Original Research

Novel X-linked inhibitor of apoptosis protein inhibitors as probes of apoptosis in biology and medicine

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Abstract

We report here a series of new inhibitors of the X-linked inhibitor of apoptosis protein (XIAP protein) based on the SMAC-tetrapeptide AVPI. The structural novelty of these molecules is the presence of the proline mimetic exo-2-azabicyclo[2.2.1]heptane-3-carboxylic acid, leading to analogs with similar activity to the natural ligand peptide. The structure–activity relationship and computational docking studies support the convenience of this unnatural amino acid as a building block to develop new peptides or small molecules targeting the XIAP-BIR3 domain.

Keywords: XIAP-BIR3, AVPI, proline mimetic, exo-2-azabicyclo[2.2.1] heptanes-3-carboxylic acid

Experimental Biology and Medicine 2011; **236**: 247–251. DOI: 10.1258/ebm.2010.010284

Introduction

In many cancers, the apoptosis mechanism is compromised by the upregulation of antiapoptotic proteins, resulting in a resistance to proapoptotic signals. The inhibitor of apoptosis proteins (IAPs) has been identified as a control point in the execution of cell death, blocking cellular apoptosis through the mitochondria pathway.

The IAPs were initially identified in the baculoviruses and since then a series of IAPs have been identified in nematode, yeast, drosophila cells and mammalian cells.[1] Among these IAPs, the X-linked IAP (XIAP) is shown to have antiapoptotic activity in mammalian cells[2] and has attracted the attention of the biomedical research community and pharmaceutical industry as a potential therapeutic target.

XIAP contains three BIR (Baculovirus IAP repeat) motifs at the N-terminus and a RING zinc-finger near the C-terminus. Precedents have shown that the BIR2 domain of XIAP inhibits the executioner caspases 3 and 7 and the BIR3 domain binds directly to the small subunit of initiator caspase 9.[3] The caspase inhibitory activity of XIAP can be antagonized by a mammalian protein called Smac (also known as DIABLO).

Smac is localized in mitochondrial membrane and is released into the cytosol during apoptosis. Smac promotes caspase activation by binding to XIAP and abrogating its inhibitory effects.[4,5] The high-resolution crystal structure of Smac complexed with the BIR3 domain of XIAP demonstrates that the N-terminal AVPI sequence is crucial for the binding of Smac to XIAP BIR3[6] (Figure 1a). These four residues share a significant homology with the N-terminus (AVPF) of caspase 9 and compete with the caspase for the same binding groove.[7] Structural analysis and point mutations of the AVPI peptide have shown that Ala 1 makes the largest contribution for the specific recognition to XIAP. The other three residues (Val 2, Pro 3 and Ile 4) interacting with the hydrophobic residues in the BIR3 surface groove appear to be less critical than Ala 1 and can be replaced by other appropriate residues.[6,8–10] Using the Smac N-terminal tetrapeptide AVPI as a lead compound, several kinds of Smac mimetics have been developed, including a series of bicyclic compounds with a lactam ring and some other non-peptidic compounds.[11]

Methods

Design of AVPI mimetics

In this study, we have designed and synthesized a new series of Smac AVPI (1a–e) mimetics, using the conformationally constrained proline mimetic exo-2-azabicyclo[2.2.1]heptane-3-carboxylic acid as the central core (Figure 2).

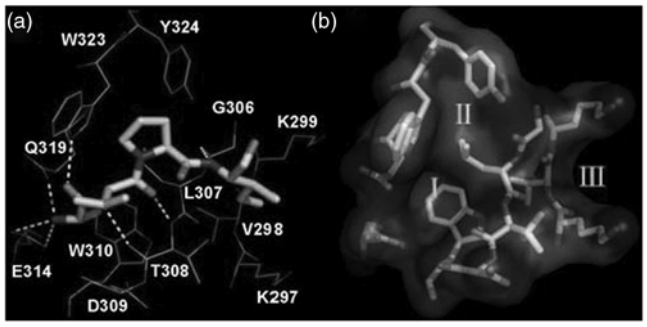


Figure 1 (a) N-terminus (AVPI) of Smac binding to BIR3 domain of XIAP (PDB: 1G73). Hydrogen-bonds are shown by the dotted lines; (b) Residues of this binding groove are shown in the surface model. I, II and III are three parts of the binding groove

As shown in Figure 1a, the proline residue of the AVPI peptide is positioned in the hydrophobic pocket formed by Trp 323 and Tyr 324. The use of this proline mimetic was envisioned to improve the CH-pi interactions between the bicyclic scaffold and the aromatic residues (Trp 323 and Tyr 324) present in the binding pocket. On the other hand, molecules containing this bicyclic scaffold should have advantageous therapeutic application for diminishing the peptidic character of the analogs and improving their proteolytic stability and pharmacokinetics.

