different *in vivo* [67], and, consequently, *in vitro* data may not predict their efficacy when administered systemically. Finally, mutations present in IAP genes [86] could affect the efficacy of SM treatment.

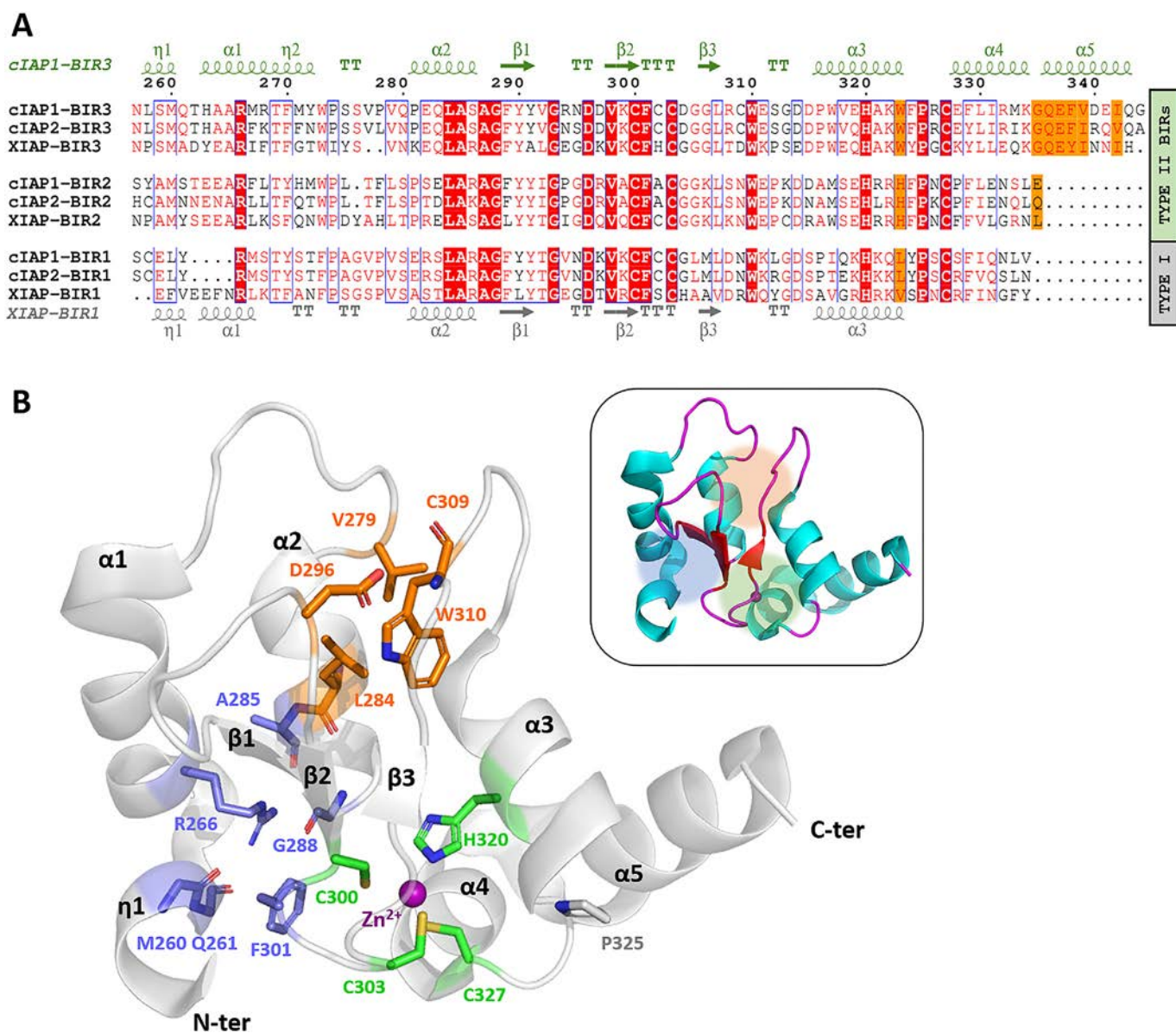
#### 5. Structure and Function of BIRs: The BIR Fold

