CHAPTER 13

The Discovery and Development of Smac Mimetics—Small-Molecule Antagonists of the Inhibitor of Apoptosis Proteins

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Contents

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1. INTRODUCTION

Apoptosis, or programmed cell death, is a critical cellular function for maintenance of normal tissue development and homeostasis as well as for the routine surveillance of cellular dysfunction by the immune system. Apoptosis can be activated by a number of internal and external signals including irradiation, chemotherapy, oxidative stress, and death receptor ligand binding. The key mediators of apoptosis are the caspases, and not surprisingly, the proteolytic activation of caspases as well as the maintenance of activated caspase function is a highly regulated process; the former being partly mediated by the apoptosome, while the latter is controlled by the inhibitor of apoptosis proteins or IAPs [1]. Although originally discovered in baculovirus-infected insect cells, several mammalian IAPs have now been identified. These include the X-linked IAP (XIAP), the cellular IAPs (cIAP-1 and -2), melanoma-linked IAP (ML-IAP), and neuronal IAP (NIAP), among others.

Apoptosis is initiated by either an intracellular (intrinsic) or extracellular (extrinsic) signaling event. Following an apoptotic stimulus, the intrinsic pathway is propagated through cytochrome *c* release from the mitochondria which, together with Apaf-1 and dATP, forms the apoptosome complex (Figure 1). The apoptosome is responsible for converting procaspase-9 to its active form *via* autocatalytic processing. Once activated, caspase-9 serves to cleave procaspase-3 to the effector caspase-3, which then goes on to perform the heavy lifting of apoptosis. The discovery that XIAP could bind to and inhibit activated caspases and that XIAP was present at elevated levels in many cancers led to the hypothesis that tumor resistance to chemotherapy may be the direct result of caspase inhibition by XIAP [3].

A major advance in this field was the discovery of the mitochondrial protein Smac/DIABLO, which is released from the mitochondria upon apoptosis-inducing signals and was shown to neutralize the effects of XIAP on both caspase-9 and caspase-3 inhibition [4]. Thus, the paradigm of apoptosis regulation through caspase inhibition by XIAP—to block apoptosis—and caspase derepression by Smac/DIABLO—to restore apoptosis—was first demonstrated.

2. ORGANIZATION OF INHIBITOR OF APOPTOSIS PROTEINS

IAPs are multidomain and multifunctional proteins (Figure 2) [5]. ML-IAP comprises a single N-terminal baculovirus IAP repeat (BIR) domain and a C-terminal RING domain. XIAP comprises an N-terminal BIR domain (BIR1), two additional BIR domains (BIR2 and BIR3), a ubiquitin (Ub)-associated domain (UBA), and a C-terminal RING domain, which

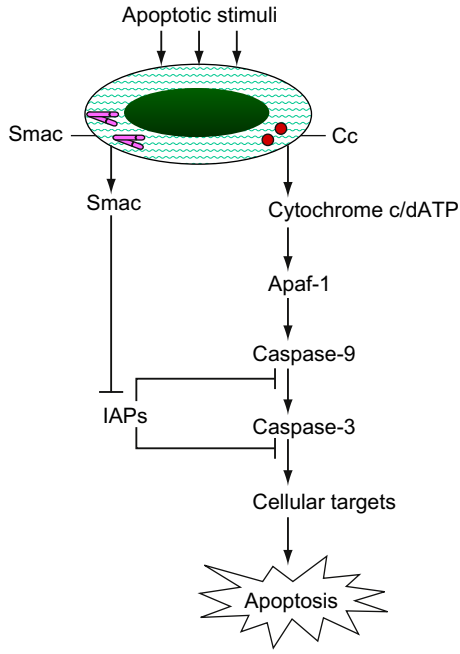


Figure 1 The intrinsic apoptotic cascade (adapted from Ref. [2] with permission from publisher).