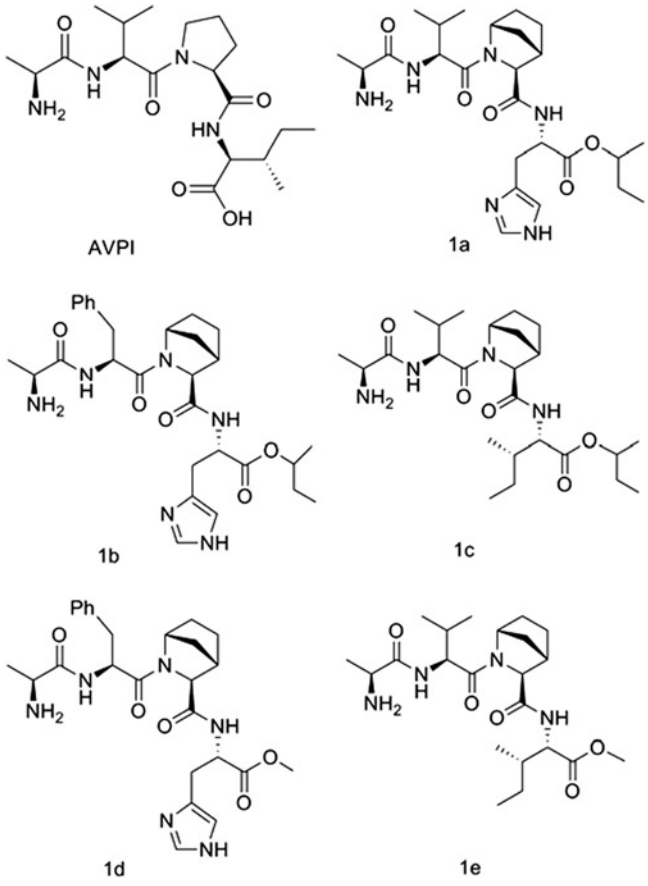


Figure 2 Structures of AVPI and its mimetics

Docking study of compound 1c with XIAP Bir3 domain

The binding groove of the XIAP-BIR3 domain can be divided into three fragments (Figure 1b). Fragment I is composed of Thr 308, Trp 310, Glu 314 and Gln 319, which is important for the charged N-terminal alanine of the AVPI peptide and analogs. The hydrogen bonds between the ligand and Thr 308 and Glu 314 are critical elements for recognition and binding. Fragment II is a hydrophobic pocket formed by Leu 307, Trp 323 and Tyr 324, which is occupied by the Pro residue of the Smac peptide. Finally, Fragment III is composed of Lys 297, Val 298 and Lys 299. Structure–activity relationship (SAR) studies have demonstrated that molecules containing large hydrophobic groups interacting with this pocket (Fragment III) exhibited high binding affinity.[8]

Considering that Fragment II might be occupied by exo-2-azabicyclo[2.2.1]heptane-3-carboxylic acid residue and a larger hydrophobic group in Fragment III should improve the binding with the protein, we have performed a docking study of compound 1c with XIAP Bir3 domain (Figure 3).

In our model, the key hydrogen bonds follow an identical pattern as the tetrapeptide AVPI derived from the natural ligand Smac. Furthermore, an additional hydrogen bond is formed between the nitrogen atom of the designed compound and a hydroxyl group of Thr 308. The exo-2-azabicyclo[2.2.1]heptane group is surrounded by Lys 307, Trp 323 and Tyr 324. One of the hydrophobic groups $-CH(CH_3)CH_2CH_3$ inserts in the hydrophobic pocket formed by Lys 297, Val 298 and Lys 299. The conformational analysis of the designed compound 1c reveals close similarity to the Smac AVPI binding in the XIAP Bir3 domain.

In order to verify if the sec-butyl group at C-terminal has a positive effect in the binding activity, we have replaced it by a methyl group generating the compounds 1d and 1e (Figure 2).

