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Design and synthesis of novel azapeptide activators of apoptosis mediated by caspase-9 in cancer cells

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ABSTRACT

A set of azapeptides was designed based on the Ala-Val-Pro-Ile peptide (derived from Smac protein) to activate caspase-9 and induce apoptosis in breast cancer cells. The diversity-oriented synthesis of the aza-peptides **5**–**9** was accomplished by alkylation of the aza-residue of aza-Gly-Pro dipeptide **15** using potassium *tert*-butoxide and a range of different alkyl halides. The resulting protected aza-dipeptide building blocks were then introduced into mimics **5**–**9** using standard coupling conditions. Biological evaluation of **5**–**9** was performed in MDA-MB-231 breast cancer cells, and indicated that the aza-Gly and aza-Phe analogs **5** and **7** were most efficient in inducing cell death by a caspase-9 mediated apoptotic pathway. Revealing a relationship between azabicycloalkanone and aza peptide mimics, novel AVPI mimics were synthesized which exhibit utility for studying structure–activity relationships to develop leads for activating apoptosis in cancer cells.

Apoptosis is a controlled sequence by which cells signal their own termination. Programmed cell death is crucial for normal tissue development and homeostasis,[1,2] because it permits elimination of aging and abnormal cells to maintain organism integrity. In contrast to normal cells, cancer cells 'switch off' apoptosis, develop and divide indefinitely.[3,4]

Programmed cell death is a multistep process highly regulated and primarily under the control of two type of proteins: the caspase enzyme family and their modulators, so-called IAPs (inhibitors of apoptosis proteins).[5] Among the latter, X-linked IAPs (XIAPs) are key regulators of death-receptor- and mitochondria-mediated apoptosis pathways. They also constitute attractive targets because of their roles in the resistance of cancer cells to apoptosis.[6,7] The XIAP contains three baculoviral IAP repeat (BIR1–3) domains, which bind and inhibit the activity of caspases-3, -7 and -9.

Smac (second mitochondria-derived activator of caspase) is a potent pro-apoptotic protein released from mitochondria in response to apoptotic stimuli.[8,9] Smac promotes apoptosis in cells by binding to the BIR3 domain of XIAP primarily via its N-terminal four residues Ala-Val-Pro-Ile,[10,11] and liberating consequently caspase-9. Mimics based on a constrained version of the Smac AVPI tetra-peptide have thus been targeted to inhibit the XIAP-BIR3 interaction and induce apoptosis.[12–16] Among the constrained analogs, those containing fused 6,5-, 7,5- and 8,5- bicyclic turn surrogates as replacements of the Val-Pro dipeptide have exhibited notable potency (e.g., **2**–**4**, Fig. 1) and have demonstrated that a β-turn is preferable for affinity and activity (Fig. 1).[12–16] Although such azabicyclo[*X.Y.*0]alkan-2-one amino acid analogs represent an important class of Smac mimics, their synthesis demands multiple steps restricting analog development.

Semicarbazides are aza-amino acids. In peptides, aza-amino acid residues can exert conformational constraints due to the urea and hydrazine, which induce turn conformations. Moreover, the introduction of an aza-residue into a peptide can improve

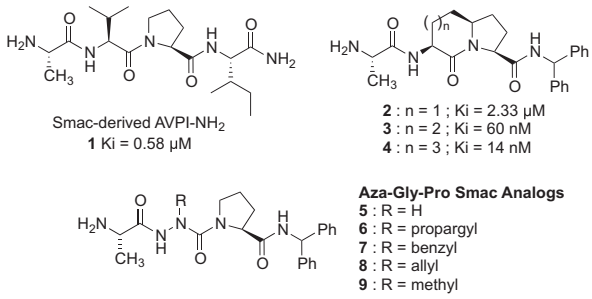

Figure 1. AVPI and peptide mimic activators of caspase-9.