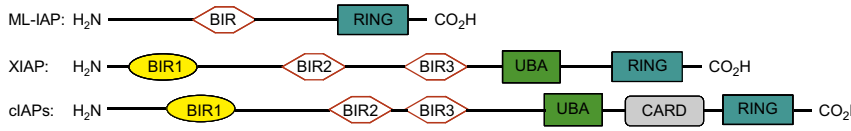


Figure 2 Domain architecture of ML-IAP, XIAP, and the cIAPs.

functions as a ubiquitin E3 ligase [6]. Caspase-9 inhibition is mediated through the BIR3 domain, while caspase-3 binds to and is inhibited by the linker region proximal to the BIR2 domain. The BIR1 domain accommodates interactions with various adaptor proteins [7,8]. The UBA domain of IAPs is essential for mediating binding to Ub-conjugated proteins and thus linking these binding interactions to downstream biochemical processes [9].

The cIAPs have a similar domain structure but include a caspase-recruitment domain (CARD). Tremendous progress has been made in understanding the mechanistic contribution of each of these domains as well as how they work cooperatively to mediate IAP functions [10]. Several regions of specific IAPs have been crystallized and the structures reported [11].

3. SMAC, XIAP, AND CASPASE-9: STRUCTURE AND MECHANISM

The mature Smac monomer exists as a three-helix bundle, and two monomer units are packed into a homodimeric tertiary structure (Figure 3) [2]. Disruption of the dimer interface through discrete mutation results in a weakened ability of Smac to promote the enzymatic activity of caspase-3. Wild-type Smac interacts independently with the isolated BIR2 and BIR3 domains of XIAP but not with the BIR1 domain despite strong sequence homology. Importantly, deletion of the four N-terminal residues from Smac, Ala-Val-Pro-Ile (AVPI), results in the loss of its ability to promote caspase-3 activity. Conversely, short N-terminal peptides derived from the Smac sequence retain this function. When co-crystallized with XIAP BIR3, the four N-terminal residues of Smac pack into a surface groove of the BIR3 domain *via* a combination of hydrogen bonding and van der Waals interactions [12].

The four N-terminal residues of caspase-9, Ala-Thr-Pro-Phe (ATPF), occupy the same surface groove on XIAP BIR3 as the AVPI sequence of Smac. This caspase-9/BIR3 interaction precludes entry of substrate peptides into the active site of caspase-9. Structurally, therefore, the XIAP/caspase-9/Smac paradigm can be summarized as: (1) activated caspase-9 is inhibited through binding of its N-terminus (ATPF) to XIAP BIR3; (2) Smac competes with caspase-9 for XIAP BIR3 binding thereby releasing active caspase-9 and reconstituting the apoptotic program. The structural details of caspase-3 derepression by Smac are considered to be the result of either homodimeric Smac binding cooperatively to the BIR2 and BIR3 domains of XIAP or *via* steric inhibition of the caspase-3/XIAP interaction by the BIR3-anchored Smac protein. Thus, the protein–ligand interactions defined by the co-crystal structure of Smac/XIAP BIR3 would serve as the starting point for the development of small-molecule antagonists of the Smac/XIAP interaction termed IAP antagonists or Smac mimetics [13].

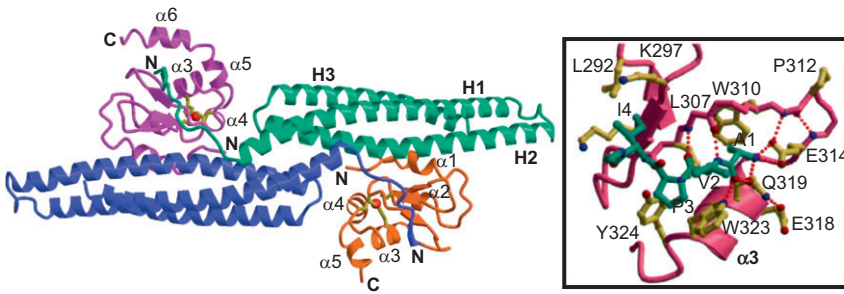
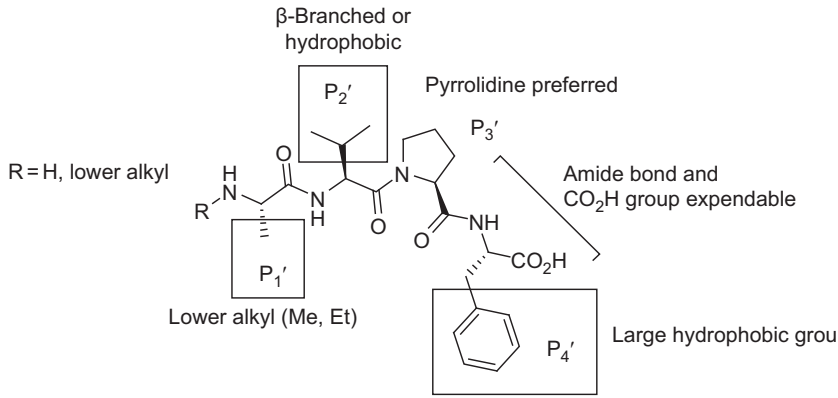