The BIR domain is composed of 70–80 amino acids (Fig. 2, A) building a central antiparallel  $\beta$ -sheet composed of 3 strands ( $\beta$ 1–3) surrounded by 5  $\alpha$ -helices (mainly facing one side of the  $\beta$ -sheet). BIRs are Zn-finger domains similar to the C2H2-type [88]. The zinc binding section (Fig. 2, B, green residues and hotspot) is composed of a  $\beta$ -hairpin ( $\beta$ 2– $\beta$ 3) followed by an  $\alpha$ -helix ( $\alpha$ 3) forming together a left-handed bba-unit. The zinc atom is coordinated by a CCHC pattern (motif CFXC(X)<sub>16</sub>H(X)<sub>4</sub>PXC) by one histidine (in  $\alpha$ 3) and three cysteine residues (two at the end of  $\beta$ 2 and the last between  $\alpha$ 3 and  $\alpha$ 4). Beyond the presence of the Zn binding residues, the fold is stabilized by the presence of two 'hot-spots' of conserved residues (residue numbered as in BIR3, Fig. 2): 1. R266–A285–G288–F301; 2. L284–D296–W310. In the first structural padlock, the N-terminal conserved residue R266 is hydrogen bonded to main chain atoms of M260, Q261 and (conserved) A285, and it is in van der Waals interaction with conserved G288 and F301. Such network of interactions stabilizes the N-terminal end of the first 2 (or 3)  $\alpha$ -helices with the  $\beta$ 1-turn- $\beta$ 2 (Fig. 2, B, light blue residues). The second 'hot spot' (Fig. 2, B, orange residues) is mainly due to hydrophobic interaction clustering together conserved L284, W310 and quasi-conserved V/L279 closed toward the solvent side by the hydrogen bond between conserved D296 and main chain nitrogen of amino acids 309 and 310.

#### 6. Structure and Function of BIRs: The IBM Groove

Type II BIRs are characterized by the presence of a surface cleft involved in the binding of IBM tetrapeptides: the IBM groove. It has been demonstrated that tetrapeptides and SMs bind to BIR3 in the conserved IBM cleft located between the  $\beta$ 3 strand and the  $\alpha$ 3 helix of IAPs (*i.e.* cIAPs and XIAP, Fig. 3). The cleft is composed of two cavities: the





