IAP antagonists: promising candidates for cancer therapy

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A promising strategy in cancer therapy aims to promote apoptosis in cancer cells. Targeting inhibitor of apoptosis proteins (IAPs) with small-molecule inhibitors has attracted increasing interest in triggering cancer cell death. It is considered to have great potential for cancer drug discovery because IAPs block apoptosis at the core of the apoptotic machinery and are aberrantly expressed in various tumors. This review focuses on the current development of small-molecule IAP antagonists for cancer therapy.

Apoptosis is a key regulator of physiological growth control and regulation of tissue homeostasis [1]. Apoptosis, or programmed cell death [2], represents a sequence of events that finally lead to the elimination of old, unnecessary and unhealthy cells without releasing harmful substances into the surrounding area [3].

The apoptotic machinery is a promising target for drug discovery and development because many human diseases are linked to dysregulated cell death, including apoptosis. Excessive apoptosis is characteristic of strokes and neurodegenerative disorders [3]; insufficient rates are characteristic of, for example, cancer [3]. Inhibition of apoptosis enhances the survival of cancer cells and facilitates their escape from cytotoxic therapies [4]. Major contributors in this context are the inhibitor of apoptosis proteins (IAPs), which block cell death in response to diverse stimuli through interactions with inducers and effectors of apoptosis [5]. IAPs are also potent regulators of nuclear factor κ-B (NF-κB) and tumor-necrosis factor (TNF) receptor signaling pathways. IAPs are overexpressed in many human malignancies and promote tumor maintenance [5]. The central role of IAPs in cancer renders these proteins promising targets for therapeutic intervention [5–8]. Structure and function of IAPs [6,9–18] are described in Box 1; second mitochondria-derived activator of caspases (Smac), the main endogenous regulator of IAPs [19–25], is described in Box 2. In the following paragraphs, we focus on the discovery of small-molecule IAP antagonists.

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IAP-BIR3 antagonists

Monovalent Smac mimetics

Smac interacts with IAPs via its N-terminal tetrapeptide, Ala1-Val2-Pro3-Ile4 (AVPI) [22]. Using this structural information, several groups designed small molecules mimicking AVPI to derive proteolytically stable compounds.

Oost *et al.* [26] used several peptide libraries to determine structure–activity relationships (SARs) for the binding of the individual residues of the N-terminal tetrapeptide. The pentapeptide N terminus AVPFY of the functional Smac homolog HID has a higher binding affinity for X-chromosome-linked IAP (XIAP)-baculoviral IAP repeat (BIR)3 than AVPI [11]. Thus, it was used as starting point. Four libraries were prepared in which each of the first four residues was varied while the other four were unchanged. Approximately 120 compounds were evaluated for binding to XIAP-BIR3. Modifications of the Ala1 residue indicate that the natural methyl or an ethyl group are most preferred for binding. Single methylation of the terminal NH_2 is well tolerated; dimethylation is detrimental. Exchange of Val2 mostly causes little or no loss of affinity. Replacing the five-membered ring in the Pro3 residue with four- or six-membered rings results in a five- to seven-fold loss in binding affinity and a greater loss with other residues. Introduction of a hydrophobic group to the five-membered ring in Pro3 can slightly improve the binding affinity. Among modifications to the Ile4 residue, hydrophobic entities such as phenylalanine and phenylglycine are most preferred. To reduce the peptide character of the pentapeptide lead and to improve the binding affinity, more than 150 capped

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BOX 1

IAP family members

IAP proteins regulate apoptosis by direct inhibition of caspases and modulation of, and by, the transcription factor NF-κB. IAPs, first described in baculoviruses [9], are highly conserved throughout evolution from Drosophila to vertebrates [6].

Eight mammalian IAPs [6] are known at present (Figure I): X-chromosome-linked IAP (XIAP), cellular IAP1 and IAP2 (cIAP1 and cIAP2), neuronal apoptosis inhibitory protein (NAIP), survivin, BRUCE, livin, and testis-specific IAP (Ts-IAP). IAPs differ extremely in size, ranging from 102 amino acids (survivin) to 4845 (BRUCE).

Identifying features among the structural motifs of IAPs [10] are the baculoviral IAP repeat (BIR) domains; they are 70–80-amino-acid cysteine- and histidine-rich domains that chelate zinc ions [11,12]. The number of BIR domains in a given IAP varies from one to three. They are located in the N-terminal region and mediate the interaction with caspases. Beyond caspases, BIR domains bind such diverse proteins as borealin and aurora B kinase (cell division), TAB1 (BMP signaling), AIF (reactive oxygen species production) and TRAF2 (TNF-receptor superfamily intracellular signaling) [13].

Beyond the BIR motif, a C-terminal really interesting new gene (RING) zinc finger, a caspase recruitment domain and ubiquitin-associated (UBA) domains are found in individual IAPs [10].

The best-investigated and most potent member of the IAP family is XIAP; it contains three BIR domains, a RING motif and a UBA domain [10]. XIAP directly inhibits both initiator and effector caspases via its BIR domains. The second and third BIR domains of XIAP mediate inhibition of effector caspase-3 and caspase-7 and inhibition of initiator caspase-9 through distinct mechanisms. The XIAP-BIR2 domain, as well as linker residues N-terminal to XIAP-BIR2, contribute to inhibition of effector caspases [14]. By contrast, the XIAP-BIR3 domain is required for inhibiting initiator caspases [15].

In addition to its role as a direct caspase inhibitor, XIAP can block apoptosis through the E3 ligase activity of its RING domain that mediates proteasomal degradation of proapoptotic proteins – for example, caspases [16]. Furthermore, XIAP promotes NF-κB activation by enhancing the translocation of NF-κB from the cytoplasm into the nucleus, by increasing the degradation of inhibitor κB protein, and through its association with TAK1 kinase and its cofactor TAB1 [17,18], although the latter has been shown only in overexpression studies.