Figure 3 The structures of the Smac homodimer and AVPI bound to the XIAP BIR3 domain (adapted from Ref. [12] with permission from publisher).

4. STRUCTURE–ACTIVITY RELATIONSHIPS OF SMAC MIMETICS

In general, the N-terminal residue (P_1') is Ala or N(Me)Ala [14]. A basic residue is required to maintain a salt bridge interaction with an acidic residue within the IAP binding pocket. Although both of these residues afford analogs with good binding to BIR3 domains, only N(Me)Ala substitution allows for robust cellular responses. NMR-derived and co-crystal structures of Smac mimetics bound to XIAP BIR3 indicate that the side chain of the P_2' residue is projected away from the protein; therefore, many side chains are tolerated at this position. Large hydrophobic groups like *tert*-leucine or cyclohexylglycine afford analogs with excellent binding, as do other β-branched amino acids. The P_3' residue is typically proline or substituted pyrrolidine, which allows for an important van der Waals interaction between the ligand and Trp323 of the BIR3 binding site. Specific substitutions on the pyrrolidine ring are tolerated, and many constrained compounds that link either the P_2' and P_3' groups or the P_3' and P_4' groups have been prepared. A large, hydrophobic substituent is preferred at the P_4' position. The P_3'–P_4' amide bond and C-terminal acid or amide moieties are dispensable for biological activity.

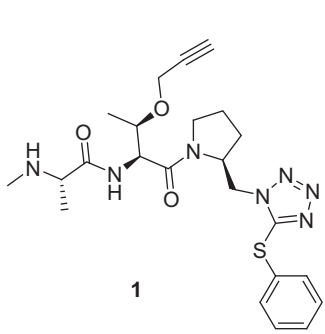


5. BIVALENCY AND SMAC MIMETIC FUNCTION

5.1. The role of cIAP-1 and death receptors

Since Smac is recognized to act as a dimer, it was expected the bivalent Smac mimetics may have improved properties relative to their monovalent counterparts [2]. This was demonstrated to be the case when an attempt to chemically manipulate the alkyne moiety of **1** afforded the homodimerization product **2** [15]. Although **2** was shown to bind XIAP

BIR3 with comparable affinity to **1**, unexpectedly **2** caused extensive cell death when dosed in combination with the TNFα-related apoptosis-inducing ligand (TRAIL); whereas **1** was much less active under these conditions.



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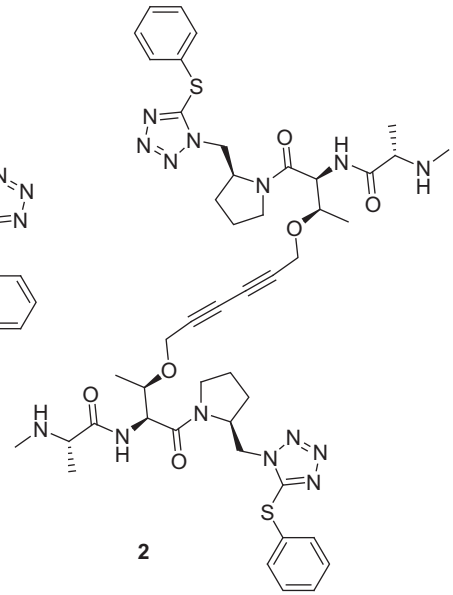