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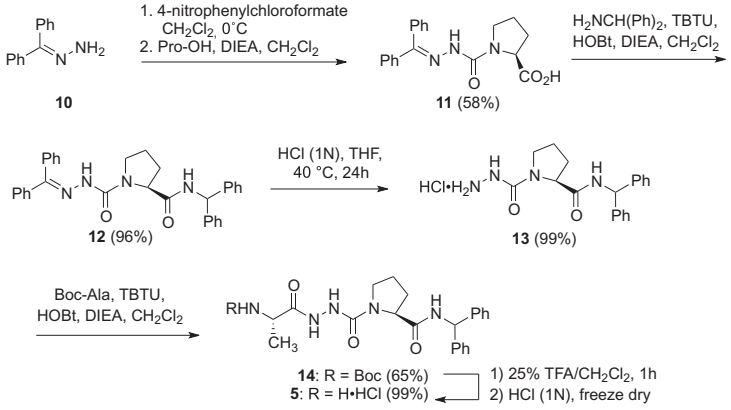
metabolic stability, because the urea is more resistant to chemical and enzymatic degradation than its amide counterpart.[17] Recently, we have demonstrated the relationship between the azabicyclo[4.3.0]alkan-2-one amino acid (indolizidinone amino acid, I^{2}aa) and aza-amino acyl proline residues as turn mimics.[18,19] For example, replacement of the Gly^{33}-Pro^{34} moiety in [D^{31}, P^{34}, F^{35}-]calcitonin gene-related peptide$^{27-37}$ by AzaGly^{33}-Pro^{34} and I^{2}aa$^{33-34}$ gave respectively 10- and 7-fold increases in antagonist potency.[18] Subsequently, in our efforts to develop molecules, which can inhibit uterine contractions and delay preterm labor, we have recently reported that replacing the central I^{2}aa residue of the prostaglandin F2α modulator PDC113.824 by the azaGly-Pro dipeptide can maintain activity and efficacy.[19] Considering the potential to explore the importance of valine for binding and activity by the preparation of aza-analogs of AVPI, we report the design and synthesis of a new series Smac mimics and their biological evaluation as caspase-9 activators that induce apoptosis in breast cancer cell lines (e.g., **5**–**9**, Fig. 1).

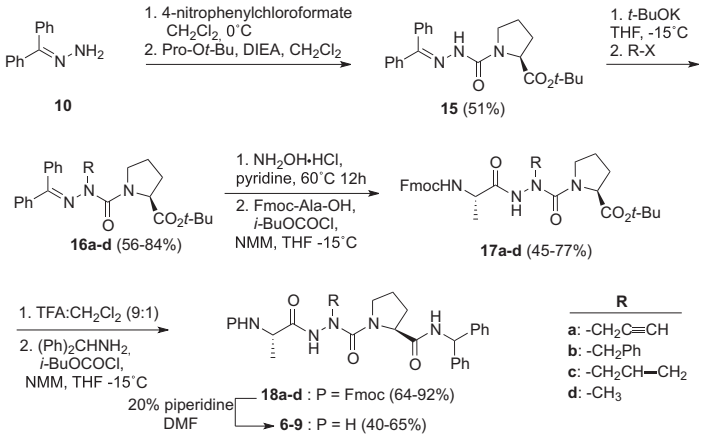
The synthesis of azaGly-Pro dipeptide **11** and its use for the synthesis of Smac mimic **5** were previously reported (Scheme 1).[20] Acylation of proline with the activated benzhydrylidine carbazate generated from benzophenone hydrazone and *p*-nitrophenyl chloroformate gave azaGly-Pro dipeptide **11**, which was coupled to diphenyl methyl amine. Synthesis of azaGly-Pro Smac mimic **5** was then completed by removal of the benzophenone protection, acylation with Boc-Ala, and Boc group removal. Aza-peptide **5** was thus obtained in five steps and 35% overall yield.[20]

Diversification of the aza-glycine residue was accomplished by a route featuring alkylation of benzophenone-protected azaGly-Pro-O*t*-Bu dipeptide **15** (Scheme 2).[21] Treatment of aza-dipeptide **15** with *tert*-butoxide and alkylation with different alkyl halides (propargyl bromide, benzyl bromide, allyl bromide and methyl iodide) afforded the corresponding aza-amino acyl proline dipeptides **16a**–**d** in 56–84% yields.[21] The benzhydrylidene protection was then removed by treatment with hydroxylamine hydrochloride in pyridine and Fmoc-Ala was coupled to the resulting semicarbazides using *iso*-butyl chloroformate and *N*-methyl morpholine to give the aza-tripeptides **17a**–**d** in 45–77% yields. Cleavage of the *tert*-butyl ester with trifluoroacetic acid in dichloromethane, coupling to diphenyl methyl amine with the same coupling conditions, and Fmoc group removal with a solution of 20% piperidine in DMF yielded Smac mimics **6**–**9** in 40–65% overall yields from **18a**–**d** (Scheme 2). In addition, Ala-Val-Pro-Ile-NH_2 was prepared as a positive control by standard Fmoc-based solid-phase peptide synthesis on Rink resin in 25% overall yield (see Experimental section).[22]