Synthesis

The synthetic route of compounds 1a–e is shown in Scheme 1. Compounds 2a and 2b were synthesized using a typical procedure for peptide coupling. The condensation of Fmoc-His(Trt)-OH and Boc-Ile-OH with 2-butanol followed

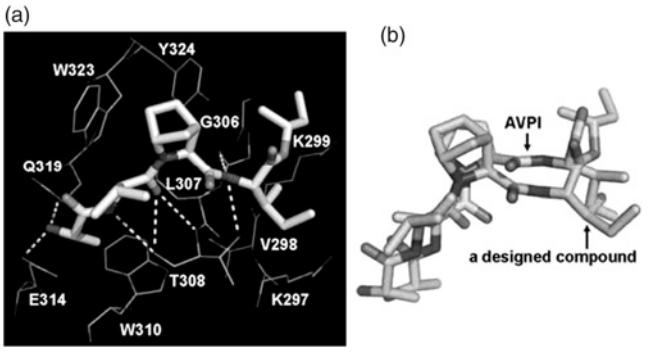
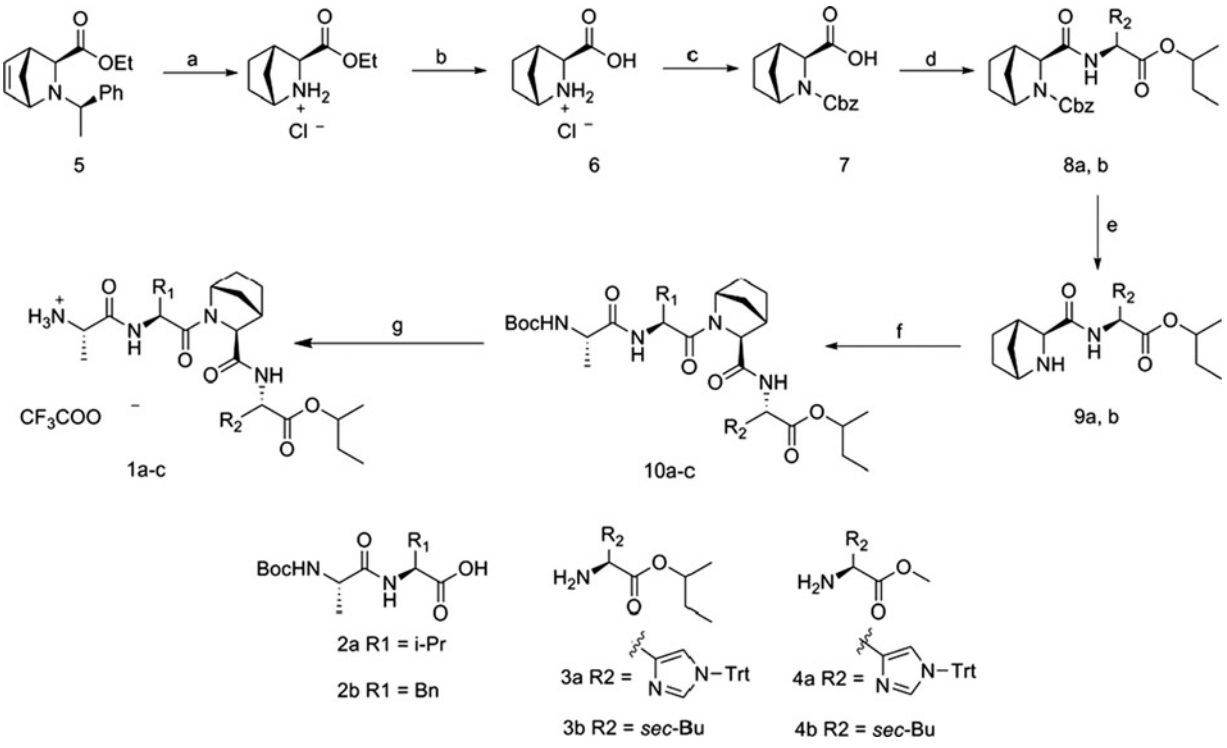


Figure 3 Docking result of compound 1c with XIAP BIR3 domain. (a) Hydrogen bonds are shown by the dotted lines. (b) Superimposition of the designed compound with N-terminus (AVPI) of Smac