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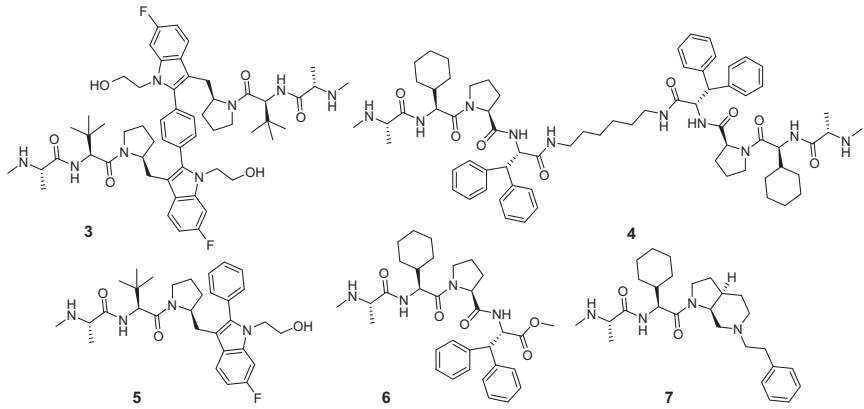
More importantly, however, was the revelation that the cIAP-1 and cIAP-2 were also critical intracellular targets of **2**. Both cIAP-1 and cIAP-2 are present in the TNFα receptor-1 (TNFR1) signaling complex, but it is unclear whether their specific function is inhibition of caspase-8, promotion of NF-κB and JNK signaling, or both. Treatment of HeLa cells with **2** in combination with TNFα resulted in significant apoptosis. Caspase-8 activation was observed within 2 h of **2** treatment and PARP cleavage, a measure of caspase-3 activity, within 4 h. These observations suggested that cIAP-1/2 function to block caspase-8 activation at the TNF receptor level and that antagonism of cIAP-1/2 by a Smac mimetic reverses caspase-8 inhibition leading to apoptosis.

The next piece of the puzzle was provided by four independent laboratories using several chemically unique Smac mimetics [16–19]. In addition to compounds **1** and **2**, bivalent analogs **3** (compound A) and **4** (BV6) as well as monovalent ligands **5** (compound C), **6** (MV1), and **7** (LBW-242) were employed in these studies.



Key observations provided by these studies were as follows: (1) cIAP-1/2 acts to protect cells from TNFα-induced apoptosis; (2) Smac mimetics elicit auto-ubiquitylation and subsequent proteosomal degradation of cIAP-1/2. Smac mimetic-sensitive cell lines undergo apoptosis owing to the presence of an autocrine TNFα loop. In these cell lines, Smac mimetic-induced apoptosis could be blocked upstream by the addition of neutralizing TNFα antibody or downstream by proteasome inhibitors.

cIAP-1 acts primarily as a ubiquitin E3 ligase necessary for maintaining protein levels of the key components of death receptor signaling pathways including the receptor interacting kinase (RIP1) [20], the NF-κB-inducing kinase (NIK) [21], cIAP-2 [22], and the TNFα receptor-associated factor (TRAF2) [23]. Smac mimetic-induced loss of cIAP-1 results in the dysregulation of death receptor signaling such as activation and/or inhibition of the canonical and noncanonical NF-κB pathways [24]. Thus, a comprehensive understanding of Smac mimetic-induced tumor cell killing revealed a much more complicated mechanism of action (Figure 4).

5.2. The XIAP BIR2–BIR3 binding hypothesis

[8,5]-Bicyclic Smac mimetic **8** (SM-122) binds to both XIAP BIR3 ($K_i = 26$ nM) and XIAP BIR2 ($IC_{50} = 5.6$ μM) [25]. Using computational models of **8** independently ligated to the BIR2 and BIR3 domains of XIAP, a suitable position for attachment of a linker group was identified. Bivalent Smac mimetic **9** exhibited excellent binding to an XIAP BIR2-BIR3 protein construct ($IC_{50} = 1.39$ nM), and a series of gel filtration and NMR experiments provided support for simultaneous occupation of the BIR2 and BIR3 domains. Importantly, these biophysical studies were conducted using IAPs devoid of either the N-terminal BIR1 domain which has