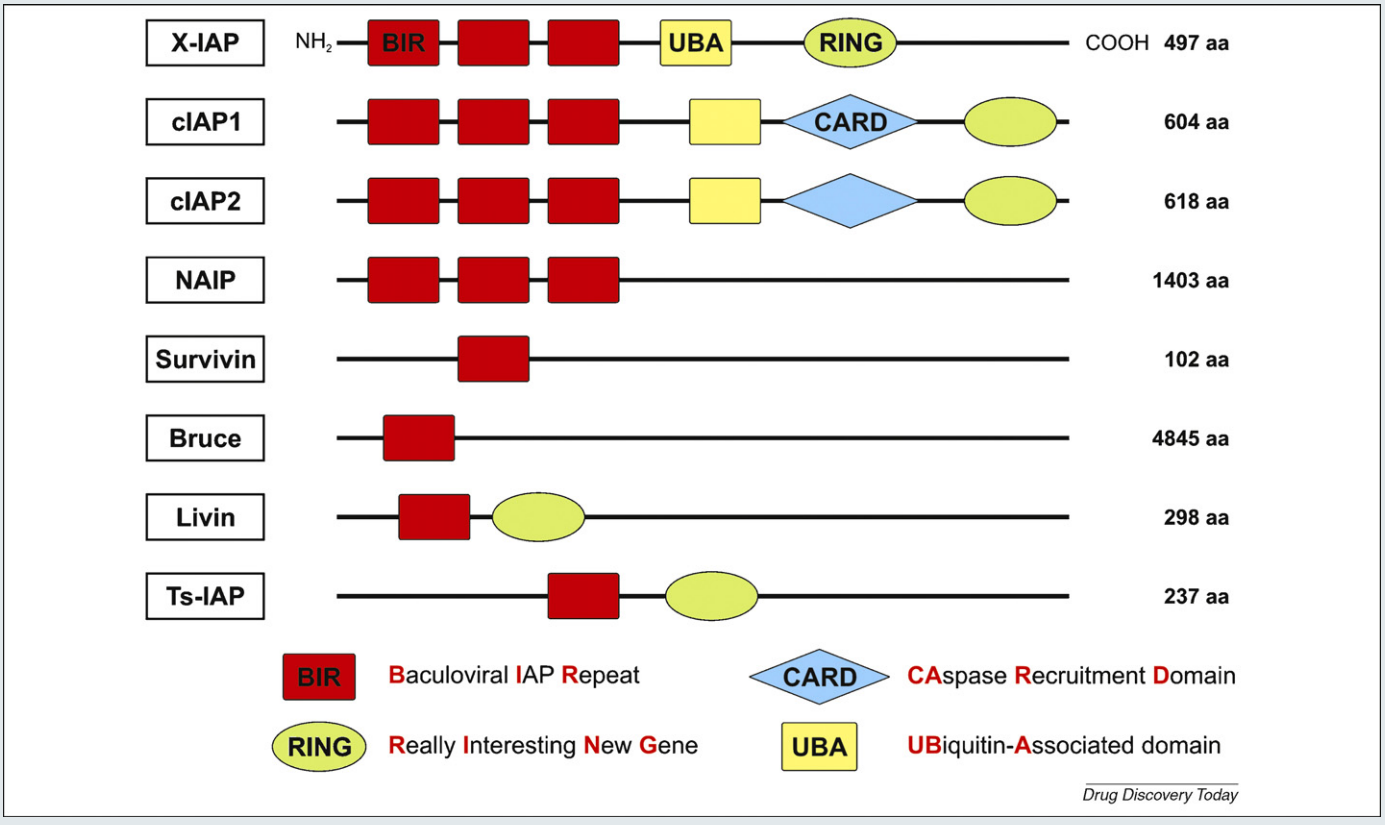

FIGURE I

The eight mammalian IAPs.

tripeptides were synthesized. Compounds 1–4 (Figure 1) exhibit low nanomolar affinities to XIAP-BIR3; 1–3 inhibit cell growth in the MDA-MB-231 cell line and effectively induce cell death [26].

Conformationally constrained, bicyclic Smac mimetics (Figure 1) were first reported by the group of Wang [27–30]. Study design was guided by modeling studies and experimental data on the AVPI/XIAP-BIR3 complex: experimental structure showed the isopropyl moiety of Val2 to be exposed to the solvent and to lack interaction with XIAP-BIR3; hydrophobic interactions with Trp323 in XIAP-BIR3 were found for the five-membered ring of Pro3. Modeling studies predicted a close similarity between the conformation of AVPI in complex with XIAP-BIR3 and the conformation of bicyclic lactams obtained by fusion of Val2 and Pro3 side chains. Such cyclization creates a new chiral center; of the two [6,5]-bicyclic stereoisomers 5 and 6, the *R*-isomer 6 represented the eutomer for binding to XIAP-BIR3 [30].

BOX 2

Smac – endogenous inhibitor of IAP

The activity of IAPs is regulated by IAP-binding proteins such as second mitochondria-derived activator of caspases (Smac) [19], also termed 'DIABLO' (direct IAP-binding protein with low p*I*) [20]. The crystal structure of Smac in a complex with XIAP-BIR3 has been determined by the group of Shi [12], whereas Fesik *et al.* established its NMR solution structure [11]. Crystal and solution structures clearly revealed that the four N-terminal residues, Ala1-Val2-Pro3-Ile4 (AVPI), in Smac recognize and bind to a surface groove on XIAP-BIR3 (Figure I).

Beyond eliminating the inhibitory effect of IAPs on caspases, Smac antagonizes the ubiquitin ligase activity of IAPs [24] and thereby stimulates their autoubiquitination and degradation [25].