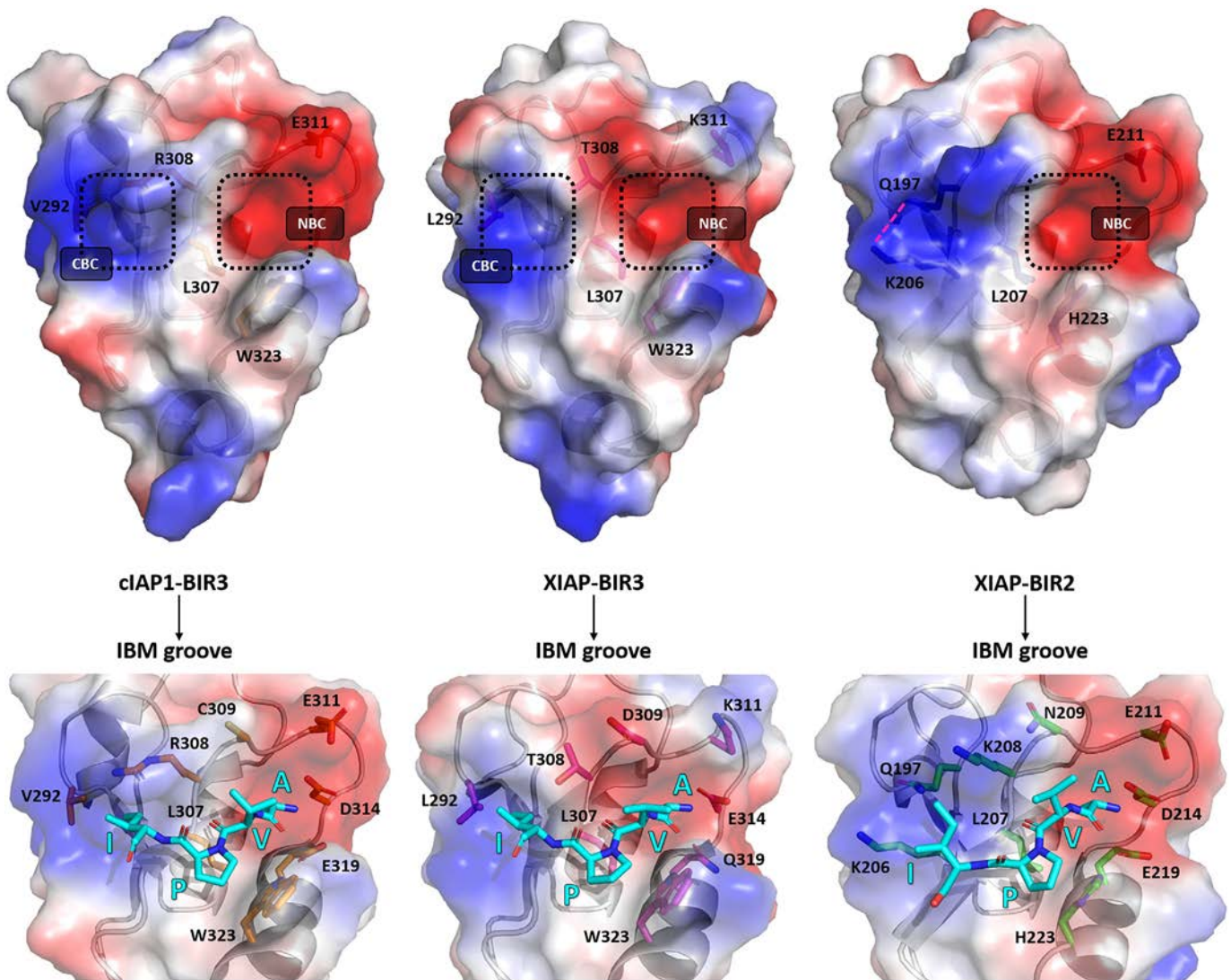
**Fig. 2.** The BIR domains: from the primary to the tertiary structure. (A) The sequence alignment of BIRs from cIAP1, cIAP2 and XIAP shows conserved motifs (highlighted in red when fully conserved, written in red when partially conserved), both among different BIRs of the same IAPs (Type I BIR1, compared to Type II BIR2 and BIR3) and among the same BIRs of different IAPs homologues (i.e. the BIR3 of cIAP1, cIAP2 and XIAP). The corresponding secondary structures are reported in green (cIAP1-BIR3 as a reference for Type II BIRs) and in grey (XIAP-BIR1 as reference for Type I BIRs). The alignment was performed with ESPRIPT (<http://espriti.ibcp.fr>) [122]. (B) Structural “hot-spots” on BIRs stabilizing the BIR fold. Beyond the Zinc binding patch (residues in green, Zinc atom in purple), the second hot spot (residues in light blue) involves the fully conserved residues (highlighted in red in the sequences alignment) in the  $\eta$ 1- $\alpha$ 1- $\alpha$ 2- $\beta$ 1- $\beta$ 2 elements. The third hot spot is stabilized by V279, L284, D296, C209 and W310 in orange sticks (Reference structure: cIAP1-BIR3, PDB id: 3MUP [123]). The secondary structure elements (cyan helices, red sheets and magenta loops) in the 3D structure are more clearly reported in the box, together with the location of the hot-spots.

N-terminal and the C-terminal binding cavities (NBC and CBC), named according to the portion of the tetrapeptide bound, and separated by a bulge (conserved L307 at the end of  $\beta$ 3; Fig. 3). As expected, NBC is negatively charged while CBC has medium-low positive charge. The NBC is paved by the conserved Trp310 and is roughly lined by residues G306, R/T308, C/D309, E/K311, D/E314, E/Q319 and W323, in cIAP1/XIAP-BIR3, respectively (Fig. 3). The CBC corresponds to the N-terminal end of  $\beta$ 1 closed by V/L292, in cIAP1/XIAP-BIR3, respectively.