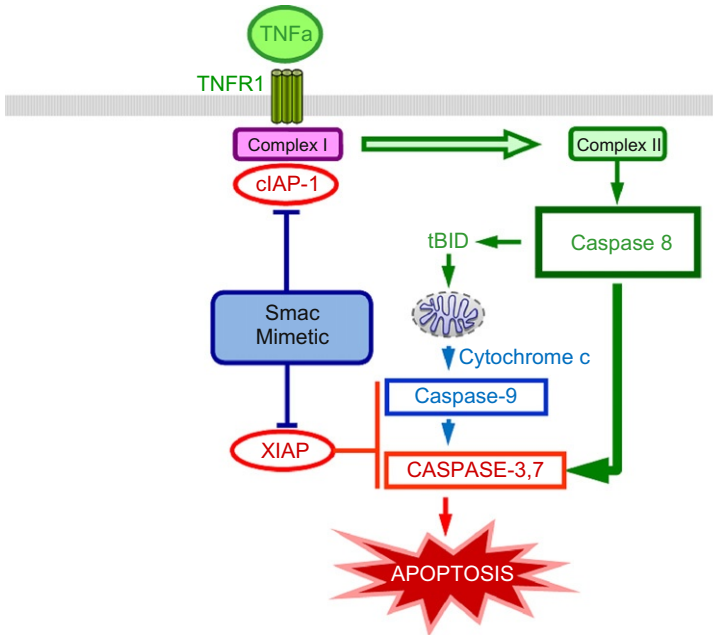
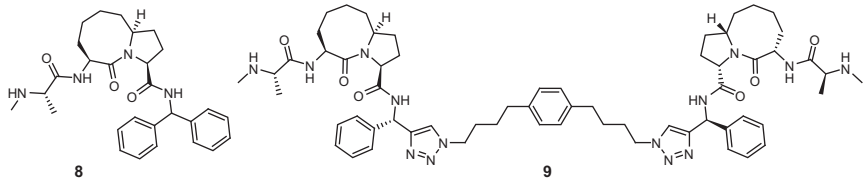


Figure 4 Schematic summary of Smac mimetic mechanism(s) of action.

been implicated in XIAP/TAB1 [7] and cIAP/TRAF [8] interactions or the C-terminal RING domain which has a key role in protein–protein dimerization and E3 ligase activity [26].



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5.3. The cIAP BIR3–BIR3 binding hypothesis

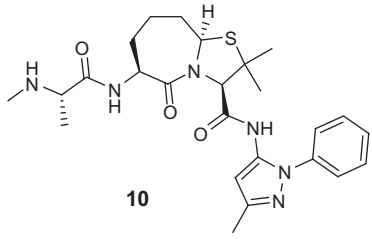
Bivalent Smac mimetic **4** (BV6) was also reported to simultaneously occupy the BIR2 and BIR3 domains of an XIAP BIR2–BIR3 construct [16]. In contrast, however, analytical ultracentrifugation suggested that **4** interacted with cIAP-1 BIR2–BIR3 *via* cross-linking of two BIR3 domains. Recent crystallographic studies using a cIAP BIR3–UBA–CARD–RING construct support the concept that the E3 ligase activity of cIAP-1 is suppressed by BIR3/RING interactions [27]. This assumes that Smac

mimetic binding to BIR3 unmasks the RING domain for participation in RING–RING dimerization in advance of ubiquitin transfer. One proposal suggests that the kinetics of Ub transfer is enhanced by a bivalent Smac mimetic-mediated cIAP BIR3–BIR3 cross-link which serves to stabilize the RING–RING dimer [28].

6. RECENTLY DESCRIBED SMAC MIMETICS

6.1. cIAP-selective Smac mimetics

Penicillamine-derived analog **10** is selective for the ML-IAP BIR domain versus XIAP BIR3 ($K_i = 50$ and 770 nM, respectively) [29]. The bias toward ML-IAP BIR binding was attributed to the replacement of Tyr324 in XIAP with Phe148 in ML-IAP—the *para*-hydroxy group of Tyr324 sterically interferes with the pro-R methyl group on **10** thus disfavoring XIAP binding. Since cIAP-1 contains the same Tyr-to-Phe replacement, **10** was shown to maintain good binding to cIAP-1 BIR3 ($K_i = 50$ nM). Antagonist **10** inhibits Smac/ML-IAP binding in a dose-dependent manner, causes single agent cell killing in MDA-MB-231 cells, induces apoptosis in A2058 melanoma cells as measured by caspase-3/7 activation, and demonstrates additivity when administered in combination with doxorubicin.