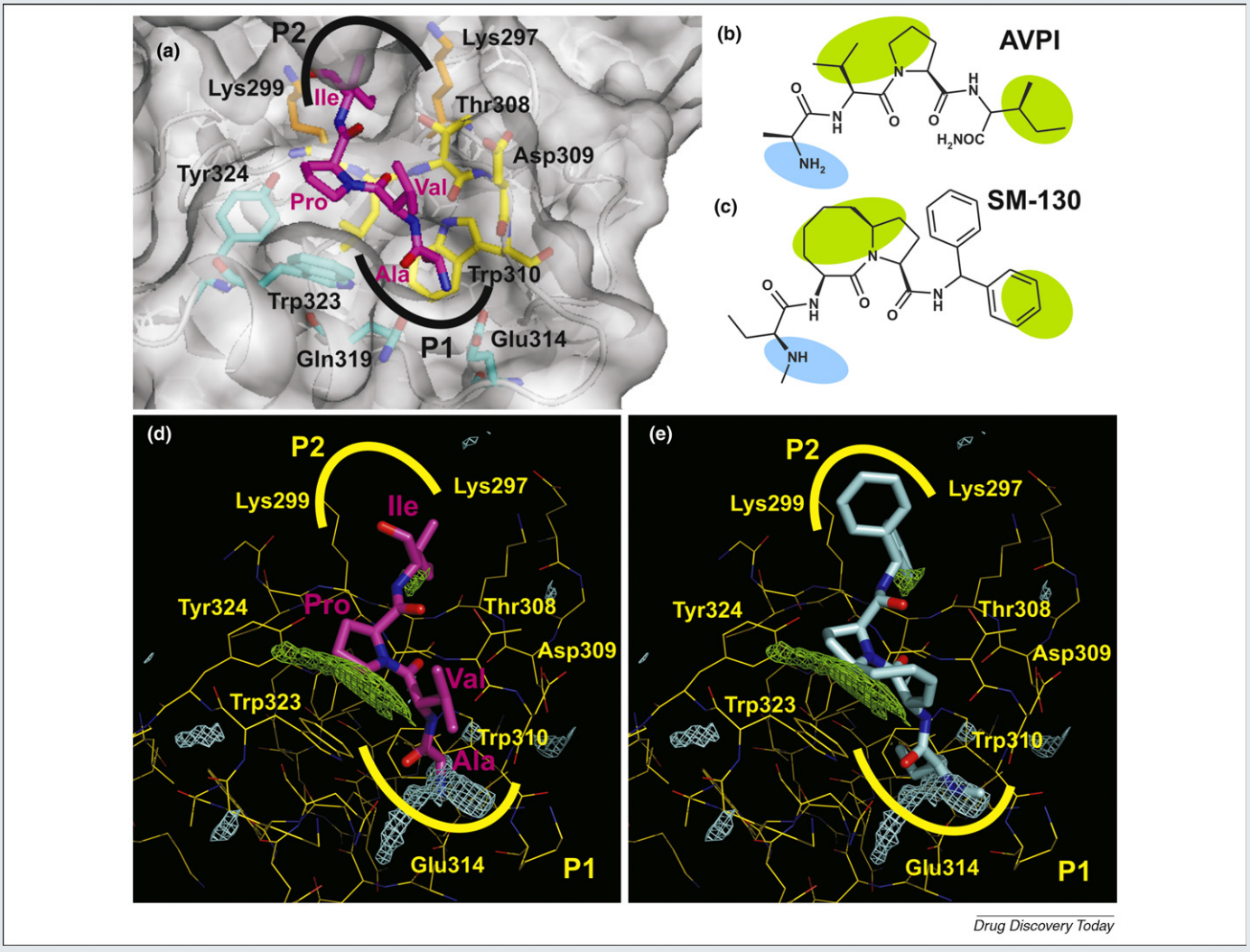

FIGURE I

Comparison of the XIAP-BIR3 binding pocket in complex with AVPI and with SM-130. (a) The binding pocket of the crystal structure (PDB code 1G73). AVPI is shown in magenta and the BIR3-pocket is shown in gray. The residues involved in the interaction with AVPI are reported in yellow (Gly306, Leu 307, Thr308, Asp309 and Trp310), orange (Lys297 and Lys299) and cyan (Glu314, Glu319, Trp323 and Tyr324). The pockets P1 and P2 are named as in Ref. [39]. Several crystal structures of XIAP-BIR3 complexes with mono- and bivalent Smac mimetics have been published recently (2JK7 [26], 2OPY, 2OPZ [59], 2VSL [50], 3CLX, 3CM2, 3CM7 [33] and 3EYL [38]). Among them, 2JK7 reports the complex with SM-130. (b) The 2D structure of AVPI. (c) The 2D structure of SM-130. This exhibits reduced flexibility versus AVPI.

The BIR3 pockets in 1G73 (a) and 2JK7 (d and e) were aligned to enable comparison of their ligand complexes. The pocket P1 and P2 is yellow colored in sections (d) and (e) with the exogenous SM-130 [(d), magenta] and endogenous AVPI [(e), cyan] ligands. This alignment allows the reader to compare the interaction modes of the two ligands with XIAP-BIR3. In addition, molecular interaction fields (MIFs) obtained with the program GRID [60] represent how the protein cavity faces the presence of either hydrophilic (using the probe OH2) or hydrophobic (using the probe DRY) counterparts. In this way, the ligands can be compared with the hydrophobic and hydrophilic fields, and a good superimposition of ligand atoms with the fields represents a favorable protein–ligand interaction. This analysis indicates two important hydrophobic (green-colored) regions and one important hydrophilic (cyan-colored) region.

The hydrophilic region is generated by the oxygens of Glu314 and Gln319, the indole NH group in Trp323, and the carbonyl and amino groups of Thr308. There, both the free NH_2 group of AVPI and the NH group of SM-130 form strong H-bonds to Glu314 and Gln319 on BIR3 (note that the nitrogen is deeply surrounded by the cyan hydrophilic MIF). Among the hydrophobic regions, the larger is due to the side chains of Trp323 and Tyr324, whereas the smaller hydrophobic pocket is formed by the side chains of Leu292 and Val298 and the hydrophobic portion of the side chains in Lys297 and Lys299. The side chains of Val2 and Pro3 of AVPI and the large bicyclic part of SM-130 lie over the larger hydrophobic region, whereas the side chain of Ile4 and one phenyl ring of SM-130 lie over the smaller region. These three regions could be considered as the pharmacophore fundamental for the interaction; a schematic view of the pharmacophore is reported in sections (b) and (c).