The pro-apoptotic potential of Smac mimics **6**–**9** was examined in MDA-MB-231 breast cancer cells at two different concentrations: 50 μM and 100 μM (Fig. 2). At 100 μM, AVPI-NH_2 and Smac mimics **5**–**9**, all induced cell death at levels (~50%) similar to the topoisomerase II inhibitor Etoposide, which blocks DNA replication.[23] At 50 μM, the relative activities of Smac mimics **5**–**9** became apparent. Relative to vehicle (DMSO), the parent peptide AVPI-NH_2 weakly induced cell death (11.5%) at 50 μM. Aza-propargyl and aza-allylglycine analogs **6** and **8** exhibited no activity, and aza-alanine analog **9** was only slightly more active relative to vehicle. In contrast, aza-glycine and aza-phenylalanine analogs **5** and **7** promoted cell death more effectively than the parent peptide at the same concentration.



Scheme 1. Synthesis of azaGly-Smac mimic **5**.



Scheme 2. Synthesis of Smac mimic **6**–**9**.

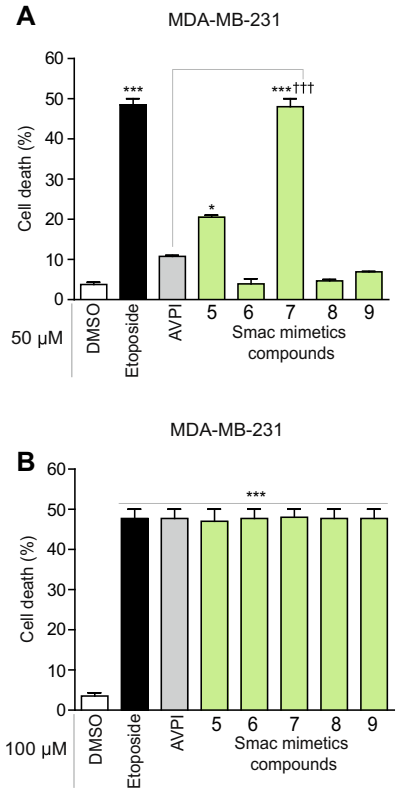


Figure 2. Smac mimic induced cell death in MDA-MB-231 cell line. (A) Cells were serum starved for 6 h and then treated with DMSO, etoposide (50 μM), AVPI-NH_2 (50 μM) or indicated Smac mimetic compounds for 16 h using a final concentration of 50 μM. After incubation, cells were trypsinized, stained with Trypan blue and counted. (B) MDA-MB-231 cells were treated as in A and incubated with all compounds at a final concentration of 100 μM. Data are expressed as percentage of cell death (%). These results are the mean ± SEM of three independent experiments. $^{***}P < 0.001$, $^{*}P < 0.05$ are values compared to the paired control DMSO condition and $^{†††}P < 0.001$ are values compared to the paired AVPI-NH_2 condition.

Moreover, aza-phenylalanine analog **7** enhanced cell mortality with similar efficacy as Etoposide. In summary, although all Smac mimics induced cell death at the higher concentration, the two aza-analogs **5** and **7** proved to be more effective than AVPI-NH_2 at 50 μM.

The activation of caspase-9 and caspase-3 via proteolytic cleavage is crucial for the regulation of the apoptotic program. Therefore, we examined conversion of their pro-forms into active proteins by Western blotting (Fig. 3). High binding affinity between Smac protein and XIAP is contingent on the interaction of its AVPI peptide with the BIR3 domain of XIAP, which induces caspase-9 activity. Thus, we expected Smac mimicry would increase levels of caspase-9 in the Western blot (Fig. 3). Moreover, caspase-3 activation is induced by caspase-9, thus intensity of the band for caspase-3 should correlate with caspase-9 and Smac mimic activity.