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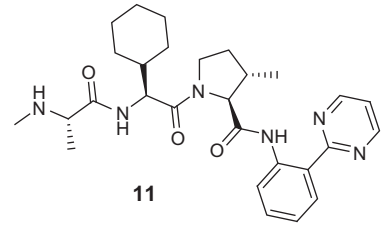

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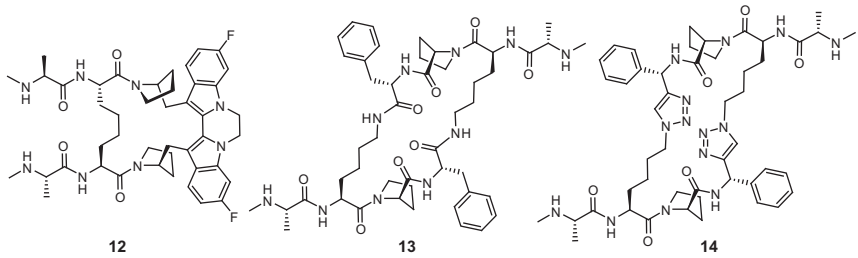
The highly cIAP-1-selective Smac mimetic **11** exploited the capacity of the cIAP-1 BIR3 to tolerate *S*-methyl substitution at the P_3' position, while the 2-pyrimidinyl group made use of both steric and electronic interactions within the P_4' binding site [30]. Together, these SAR elements enhance binding of **11** to cIAP-1 while dramatically reducing its affinity for XIAP BIR3 ($K_i = 0.016$ *vs.* >34 μM, respectively).

6.2. Macrocyclic Smac mimetics

A series of bivalent Smac mimetics culminated in the discovery of macrocyclic Smac mimetic **12** linked *via* the P_2' and P_4' residues [31,32]. Macrocycle **12** binds with subnanomolar affinity to both the XIAP and cIAP-1

BIR3 domains and effects tumor cell killing in the SK-OV-3 ovarian cancer cell line (CC_{50} = 6 nM) [33].

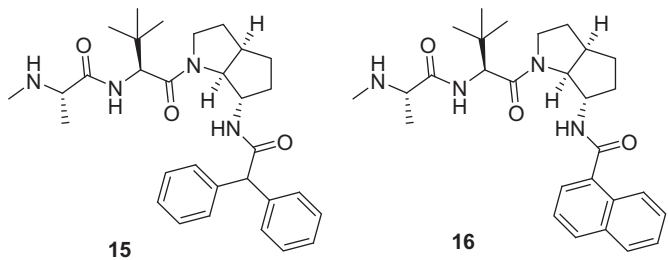


Cyclic peptide **13** binds to BIR2–BIR3-containing XIAP constructs with a biphasic dose–response curve representing two binding sites (IC_{50}s = 0.5 and 406 nM) [34]. Gel filtration experiments indicate that **13** forms a 1:1 complex with BIR2–BIR3 XIAP. Co-crystallography of **13** with the XIAP BIR3 domain afforded a 2:1 BIR3:**13** structure, which was utilized to build a model of the BIR2–BIR3/**13** complex.

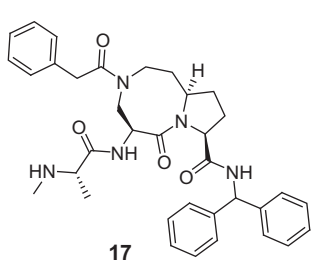
In an effort to reduce the peptide character of **13**, Smac mimetic **14** was prepared [35]. Interestingly, analysis of the binding curve associated with **14** and BIR2–BIR3 XIAP afforded IC_{50} values of 0.43 and 23 nM for the two binding sites. When tested against the BIR2-only XIAP, the IC_{50} value rose sharply to 3.2 μM (compared to 53.9 μM for **13**) suggesting that other protein–ligand interactions may be involved in binding [36].