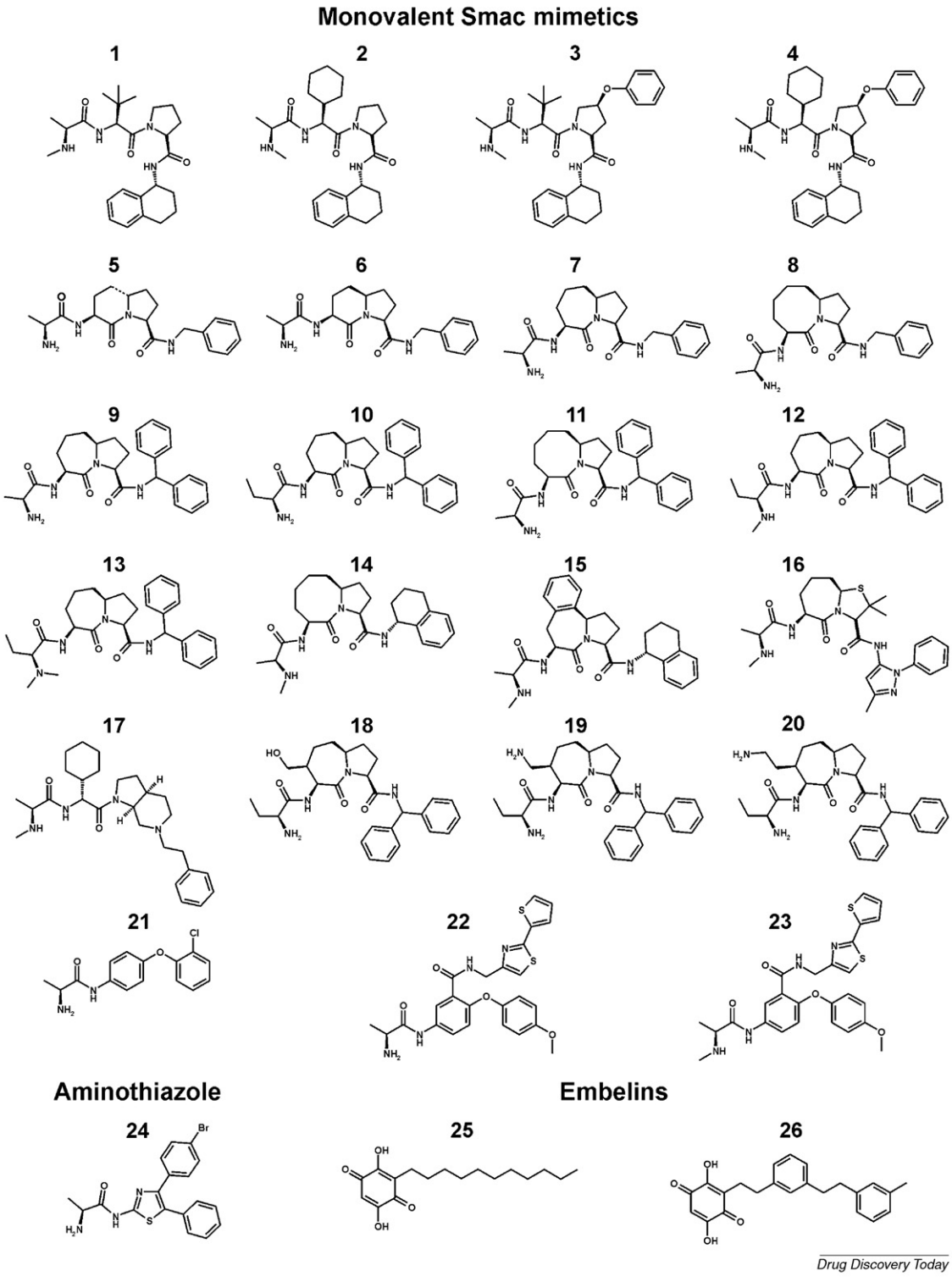

FIGURE 1

Chemical structures of main IAP-BIR3 antagonists.

Confirming modeling studies, expansion of the [6,5]-bicyclic ring to [7,5] or [8,5] rings optimizes binding affinity, as shown for *R*-configured compounds 7 and 8 [28–30]. In the light of SAR data on Smac mimetics containing a [6,5] ring, compounds 9–11 were designed, which exhibited further improved binding affinities to XIAP-BIR3. These compounds exhibited weak activities in cell-based assays using sensitive cancer cell lines, however. This indicates low cell permeability, probably owing to the primary amine group in 9–11. Replacing the primary with a secondary or tertiary amine in 12 and 13 [29,30] showed that high-affinity binding to

XIAP-BIR3 is only retained with the secondary amine. In addition, introduction of the secondary amine strongly improves cellular activities, such as inhibiting cell growth in the MDA-MB-231 human breast cancer cell line. Additional modifications yielded 14 (Figure 1), which exhibits a K_i value of 14 nM to XIAP-BIR3 and potently inhibits cell growth in several cancer cell lines [30].

Novel tricyclic Smac mimetics were used to investigate whether further conformational restriction is tolerated for IAP binding [31]. These structures have a phenyl ring fused to the seven-membered ring; the most potent compound – 15, or WS-5 (Figure 1) – binds to XIAP, cellular IAP (cIAP)1 and cIAP2 with low nanomolar K_i values. Binding is stereoselective; the tetrahydronaphthyl group of the eutomer exhibits *R*-configuration. This confirms modeling predictions that showed that only the *R*-configured structure can interact effectively with the hydrophobic pocket in XIAP-BIR3 [31].

Zobel *et al.* [32] designed conformationally constrained Smac mimetics, such as 16 (Figure 1), starting from SAR data for tetrapeptide (P1–P2–P3–P4) binding to the BIR domains of melanoma inhibitor of apoptosis (ML-IAP) and XIAP; this information was translated into peptide isosteres exhibiting rather high binding affinity for IAP-BIR domains. Compounds like 16 contain a [7,5]-bicyclic lactam that conformationally constrains the P2–P3 portion of these molecules and a five-membered heterocycle that optimally positions an aromatic ring in the P4 position. Compound 16 binds to cIAP1-BIR3 with a K_i value of 50 nM, disrupts key IAP protein–protein interactions, causes activation of effector caspases, and exhibits both single-agent cell killing and additivity with doxorubicin in cancer cell lines [32].