Scheme 1 Reagents and conditions: (a) (i) Pd/C, H_2, room temp., 18 h; (ii) Con. HCl. (b) Aqueous HCl, reflux, 5 h. (c) CbzCl, sat. $NaHCO_3$ in H_2O, room temp., five hours. (d) 3a, b, HOBt, DCC, DIPEA, DCM, 0 °C to room temp., seven hours. (e) Pd/C, H_2, EtOH, room temp., 1.5–2 h. (f) 2a, b, HOBt, DCC, DIPEA, THF, 0 °C to room temp., 12 h. (g) 20% TFA in DCM, room temp., 1–2 h

by removal of the Fmoc or Boc protective group resulted in compounds 3a and 3b, respectively. Compound 5 was obtained from a stereoselective aza-Diels Alder reaction.[12] Hydrogenation of this compound followed by acidolysis led to compound 6. Compound 6 was protected with CbzCl to yield compound 7 and then coupled with 3a and 3b to obtain compounds 8a and 8b, respectively. After deprotection, compound 9a was coupled with 2a and 2b to give compounds 10a and 10b, respectively. Compound 9b was coupled with 2a to give compound 10c. Removal of the Boc and trityl protective groups provided the target compounds. Compounds 1d and 1e were synthesized following the same procedure, although their precursors were 4a and 4b, respectively.

Results

2D-NOESY

It is known in the literature that peptides containing proline in their structures can adopt either *cis* or *trans* conformation. We were able to confirm that the proline mimetic residue is stabilizing our analogs exclusively in the *trans* conformation through a two-dimensional nuclear overhauser enhancement spectroscopy experiment. Compound 10a presents a strong NOE effect between the α-proton of the valine residue and the bridgehead proton of the bicyclic scaffold. On the other hand, in a *cis* conformation the α-proton of the valine should present a strong NOE interaction with α-proton of the exo-2-aza-bicyclo[2.2.1]heptane residue. However, this effect cannot be found in the NOESY spectra. Although weak, a NOE effect between the methyl groups of the valine and the bridgehead proton can also be observed. These experimental evidences are in agreement with the optimized model using molecular mechanics (MM + forcefield), where the calculated distances corroborate the NOE effects observed in the NOESY resonance (Figure 4).

Fluorescence polarization assay and binding activity

The basis of the assay is disruption of fluorescence polarization resulting from binding of a XIAP-BIR3 (bacoloviral

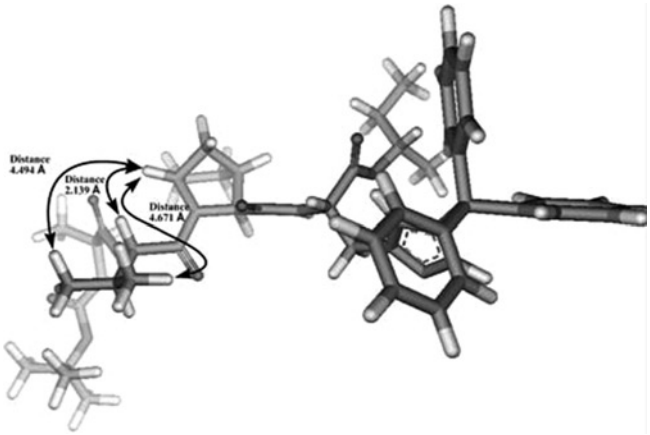


Figure 4 Optimized model of compound 10a using molecular mechanics

Table 1 XIAP binding assay results for Smac and the designed Smac analogs

Compounds	IC_{50} (μmol/L)	R2*
1a	0.40	0.9488
1b	4.52	0.9102
1c	4.36	0.9145
1d	2.34	0.9695
1e	5.47	0.9638
SMAC	0.20	0.9642

*Ran a 16-point curve with two-fold dilutions from 250 to 0.0077 μmol/L on XIAP binding

IAP repeat, Bir3) domain protein derived from two of the three conserved caspase binding BIR domains of XIAP to a rhodamine tagged 7-mer N-terminal SMAC peptide (tracer). Compounds that disrupt the binding of the tracer to the BIR3 domain may have activity that mimics the physiological interaction of XIAP and the SMAC protein, possibly resulting in the same physiological outcome. Compounds 1a–e and Smac peptide (AVPI) were evaluated for their binding affinities to XIAP using the fluorescence polarization assay (FPA). In this binding assay, only compound 1a was as potent as the AVPI peptide and the other analogs were 10–20 times less active (Table 1).