The overall features of the IBM groove are conserved in cIAP- and XIAP-BIR3, but subtle aminoacid substitutions can modulate the affinity for different SMs. In particular, in cIAP1-/XIAP-BIR3 the IBM cleft is more/less wide depending on quasi-conservative amino acids substitution: V/L292 at the end of  $\beta$ 1 (CBC) and D/E314 (NBC). In addition, the negative charge on NBC is higher in cIAPs due to substitution E/Q319 and E/K311 in cIAPs/XIAP, respectively. The different features of the

IBM cavity were exploited for the selection of SMs with different affinity for cIAP- and XIAP-BIR3, as reported in Corti et al. [89] and in Ndubaku et al. [90]. Such selective molecules can be used to study different effects in apoptosis modulations in several cell lines and develop cIAPs-targeted therapies with reduced side-effects (mainly due to XIAP inhibition). Interestingly, during optimization of IAP antagonists, Vamos and colleagues designed potent compounds which resulted selective for the unique BIR domain of ML-IAP [91]. Given the peculiar role of ML-IAP in tumorigenicity [92], such compounds could be useful to characterize novel different approaches adopted by IAPs to regulate cell death.

The IBM groove in BIR2 is different from that in BIR3 mostly for a shallow CBC due to the H-bond between Q197 and K206 that closes part of this cleft (Fig. 3). In the BIR3 domain, such residues are D/K297 and G/G306 in cIAP/XIAP, respectively. The crucial role of K206 in reducing BIR2 affinity for the Smac tetrapeptide AVPI was demonstrated by



**Fig. 3.** Structural features of the IBM groove of type II BIRs hosting the tetrapeptide AVPI. Electrostatic surfaces ( $\pm 70$  keV) of cIAP1-BIR3 (PDB id: 3D9U), XIAP-BIR3 (PDB id: 1G73) and XIAP-BIR2 (PDB id: 4J46). The IBM groove runs from the negatively charged N-terminal binding cavity (NBC) to the positively charged C-terminal binding cavity (CBC), separated by the conserved Leucine 207/307 (in BIR2/BIR3, respectively). In XIAP-BIR2 the CBC is shallower than in BIR3 due to the interaction between Q197 and K206 (magenta hatches). On the bottom, a zoomed view of the IBM groove of each BIR domain, with the residues interacting with AVPI (in cyan sticks) highlighted in orange, magenta and green sticks, for cIAP1-BIR3, XIAP-BIR3 and XIAP-BIR2, respectively. Structures drawn with PyMOL (<https://pymol.org/2/>; The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

mutagenesis experiments [93]. The XIAP-BIR2 IBM groove has been shown to interact with a basic patch (R36 and R41 residues) of RIPK2 by Hrdinka and colleagues [94], thus being involved in NOD2 signaling. The role of different substituents in position 4 of AVPI tetrapeptide was extensively analyzed, in particular to enhance BIR2 vs BIR3 selectivity. As a result, bulky and flat chemical groups (like phenylhydrazine or 1-naphthyl moieties) appear to be better suited for the shallow CBC, enhancing both BIR2 affinity and selectivity [95]. In the last years, through a structure-based approach, novel XIAP-directed compounds were developed [96,97] by exploiting the increased selectivity for the BIR2 domain of XIAP compared to the BIR3 of XIAP and cIAP1. These compounds were shown to promote apoptosis without inducing cIAP1 degradation [98].

## 7. Type I BIR Domains: BIR1

Type I BIR domains do not inhibit caspases, but interact with adaptor proteins activating different survival pathways. In particular, XIAP-BIR1, in a dimeric form, recognizes TAB1 (TAK binding protein 1), a kinase activator, promoting the NF- $\kappa$ B survival pathway [99]. This likely occurs

through a structural mechanism proposed by Lu and colleagues [100], where BIR1- and RING-mediated dimerization of XIAP allows the recognition and dimerization of TAB1 and subsequent dimerization and activation of TAK1 and NF- $\kappa$ B. cIAPs are recruited to the TNF receptor signaling complex through the interaction of cIAPs-BIR1 with elongated proteins named TRAFs (TNF receptor-associated factors). This binding event is essential for cIAPs activity, which consists of the ubiquitination of substrates involved in the canonical and non-canonical NF- $\kappa$ B pathways, where cIAPs positively or negatively regulate cell survival [99].