LBW242 (compound 17, Figure 1) induces apoptosis in multiple myeloma (MM) cells resistant to conventional and bortezomib therapies [33,34]. In human MM xenograft mouse models, 17 is well tolerated, inhibits tumor growth and prolongs survival. Combining 17 with tumor-necrosis-related apoptosis-inducing ligand or the proteasome inhibitors bortezomib and NPI-0052, as well as with the conventional anti-MM agent melphalan, induces additive or synergistic anti-MM activity, respectively [35]. In a subset of highly sensitive tumor cell lines, activity of 17 depends on TNFα signaling. Mechanistic studies indicated – although performed only in one cell line – that, in this context, XIAP is a positive modulator of TNFα induction, whereas cIAP1 negatively regulates TNFα-mediated apoptosis [36].

Starting from known conformationally constrained Smac mimetics such as 12, Mastrangelo *et al.* [37] generated 4-substituted azabicyclo[5.3.0]alkanes with enhanced affinity to XIAP-BIR domains. Crystal structure analysis of XIAP-BIR3 in complex with 18 (Smac005) and 19 (Smac010) resulted in design and synthesis of 20 (Smac037) (Figure 1). Subsequently, the crystal structure of 20 bound to XIAP-BIR3 was shown to agree nicely with the prior *in silico* model [38]. In addition, 18–20 were docked to the cIAP1-BIR3 domain. Binding modes to XIAP-BIR3 and cIAP1-BIR3 were found to be very similar [38]. Considering recent reports that Smac mimetics can kill cancer cells by binding to cIAP1 and cIAP2 followed by TNF-receptor-mediated apoptosis, compounds 18–20 were tested in the MDA-MB231 cell line for inducing cIAP degradation; all three compounds were effective in these tests [38].

Huang *et al.* [39] combined *in silico* docking, fragment-based drug design and nuclear magnetic resonance (NMR) spectroscopy for the derivation of potent Smac mimetics with cellular activity. They first designed a library of L-alanine derivatives by coupling it with 578 primary and 815 secondary amines. After docking, 15 compounds were synthesized and tested by NMR for BIR3 binding; compound 21 (Figure 1) was identified as a weak binder. Molecular docking studies support that several binding characteristics of 21 overlap those observed with the Smac peptide, in particular the interactions provided by the first three amino acids in AVPI. The X-ray structure of the AVPI/BIR3 complex shows that the Ala and Val residues occupy a pocket, P1, on the surface of the domain, and the side chain of the Ile residue occupies another pocket, P2 (Box 2). Thus, modifications of 21 at position 2 of the 4-phenoxy-benzene scaffold might enable the selection of an additional scaffold mimicking the interactions of the Ile residue in AVPI with the P2 pocket. Accordingly, some 900 derivatives of 21 were designed. Compounds were rank ordered by docking, and top-scoring compounds, such as 22 (Figure 1), were synthesized and tested by NMR. From this second iteration, compound 23 (Figure 1) exhibited the highest binding affinity for BIR3. As shown by modeling, 23 docks on the BIR3 surface by occupying each of the two subpockets occupied by AVPI [40]. Additional interactions between the domain and the bridging proline residue in AVPI are mimicked by the peripheral phenyl ring in 23. This compound markedly induced apoptosis in breast cancer cells [40].

Aminothiazole Smac mimetics

As described above, Oost *et al.* [26] reported on peptide-based XIAP antagonists such as 1–4. With a view toward therapeutic applicability, the authors intended to reduce the peptidic character of this initial series by improving proteolytic stability and cell permeability. SAR findings from the initial study guided the study design [26]. Because the alanine residue within AVPI was found to be essential for XIAP-BIR3 binding, it was retained in the novel series. Another important element was the peptidic bond of the valine residue, forming H-bonds to both the backbone amide and the carbonyl of XIAP-Thr308. Thus, a library was synthesized containing compounds with an alanine linked to moieties capable of forming H-bonds with XIAP-BIR3 [41]. Substituted five-membered heterocycles such as imidazoles and thiazoles were identified that favorably replace peptide fragments of the lead AVPI. The most potent compound was 24 (Figure 1), with a K_i of 0.74 μM for inhibiting the XIAP–caspase-9 interaction [41].

Embelins

Chemistry of embelins (compounds 25–26, Figure 1) differs strongly from the Smac mimetics described above. The group of Wang [42] used the docking software DOCK for structure-based virtual screening of an in-house natural product database and discovered 25 (embelin). The high-resolution structure of XIAP-BIR3 complexed with Smac served to define the binding site for database searching. The sum of electrostatic and van der Waals interactions, as calculated with DOCK, was used for scoring. The 1000 top-ranked hit candidates were rescored using the consensus scoring program X-score. The top 200 compounds after re-ranking were considered as potential XIAP inhibitors, 36 of which were tested for XIAP-BIR3 binding. Out of five actives, compound 25 exhibited highest affinity (IC_{50} value of 4.1 ± 1.1 μM) [42]. NMR analysis confirmed that 25 interacts with several crucial residues in

XIAP-BIR3 with which Smac and caspsase-9 also bind. The compound inhibits cell growth, induces apoptosis, and activates caspase-9 in prostate cancer cells with high levels of XIAP but has a minimal effect on normal prostate epithelial and fibroblast cells. The *n*-undecyl alkyl chain is crucial for XIAP binding; truncation to an ethyl group abolishes affinity. Modeling suggested that the long alkyl chain resides in the position-4 hydrophobic site of AVPI. Optimizing the bulk of the side chain via a 3-methyl phenethyl group gave the most active compound, 26 (K_i = 180 nM), among embelins [43].

IAP-BIR2 antagonists

Arylsulfonamides

XIAP-inhibiting arylsulfonamides [44] were identified in a library of approximately 160 000 compounds using a high-throughput enzymatic derepression assay in which recombinant XIAP was combined with active caspase-3 to inhibit caspase-mediated cleavage of the fluorogenic substrate Ac-DEVD-afc. The hits from the primary screen exhibited poor aqueous solubility, limiting their usefulness in cell models; hence, a small focused library based on the original hits was synthesized to search for XIAP inhibitors with improved physicochemical properties. This secondary screen yielded compounds TWX006 and the more soluble TWX024 (27 and 28 in Figure 2).