6.3. Orally bioavailable Smac mimetics

Smac mimetics **15** and **16**, designed using the software program CAVEAT, were shown to have oral bioavailability in mice when dosed as an aqueous hydroxypropyl-β-cyclodextrin/succinic acid solution [37]. Both **15** and **16** were able to bind to the ML-IAP BIR domain with good affinity (K_is = 82 and 46 nM, respectively), and the protein–ligand structures were solved by X-ray co-crystallography. Both analogs were advanced into murine xenograft studies, and **16** potently inhibited tumor growth without affecting body weight.



Diazabicycle **17** (SM-337) has nanomolar binding affinity to XIAP, cIAP-1, and cIAP-2 BIR3 domains (K_is = 8.4, 1.5, and 4.1 nM, respectively) and is capable of relieving XIAP-mediated inhibition of caspase-3/7 in a cell-free system [38]; is eight times more potent than **8** at inhibiting MDA-MB-231 cell growth (IC_{50} = 31 *vs*. 259 nM, respectively); and induces proteosomal degradation of cIAP-1 at concentrations as low as 10 nM. At 30 mg/kg, oral dosing of **17** hydrochloride in the rat afforded an AUC of 1985 ± 614 μg/L and a C_{max} which is 17-fold higher than its IC_{50}. Another diazabicycle-based Smac mimetic **18** (AT-406) was selected for clinical evaluation [39]. Smac mimetic **19** (LCL-161) was profiled against a number of ovarian, melanoma, and lung cancer cell lines as a single agent and in combination with taxol® [40] and is administered orally as tablets or in solution in an ongoing clinical trial [41].



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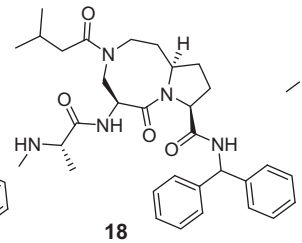

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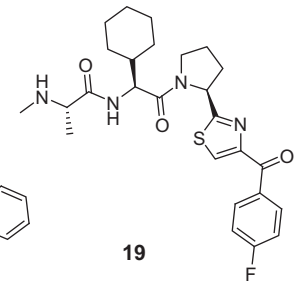

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7. PRECLINICAL AND CLINICAL EVALUATION

To date, six Smac mimetics have advanced to clinical trials: GDC-0152/RG7419, GDC-0917/RG7459, HGS1029/AEG40826·2HCl, LCL-161, TL32711, and AT-406. These compounds represent a panel of monovalent (GDC-0152, GDC-0917, LCL-161, AT-406) and bivalent (HGS1029, TL32711) Smac mimetics, and their routes of administration are either *via* intravenous infusion or *via* oral dosing.

GDC-0152/RG7419 (structure not disclosed), administered by intravenous infusion, was advanced into an open-label Phase 1 dose escalation study for patients with locally advanced or metastatic malignancies. Monocyte chemotactic protein-1 (MCP-1) is expressed during an inflammatory response and has been associated with Smac mimetic treatment in some preclinical species [42]. Although the PK parameters were dose-proportional with moderate variability, no consistent dose-dependent changes in MCP-1 levels were observed in patients following GDC-0152 treatment at the doses studied [43]. This investigation was completed in 2010.

In late 2010, an orally bioavailable Smac mimetic, GDC-0917/RG7459 (structure not disclosed) was introduced into Phase 1 clinical trials for refractory solid tumors or lymphoma.

HGS1029/AEG40826·2HCl (structure not disclosed) is administered as a 15-min infusion provided on days 1, 8, and 15 of a 28-day cycle in patients with relapsed or refractory solid tumors [44]. Of the 27 patients enrolled to date, 1 patient experienced dose-limiting toxicity after the first dose but recovered quickly and no further DLTs were observed. HGS1029 produced a dose-dependent but short-lived lymphocytopenia and, possibly, transient neutrophilia and supraventricular tachycardia. The pharmacokinetics (PK) was linear over the dose range tested and did not vary between single and multiple doses. cIAP-1 loss was observed in peripheral blood mononuclear cells (PBMCs) and was dose dependent. HGS1029 is well tolerated and dose escalation is ongoing. A second Phase 1 trial evaluating HGS1029 in patients with relapsed or refractory lymphoid malignancies was initiated in 2009.