The BIR1 domain, which displays ~45% sequence similarity with BIR3, does not display the typical IBM groove, which is characteristic of the anti-apoptotic activity of type II BIRs. In fact, the residues K102/R82 in cIAP1/XIAP, respectively, display bulkier side chains than the corresponding residues in type II BIRs (E319/Q319 in cIAP1/XIAP BIR3, E219 in cIAPs/XIAP BIR2). Furthermore, the presence of L106/V86 in cIAPs/XIAP, respectively, weaken the stacking interactions that the IBM establishes with the corresponding residues in type II BIRs (W323 in cIAPs/XIAP BIR3, H223 in cIAPs/XIAP BIR2). Therefore, according to the BIR1 crystal structures [101], K102/R82 and L106/V86 would interfere with a BIR1-caspase and a BIR1-Smac putative interaction.



The structure of cIAP2-BIR1 in complex with TRAF2 (pdb-id 3M0A; [101]) shows two different interaction patches, but mutational studies identified the BIR1 N-terminal helix-turn-helix motif as the correct interaction surface. Such motif displays a quite shallow polar surface where the most important residues (based on mutational studies) are L30, M33, E47 and R48. Notably, these residues are located on the side of BIR1 opposite to the IBM-groove homologous surface (Fig. 4, panel A, in light blue). In the same work, Zheng and colleagues show that the surface of cIAP2-BIR1 involved in the binding to TRAF2 is the same exploited by XIAP-BIR1 for the interaction with TAB1. For this reason, such conserved surface could be an interesting target for drug design purposes [102].

Both XIAP-BIR1 (PDB id: 2POI for XIAP-BIR1 alone and 2POP for XIAP-BIR1/TAB1 complex) and cIAP1-BIR1 (PDB id: 3M1D) crystal structures display a symmetrical dimeric assembly. The cIAP1-BIR1 dimer in the crystal counts only 4 stabilizing hydrogen bonds. The  $\Delta^iG$  P-value of cIAP1-BIR1 dimerization interface (0.318; <http://www.ebi.ac.uk/pdbe/pisa/>) indicates a stable interaction, but cIAPs-BIR1 were unable to dimerize even at high concentration (up to 60 mg/ml, [63]). In the crystal of XIAP BIR1, the dimer is stabilized by 20 hydrogen bonds and 18 salt bridges [100] and presents a  $\Delta^iG$  P-value of 0.905, indicating the poor hydrophobicity of the interface, often associated to crystal artifacts. Nevertheless, the XIAP-BIR1 dimer is stable also in solution and supposed to be biologically significant, since dimer-disruptive mutations resulted in a reduced ability to activate NF- $\kappa$ B [100]. The dimerization of XIAP-BIR1 was shown to be essential for XIAP-TAB1 interaction (Fig. 4, panel B), and is characterized by a strong network of electrostatic interactions. Such dimerization surface was targeted through *in silico* docking [102], identifying NF023 as an interesting moiety to be optimized for the impairment of XIAP-BIR1 dimerization and, thus, to modulate the NF- $\kappa$ B survival pathway.