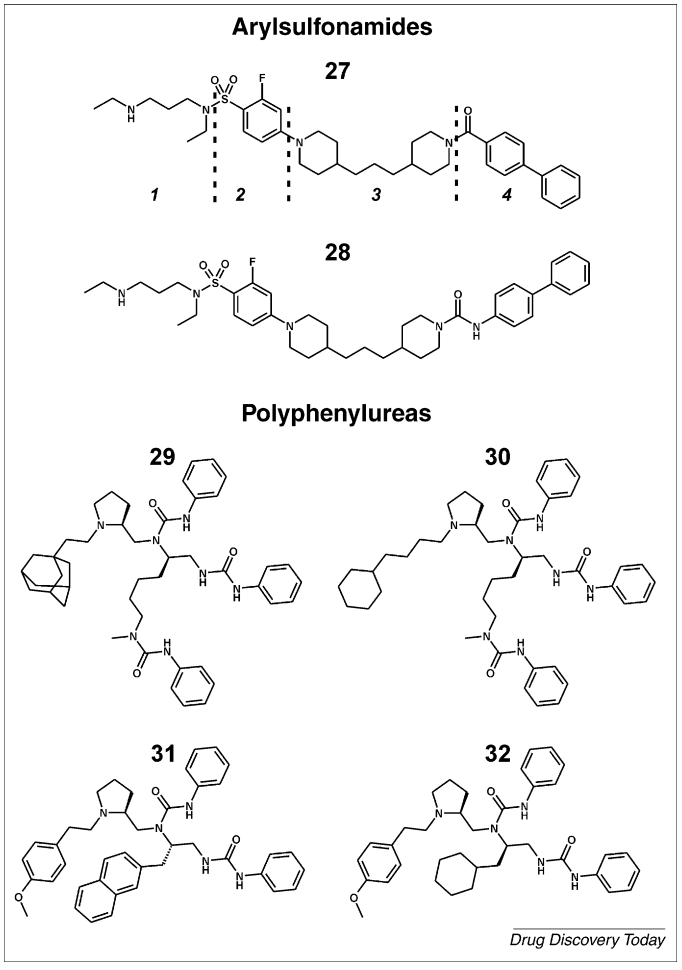

FIGURE 2

Chemical structures of main IAP-BIR2 antagonists.

The TWX molecular scaffold exhibits four fragments (Figure 2), each of which can be substituted by a series of diverse building blocks. Following SARs were derived for inhibitor activity: flexible acyclic diamines were superior to cyclic diamines when substituted in fragment 1. Ethyl substituents on either nitrogen led to increased activity relative to methyl, indicating that hydrophobicity is crucial for activity. Aryl sulfonyl groups were superior to carbonyl groups when substituted in the second fragment, suggesting that proper positioning of the H-bond donor oxygen is crucial for binding. The second and third fragments can be *para* or *meta* linked; *ortho* linkage decreases activity dramatically. An *ortho*-fluorine on the aromatic ring of fragment 2 increases activity. Concerning fragment 3, compounds with more rigid and/or shorter moieties than present in 27 and 28 exhibit considerably lower activity. The scaffold can tolerate a wide variety of large, hydrophobic groups in the fourth fragment. Smaller, more polar groups result in lower activities, indicating that fragment 4 occupies a large hydrophobic pocket. Truncated versions in which N- or C-terminal fragments were deleted resulted in a loss of activity. Molecular modeling studies predicted that 27 occupies the linker region between BIR1 and BIR2 of XIAP [44].

Polyphenylureas

Schimmer *et al.* [45] screened eleven mixture-based combinatorial chemical libraries, comprising approximately one million compounds, for putative XIAP inhibitors. One of these libraries contained ~90 000 polyphenylureas. Each mixture was screened for its effects on caspase-3 activity in the presence and absence of XIAP, scoring as a 'hit' those compound mixtures that increased caspase-3 activity by a factor of two or more when applied to XIAP/caspase-3 assays without affecting caspase-3 alone. The positive mixtures were deconvoluted by standard methods, yielding 36 individual compounds, which were screened in the same derepression assay. Eight compounds increased caspase-3 activity in XIAP-inhibited reactions by a factor of two or more [45]; four actives (29–32) gives Figure 2.

To determine the specificity of 29–32, their effects on inhibition of caspase-3, caspase-7, and caspase-9 by XIAP were compared. All compounds restored the activity of caspase-3 and caspase-7 toward normal, but none of them reversed XIAP-mediated suppression of caspase-9, indicating XIAP-BIR2 as their target site [45]. Furthermore, these compounds were preferentially toxic to primary malignant cells over normal cells and were also active in xenograft models, in which they delayed the growth of tumors of prostate, breast and colon carcinoma cells without obvious untoward toxicity to the host animal [45,46].

IAP-BIR3–BIR2 antagonists

Bivalent Smac mimetics

In bivalent Smac mimetics, the monovalent counterparts are connected via appropriate linkers. The design of bivalent Smac mimetics was expected to yield compounds with enhanced affinity to IAPs because of the following evidence: (i) natural Smac forms dimers, (ii) natural Smac binds to full-length XIAP much stronger than its N-terminal tetrapeptide AVPI, (iii) the inhibition of caspase-3 and caspase-9 activity is counteracted by much lower doses of natural Smac than AVPI, and (iv) AVPI binds to both BIR2 and BIR3 regions. Thus, compounds exhibiting two AVPI-like binding