At 100 μM concentration, Smac mimics **5**–**9** as well as AVPI-NH_2 and Etoposide, all enhanced formation of active caspases-3 and -9 (Fig. 3B). However, relative effectiveness of the AVPI analogs was better indicated at 50 μM. At this concentration, only aza-phenylalanine analog **7** produced detectable amounts of caspases-3 and -9. In addition, Etoposide resulted only in caspase-3 activation at this concentration, likely due to the short exposure to the drug. In fact, previous publications reported that a 48 h treatment was required to activate caspase-3 in MDA-MB-231 cells.[24]

Dose–response experiments were subsequently performed to determine EC_{50} values (Fig. 4). The EC_{50} values of AVPI-NH_2, and aza-glycine and aza-phenylalanine analogs **5** and **7** were respectively, 72.66 μM, 73.37 μM and 50.18 μM. In sum, at high concentration, AVPI-NH_2 and azapeptides **5**–**9**, all exhibited a pro-apoptotic potential through the activation of caspases-3 and -9. Among the aza-analogs, aza-phenylalanine derivative **7** exhibited the highest efficacy in activating caspases-3 and -9.

In conclusion, we developed a new strategy for making Smac mimics that promote cell death based on the synthesis of azapeptides. In particular, aza-glycine and aza-phenylalanine analogs **5** and **7** promoted caspase-3 and -9 activation and caused ultimately cell death. Aza-phenylalanine **7** was 25% more potent than AVPI-NH_2 in the MDA-MB-231 breast cancer cell assays. The enhanced activity of **7** may be a combination of its potential to mimic the active turn conformation of the native peptide as well as better ability to interact at the binding site in which valine is normally accommodated.

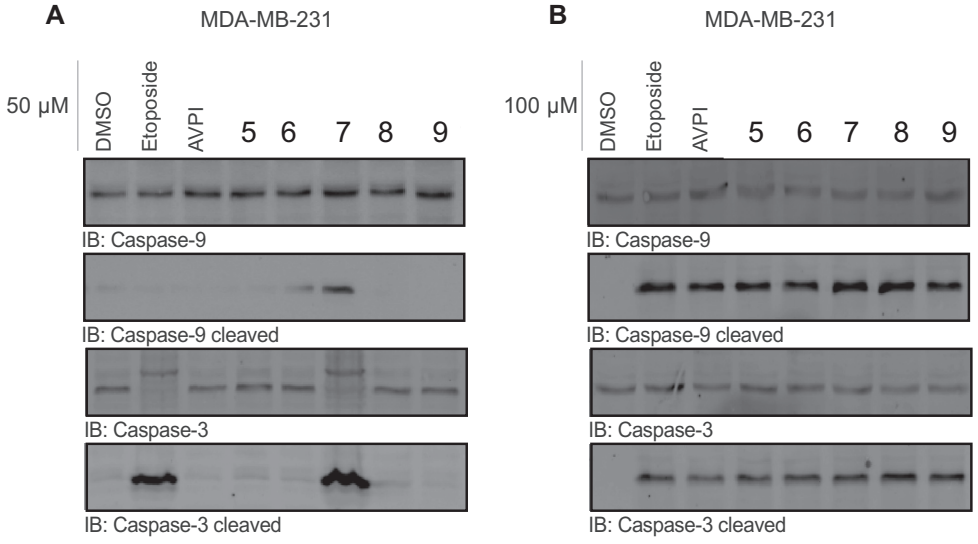


Figure 3. Smac mimic promoted activation of caspase-3 and caspase-9 in MDA-MB-231 cells. (A) Cells were serum starved for 6 h, treated with DMSO, etoposide (50 μM), AVPI-NH_2 (50 μM) and related synthetic compounds at a final concentration of 50 μM, harvested and activation of caspase-3 and caspase-9 was assessed by Western blotting. Quantifications are the mean ± SEM of three independent experiments. (B) Cells were treated as in A, where all compounds were used at a final concentration of 100 μM. Quantification is the mean ± SEM of three independent experiments.