## 8. Design of Compounds Specific for Different IAPs

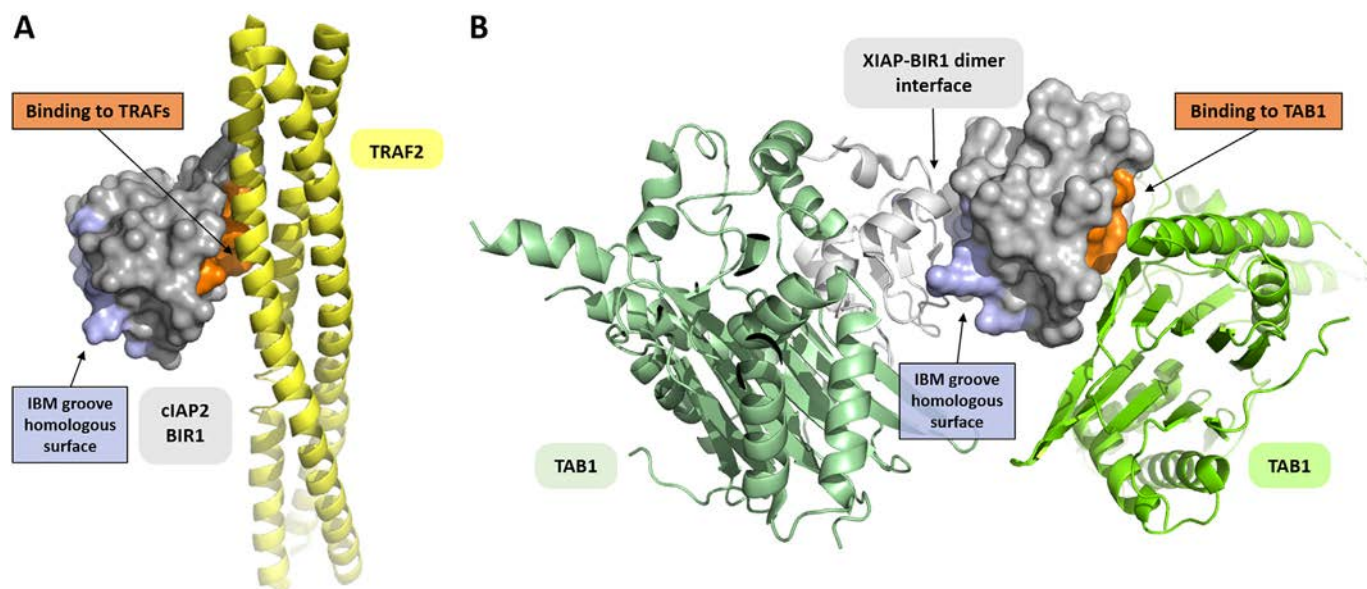
As already mentioned, IAP-directed compounds were originally designed to specifically target XIAP [28–30,103], but later works have shown that SMs cause the degradation of cIAP1/2 [60,104], and this is ultimately responsible for the killing of sensitive cancer cells, at least

*in vitro* [59]. As BIRs are extremely conserved among the different IAPs, it is challenging to synthesize compounds indeed specific, but thanks to the increased knowledge of the structural properties of BIRs and the molecular basis for the interaction between SMs and the IBM groove, novel compounds have recently been described. In particular, efforts have been made to maintain the specificity for cIAP1, while reducing the inhibition of XIAP [89]. This would maintain the capacity to kill sensitive cancer cells through the activation of the non-canonical NF- $\kappa$ B pathway, and simultaneously would reduce the overall toxicity of SM administration [77,105]. With the latter aim, Birinapant has recently been described and is currently being tested in clinical trials [79]. This compound has been shown to display increased tolerability thanks to a decreased potency against cIAP2 and XIAP [38,105]. Of note, SM treatment rapidly deplete cIAP1, that is crucial for cIAP2 degradation [62,63], and simultaneously stimulate cIAP2 expression due to the activation of NF- $\kappa$ B. In this context, cIAP2 has been proposed as a possible mechanism of resistance to SM treatment [81] and therefore it could represent a potential target in combination therapies.

Finally, to increase the efficacy of SMs, hybrid compounds have been described. Indeed, thanks to the understanding of the residues that can be modified without losing affinity for the BIRs, monomeric SMs have been linked with direct activators of caspases [106], integrin inhibitors [107], nanoparticles [108] to enhance their cytotoxic activity and bio-availability. In other approaches, the ubiquitin ligase activity of IAPs has been exploited by designing proteolysis targeting chimeras (PROTAC) [109] which, by putting in direct contact IAPs with the target protein, allow the degradation of mutant huntingtin [110], BCR-ABL [111], estrogen receptor  $\alpha$  (ER $\alpha$ ) [112] and CRABP-II [113]. These works expand the opportunities to employ IAP-directed compounds in cancer treatment.