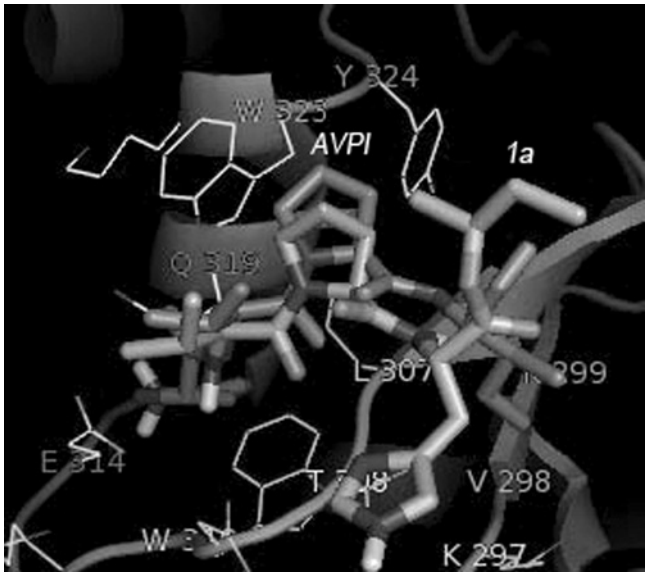


Figure 5 (a) Modeled structure of compound 1a in complex with XIAP BIR3 domain. The X-ray structure of Smac AVPI in complex with XIAP BIR3 is superimposed on the modeled structure for comparison. The figures are rendered using Pymol software[16]

Discussion

Based on the results described above, it is possible to rationalize the SAR of these analogs. Compound 1b is two times less active than its direct analog 1d, indicating that the sec-butyl ester has a deleterious effect on the binding activity. Compound 1a was less active than the SMAC-based peptide; however, this compound contains the sec-butyl substituent at C-terminal position. As mentioned before, this group does not provide the optimal interaction with the BIR3 domain, allowing the possibility of improving the activity after modifying this group. In many examples of SMAC mimetic compounds with good activity, their structure contains an aromatic residue interacting with Fragment III of the BIR3 domain. A similar trend is observed in our analog series, where the best results were achieved with compounds 1a and 1d. Both compounds present an imidazole ring (histidine residue) interacting with this fragment.

Although compound 1a assumes a similar conformation as AVPI peptide, the modeling study (using AutoDock 3.0)[13–15] has also corroborated that the sec-butyl group at C-terminal is not suitable for hydrophobic interactions supporting the FPA results. According to the modeled structure of compound 1a in complex with XIAP BIR3 domain, the sec-butyl group extends in an opposite direction compared with the AVPI peptide (Figure 5).

It is also worth noting that the prolyl peptide bonds in our analogs exclusively adopt the *trans* conformation. In many proteins prolyl *cis* isomers usually have distinct functions compared with the *trans* isomers, and the conversion between them can be catalyzed by peptidyl prolyl *cis–trans* isomerases (PPIases).[17] The prolyl *cis–trans* isomerization plays an important role in many physiological and pathological processes, such as transcription regulation,[18] embryonic development, hematopoiesis,[19] Alzheimer's disease and other neurodegenerative diseases.[20] To elucidate the molecular mechanisms in these processes, many proline analogues have been designed and synthesized.[21–23] However, all of them resulted in a mixture of *cis* and *trans* isomers, except L-5,5-dimethylproline (dmP), which was reported to constrain the prolyl peptide bond to the *cis* conformation.[24] In our study, it was interesting to find that the proline analogue exo-2-aza-bicyclo[2.2.1]heptane-3-carboxylic acid can lock the prolyl peptide bond in a *trans* conformation, which may be used as an alternative probe for the study of the mechanism and function of prolyl isomerization. Furthermore, our finding suggests that novel small molecules may be designed based on this scaffold to regulate the prolyl isomerization processes.

In summary, we have designed and synthesized five new Smac AVPI mimetics incorporating the proline surrogate exo-2-aza-bicyclo[2.2.1]heptane-3-carboxylic acid residue. Our designed compound 1a has exhibited comparable binding activity to the native peptide sequence AVPI, validating this unnatural amino acid as a building block to synthesize new inhibitors of XIAP-BIR3 domain. The SAR analysis has also provided useful information about the influence of the alkyl groups at C-terminal part of our molecules. Further optimization of the lead compound 1a and synthesis of new analogs using the exo-2-aza-bicyclo[2.2.1]heptanes-3-carboxylic acid residue are ongoing in our laboratory.

Author contributions: ZH, LZ and JCR supervised the project. AN designed the compound structures. QH, FRPC and XZ performed the chemical experiments and wrote the paper. ZL performed the experiments with QH. KW and JA did the *in vitro* biological screening.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (90713005), Raylight Corporation, the NIH NCDDG grant (U01-CA113318) and the NIH PO1 grant (CA055164).

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(Received September 20, 2010, Accepted November 16, 2010)