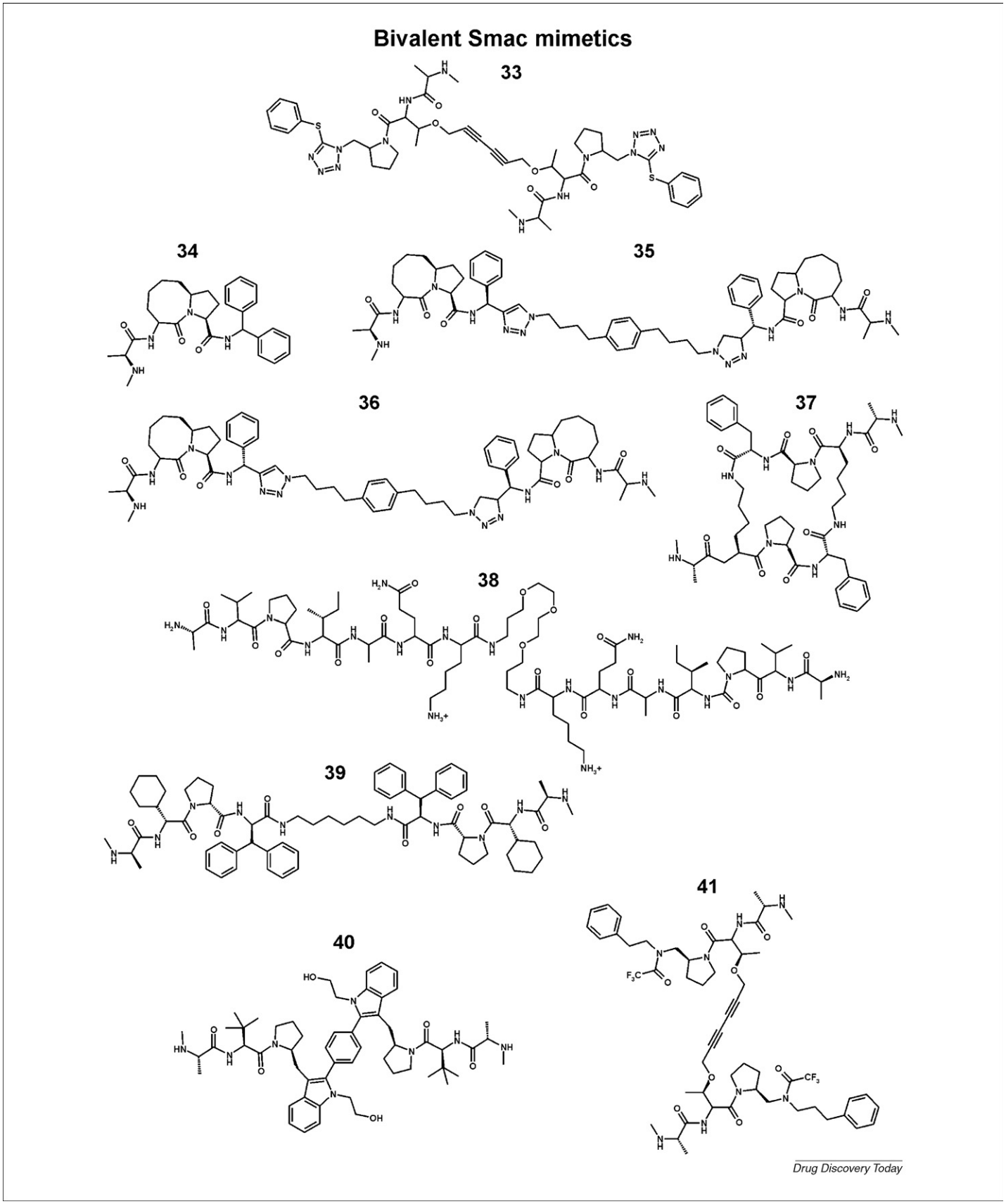

FIGURE 3

Chemical structure of main IAP-BIR3/BIR2 antagonists – bivalent Smac mimetics.

motifs might achieve optimized binding affinities to full-length IAPs.

Li *et al.* [47] described the first bivalent Smac mimetic (compound 33, Figure 3). Expectedly, 33 and its monovalent counterpart bind to XIAP-BIR3 with comparable affinities. By contrast, the affinity to full-length XIAP and the potency to counteract caspase-3 inhibition by XIAP are much stronger for the bivalent mimetic [43]. K_i value for binding of 33 to full-length XIAP is <0.7 nM [48].

The Wang group [49] designed bivalent Smac mimetics starting from conformationally constrained monovalent compounds such as 34 (SM-122) (Figure 3). First, 34 was shown to bind to BIR2 and BIR3. Then, appropriate linkers were designed via modeling studies. The pro-*R* phenyl of the benzhydryl moiety was shown to fit into a hydrophobic groove of BIR2 and BIR3, whereas the pro-*S* phenyl does not contribute to binding. Thus, this moiety is well suited for linking two molecules of 34. Modeling further indicated profound flexibility of the region between XIAP-BIR2 and XIAP-BIR3, favoring the possibility to insert a short linker. These findings led to the design of 35 (SM-164), in which the pro-*S* phenyl of the monovalent 34 was replaced by the bioisosteric [1–3]triazole. To check a putative impact of the stereochemistry around the two chiral centers vicinal to the triazole moieties, compound 36 (SM-206) was synthesized [49]. In comparison to AVPI, compounds 34 and 35 were tested for binding to full-length XIAP. Compound 35 was 270 times more potent than 34 and more than 7000 times more potent than AVPI. Compound 35 exhibits stereoselective binding; the affinity of its stereoisomer 36 is diminished 50-fold. The bivalent mimetic is also superior to the monovalent in cell-free functional assays [49].

Nikolovska-Coleska *et al.* [50] described the first cyclic bivalent Smac mimetic, 37 (Figure 3). Binding affinities of 37 to XIAP-BIR2 (K_i = 4.4 μM) and XIAP-BIR3 (K_i = 4 nM) domains differ considerably. Compound 37 binds to XIAP-BIR2-BIR3 protein with a biphasic dose–response curve, exhibiting IC_{50} values of 0.5 nM for the first site and 406 nM for the second site. Gel filtration experiments with wild-type and mutated XIAPs show that 37 interacts with two molecules of XIAP-BIR3, forming a 1:2 stoichiometric complex. By contrast, 37 concurrently interacts with both BIR2 and BIR3 in the presence of XIAP-BIR2–BIR3 and does not induce dimerization. These data confirm the results of previous studies [51,52]. Another group [53], however, showed that some chemically different bivalent Smac mimetics interact only with XIAP-BIR3 and not with XIAP-BIR2. Thus, different bivalent ligands might interact differently with XIAP-BIR2–BIR3. In cell-free functional assays, compound 37 is a potent antagonist of both XIAP-BIR3 and XIAP-BIR2–BIR3. Whereas it is six times more potent than its monovalent counterpart in antagonizing XIAP-BIR3, this factor is more than 200 for antagonizing XIAP-BIR2–BIR3. Thus, a bivalent Smac mimetic, which concurrently binds to both BIR domains and removes the inhibition of caspase-3 and caspase-7 by XIAP, antagonizes XIAP-BIR2–BIR3 much more efficiently than the monovalent counterpart [50].