## 9. Summary and Outlook

BIR domains are conserved among IAPs and are present in multiple copies, being classified in type I and type II. Nevertheless, BIRs evolved different strategies to promote IAP pro-survival role. Although the BIR fold is a distinctive hallmark of all IAPs and it is stabilized by the presence of conserved residues, crucial amino acidic substitutions make



**Fig. 4.** The surfaces of type I BIRs mediating pro-survival macromolecular complexes. The chain D from 3M0A (cIAP2-BIR1 in complex with TRAF2) was superimposed to chain B from 2POP (XIAP-BIR1 in complex with TAB1), and therefore the two panels (A, B) display the BIR1 molecules in the same orientation. (A) The structure of cIAP2-BIR1 (grey surface) in complex with TRAF2 (yellow cartoon) reveals that the interaction of TRAF2 with the BIR1 domain occurs through a set of aminoacids (orange area on BIR1) opposite to the surface homologous to the IBM groove (light blue area) of type II BIRs. (B) The orange patch of XIAP-BIR1 (grey surface) interacting with TAB1 (green cartoon) is the same observed for cIAP2-BIR1. The light blue area on XIAP-BIR1 is homologous to the IBM groove found on type II BIRs, but rather than binding to caspases or Smac/DIABLO, is involved in XIAP-BIR1 dimerization.

the difference both between type I and type II BIRs, and within the same BIR type. Type I BIRs are involved in protein-protein interactions, which in turn regulate the NF- $\kappa$ B pathway. A deeper investigation of these mechanisms could shed new light on the relevance of type I-mediated protein-protein interaction processes in cancer, thus providing further hints in rational drug design. Type II BIRs display a functional IBM groove for the direct binding of peptides, which provided the basis for SM design. Yet, homologous IBM grooves are not identical, and selective SMs have been developed in order to study the distinct processes and to propose tailored SM-based therapies.

Despite the great expectation around IAP-directed compounds, the clinical efficacy of SMs is still limited by the lacking of predictive markers of response. Moreover, these compounds have been employed for the treatment of different types of tumors, without individuating those that could majorly benefit from this kind of treatment [79]. The insufficient efficacy of IAP-directed therapy is likely due also to the fact that IAPs are not merely inhibitor of apoptosis as initially thought [38,114] and therefore the final outcome of SM treatment is the result of a complicated network of different effects. Notably, XIAP [115], cIAP1 [116] and cIAP2 [117] knockout mice have only mild phenotypes, and this is not compatible with their expected pivotal role in regulating apoptosis, both during development and in normal cell homeostasis, though it could also be the effect of their partially redundant activities. An attempt to clarify the relevance of cIAP1, cIAP2 and XIAP was made by employing double knockout mice. It was shown that cIAP1/cIAP2 and XIAP/cIAP1 double knockouts were embryonic lethal, but XIAP/cIAP2 double knockout mice were viable and fertile [118]. This evidence allowed to hypothesize that cIAP1 could play a pivotal role in mouse development, adult physiology and NF- $\kappa$ B pathway regulation. In contrast with these findings, a later work showed that XIAP/cIAP1 double knockout mice are viable [84], therefore clarifying that a deeper understating of the role of these IAPs in organism development and NF- $\kappa$ B regulation is still necessary. Nonetheless, the development of SMs importantly contributed to a significant advance in the field, and allowed the characterization of IAP roles and to investigate several molecular mechanisms of cell death, as apoptosis itself and necroptosis [77,119–121]. Moreover, SMs now represent a fundamental tool to investigate several signaling pathways such as MAPKs and NF- $\kappa$ B [64,67].

The expectation is that a further understanding of the IAP activity will soon provide the rationale for novel combinatorial therapies. In this sense, a promising combination is constituted by immune checkpoint inhibitors which may benefit from the transient pro-inflammatory environment triggered by SMs. Therefore, the employment of SMs in cancer treatment could be successful when combined with immune-based approaches.

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## Declarations of interest

None.

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