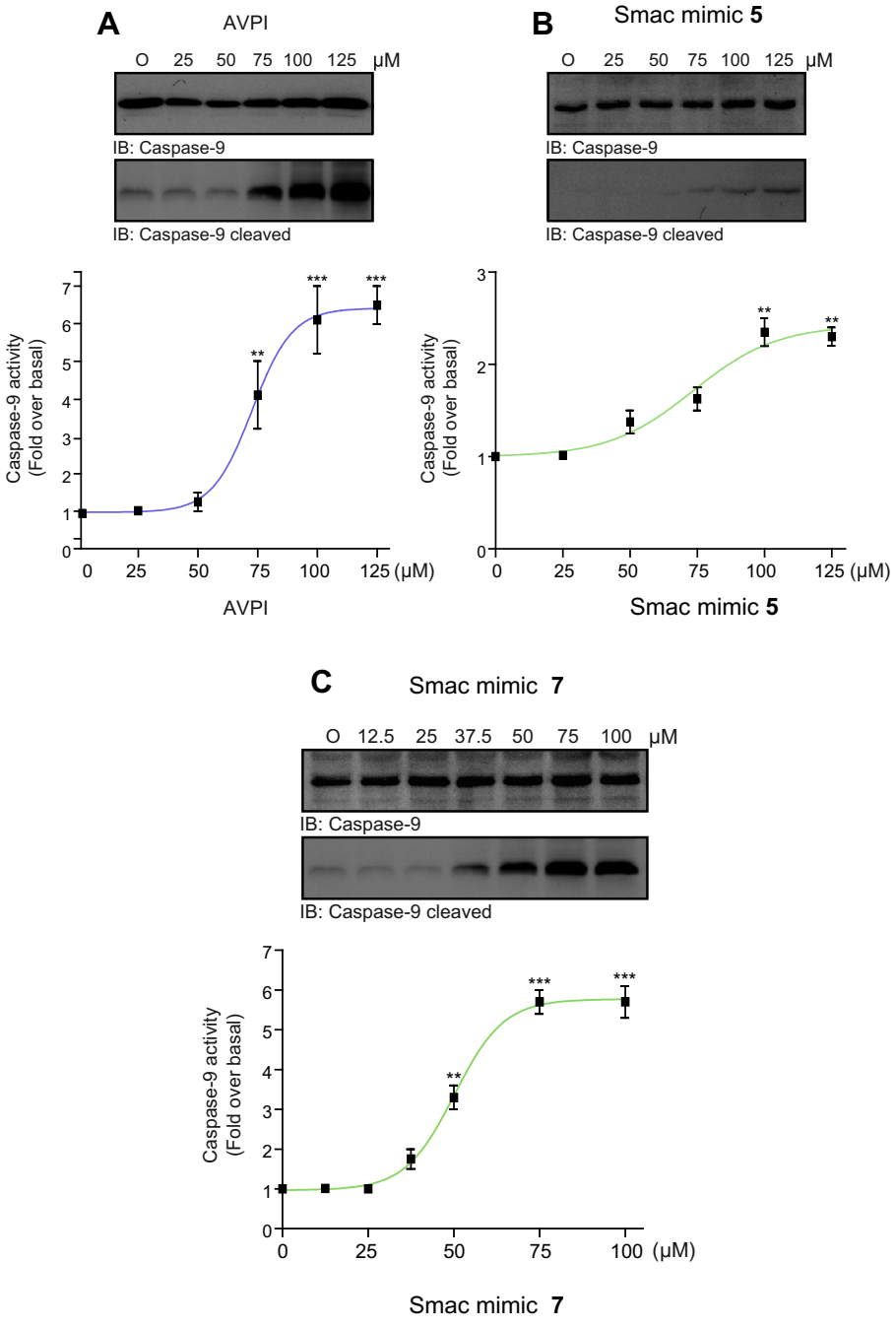


Figure 4. Smac mimic promoted activation of caspase-3 and caspase-9 in MDA-MB-231 cells. (A) Cells were serum starved for 6 h and treated with AVPI-NH_2 at indicated concentrations for 16 h and then harvested. Activation of caspase-9 was measured by Western blotting (upper panel). Quantifications are the mean ± SEM of two independent experiments (lower panel). (B and C) MDA-MB-231 cells were treated as in A and incubated respectively with **5** and **7**. Quantification is the mean ± SEM of two independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ are values compared to the paired untreated (DMSO) condition.

Considering the utility of diversity-oriented methods for generating aza-peptides, they represent a promising new class of Smac mimics for probing the structure–activity relationships at the AVPI binding site towards the development of improved therapy for treating cancer.

Acknowledgments

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Supplementary data

Supplementary data (synthetic experimental detailed procedures and analytical data for compounds **17a–d**, **18a–d**, **6–9** and AVPI-NH_2, and biological assay protocols) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.095.

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