Gao *et al.* [51] reported on dAVPI (compound 38 in Figure 3), a bivalent Smac mimetic connecting two AVPI-like monomers via a 14-atom alkoxy chain. Their study reveals in detail how dimeric Smac antagonizes caspase-3 inhibition by XIAP. Smac first interacts with XIAP-BIR3 via one N-terminal AVPI. Beyond neutralizing caspase-9 inhibition by XIAP, this interaction also primes and facilitates the second AVPI motif of dimeric Smac to interact with XIAP-BIR2; this follow-up Smac–BIR2 interaction alone effectively attenuates the ability of the adjacent linker region of XIAP to inhibit caspase-3. Thus, Smac interacts with the two BIR domains of XIAP in a sequential and dynamic manner, and such interaction cooperatively disrupts inhibition of caspase-3 by a different region of XIAP, the linker region between BIR1 and BIR2 [51].

Studies with the bivalent compounds 39–41 contributed to the understanding of how cIAP1 and cIAP2 might be involved in apoptotic events induced in tumor cells by IAP antagonists. Varfolomeev *et al.* [52] designed BV6 (compound 39 in Figure 3), monomers of which are connected by a heptyl chain. Binding of 39 to the BIR domains of IAPs leads to rapid ubiquitination and proteasomal degradation of cIAPs [52]. These authors discovered that cIAP proteins are important regulators of NF-kB signaling and ubiquitin ligases for the crucial kinase in the noncanonical nuclear factor kappa-light-chain-enhance of activated B cells pathway (NF-kB), NIK, and finally they showed that IAP antagonist-induced cell death is dependent on TNF signaling [52].

A short and rigid phenylene linker serves to connect the monomeric parts of 40 (Figure 3) [54]. The results of this study further illuminate how cIAP1 might functionally contribute toward tumor cell survival. The authors show that a main target of IAP antagonists is cIAP1 and that removal of cIAP1 through genetic deletion or application of IAP antagonists both activates NF-kB signaling that induces TNFα production and kills sensitive tumor cells through enhanced TNF-R1 death-receptor signaling and caspase-8 activation [55]. Amplification and enhanced levels of cIAP1 have been observed in several cancer types [55], and recent work has demonstrated that together with other oncogenic events, amplification of the cIAP1 gene locus is required to both initiate and enhance liver cancer growth in a mouse model [55]. Therefore, tumors might develop that rely on cIAP1 to inhibit TNFα apoptosis signaling. By targeting cIAP1 with IAP-antagonist drugs, physiological TNF-receptor apoptotic signaling might be greatly augmented, resulting in enhanced tumor cell death.

In AEG40730 (41 in Figure 3) [56], the monomers are connected via the same diyne linker already used for the first bivalent Smac mimetic 33 [47]. Compound 41 binds to the BIR3 domain with nanomolar affinity, causes profound apoptotic sensitization to death ligands and induces caspase-8-dependent apoptosis in a subset of cancer cell lines through activation of a TNF-α autocrine loop. Constitutive ubiquitination of receptor-interacting protein 1 (RIP1) is shown to represent a common feature of cancer cells, and the authors demonstrate further that cIAP1 and cIAP2 function as E3 ubiquitin ligases that induce RIP1 ubiquitination in cancer cells. Reducing cellular levels of cIAP1 and cIAP2 using compound 41 or siRNA results in a strong reduction in RIP1 ubiquitination and causes RIP1 to switch from functioning as a prosurvival scaffold molecule to a proapoptotic adaptor protein [56].

Concluding remarks

Therapeutic targeting of IAPs – for example, using small-molecule inhibitors or antisense oligonucleotides – presents a promising approach for cancer-cell-selective induction of apoptosis. In the past decade, a broad spectrum of IAP antagonists has been developed: BIR3 region antagonists, including monovalent Smac mimetics, aminothiazoles and embelins; BIR2 region antagonists

comprising polyphenylureas and arylsulfonamides; and BIR2–BIR3-region-inhibiting bivalent Smac mimetics.

Smac mimetics represent by far the largest fraction. Monovalent Smac mimetics were designed to mimic the Smac AVPI-binding motif and so target the XIAP-BIR3 domain. They exhibit high affinities, not only to XIAP-BIR3 but also to cIAP1, cIAP2 and ML-IAP proteins. Bivalent Smac mimetics, containing two AVPI-binding motifs, bind to XIAP-BIR2–BIR3 with an extremely high affinity, exceeding that of Smac protein. Bivalent Smac mimetics achieve such high affinities by concurrently targeting both the BIR2 and BIR3 domains in XIAP and can trigger autoubiquitination of RING-domain-containing IAPs, especially cIAP1 and cIAP2. Preclinical profiling studies have shown that Smac mimetics effectively sensitize cancer cells to other therapeutic agents, but they are also capable as single agents of inducing apoptosis in some but not all human cancer cell lines. To date, two Smac mimetics have reached phase I clinical development [5], and approximately ten are in an advanced preclinical development stage and are expected to enter human clinical testing soon [5].

For the future clinical development of IAP-targeting cancer therapeutics, an important avenue of research will be to explore whether pan-IAP or more selective IAP inhibitors are better suited for cancer-cell-selective induction of cell death. Few recent studies show that most efficient apoptosis induction in cancer cells is achieved by Smac mimetics that target both the cIAPs and XIAP [57,58]; however, the question has not yet been answered whether this increased potency of pan-IAP inhibitors against cancer cells is associated with enhanced toxicity on non-malignant cells and normal tissues.

We are confident that in the near future we will witness clinical justification of IAP antagonism as a therapeutic approach that will benefit cancer patients considerably.

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