LCL-161 has demonstrated single agent activity in tumor models with high basal TNFα levels and has shown synergistic activity in combination with taxanes. When evaluated in patients with advanced treatment-refractory solid tumors, oral LCL161 exhibited dose-proportional PK up to 1800 mg po [41]. Doses >500 mg achieved AUC levels at or greater than those which illicit a tumor response in preclinical models as either a single agent or in combination with standard-of-care agents. Loss of cIAP-1 was observed by Western blot analysis of PBMC-derived cell lysates as well as from a Merkel cell carcinoma biopsy; elevated levels of cleaved cytokeratin-18 were also observed at doses ≥ 320 mg. The high-dose groups (500–1800 mg) displayed increases in both serum IL-8 and MCP-1 levels, suggesting activation of NF-κB pathways. No dose-limiting toxicities have thus far been observed and a combination trial with paclitaxel is planned for 2010.

TL32711 (structure not disclosed) is a bivalent Smac mimetic which selectively antagonizes multiple IAPs and has demonstrated antitumor activity in a number of preclinical models [45]. In animals, TL32711 was well tolerated up to 60 mg/kg, the highest dose tested, on a q3d $\times$ 5 schedule. In the MDA-MB-231-derived xenograft model, the minimally efficacious dose was 1.25 mg/kg (q3d $\times$ 5) and activity was maintained in both large and small tumors. TL32711 was rapidly and extensively distributed into normal and tumor tissue but was more slowly cleared from tumor tissue relative to normal tissue. Rapid reduction in cIAP-1 was observed in tumor tissue where cIAP-1 levels remained suppressed for up to 2 weeks following a single 5 mg/kg dose. A Phase 1a dose escalation safety study in patients with refractory solid tumors and lymphoma began enrolling in 2009 [46]. TL32711 is administered as a 30-min iv infusion once weekly for 3 weeks per repeated 4-week intervals. More

recently, a Phase 1b/2a study combining TL32711 with several standard-of-care chemotherapies was initiated.

A similar dose escalation and safety study of AT-406 in patients with solid tumors and lymphomas commenced in 2010. AT-406 is to be administered orally to determine the maximum tolerated dose. Patients will receive **18** on days 1–5, and days 15–19 of a 28-day cycle.

8. CONCLUSION

Based on the unique mechanism of apoptosis induction, Smac mimetics hold promise for treating patients suffering from a wide variety of difficult to treat solid tumors and hematological malignancies [47,48]. As such, they will join a family of other apoptosis-inducing agents under clinical development including Bcl-2 antagonists, antisense XIAP, rhApo2L/TRAIL, and monoclonal antibody DR4/DR5 agonists. Much has been learned about the mechanism of Smac mimetics although it remains unclear as to the specific contributions of cIAP-1, cIAP-2, and XIAP antagonism toward tumor regression [49]. In addition, many details of IAP–ligand interactions remain unresolved such as whether bivalent IAP antagonists bind discretely to individual IAPs (BIR2–BIR3) or to homo- or hetero-IAP complexes *via* BIR3 cross-linking. Treatment with either monovalent or bivalent Smac mimetics results in the proteosomal degradation of cIAP-1, and that loss of cIAP-1 has direct consequences for TNFα and TRAIL signaling. The mechanism by which Smac mimetics synergize with specific chemotherapeutic agents is less well understood. Additionally, cIAP regulation has been implicated in other receptor signaling pathways such as TWEAK/Fn14 [50] and the NOD family of receptors [51,52] as well as direct interaction with the immune system [23,53,54]. The clinical development of both monovalent and bivalent Smac mimetics will provide an advanced look at the therapeutic benefit of this type of cancer treatment. Already, dose escalation studies have determined that many of these agents are well tolerated in patients with advanced disease while showing significant target suppression. The development of specific biomarkers for apoptosis-inducing agents like Smac mimetics will further aid in the development of these novel therapies.

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