Bioorganic & Medicinal Chemistry Letters 24 (2014) 1452–1457

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel dimeric Smac analogs as prospective anticancer agents

Ewa D. Micewicz [a], Hai T. Luong [b,†], Chun-Ling Jung [b], Alan J. Waring [c,d], William H. McBride [a], Piotr Ruchala [b,*]

[a] Department of Radiation Oncology, University of California at Los Angeles, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA
[b] Department of Medicine, University of California at Los Angeles, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA
[c] Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90502, USA
[d] Department of Physiology and Biophysics, University of California Irvine, 1001 Health Sciences Road, Irvine, CA 92697, USA

ARTICLE INFO

Article history:
Received 17 November 2013
Revised 5 February 2014
Accepted 7 February 2014
Available online 18 February 2014

Keywords:
Smac mimics
New anticancer agents
Peptides
S-alkylation of peptides
Apoptosis

ABSTRACT

A small library of monovalent Smac mimics with general structure NMeAla-Tle-(4*R*)-4-Benzyl-Pro-Xaa-cysteamide, was synthesized (Xaa = hydrophobic residue). The library was screened in vitro against human breast cancer cell lines MCF-7 and MDA-MB-231, and two most active compounds oligomerized via S-alkylation giving bivalent and trivalent derivatives. The most active bivalent analogue SMAC17-2X was tested in vivo and in physiological conditions (mouse model) it exerted a potent anticancer effect resulting in ~23.4 days of tumor growth delay at 7.5 mg/kg dose. Collectively, our findings suggest that bivalent Smac analogs obtained via S-alkylation protocol may be a suitable platform for the development of new anticancer therapeutics.

Apoptosis (programmed cell death, PCD) functions as an important mechanism controlling homeostasis, normal development, host defense, suppression of oncogenesis, and its dysfunctional regulation is associated with a variety of human pathologies, including cancer,[1–5] inflammation[6,7] and neurodegeneration.[8,9]

Inhibitors of Apoptosis Proteins (IAPs) are key regulators of apoptosis.[10–12] They contain one or more of Baculovirus IAP Repeat (BIR) domains,[12,13] which are approximately 70 amino acid long structural motifs[13,14] primarily responsible for the anti-apoptotic activity of IAPs. Specifically, they bind and inhibit various caspases, enzymes belonging to cysteine-aspartyl proteases family, which are crucial for the apoptotic process.[15] A total of eight mammalian IAPs have been identified to date with the most potent caspase inhibitor family member being XIAP (X-linked IAP),[16,17] which effectively inhibits three caspases: caspase-3, -7, and -9.[18–21] An apoptotic signaling is in turn regulated by the second mitochondria derived activator of caspases (Smac), also called a direct IAP binding protein with low pI (DIABLO),[22,23] which has been identified as an endogenous proapoptotic antagonist of IAP proteins. After its release from the mitochondria into cytosol, and subsequent processing by proteases (removal of 55 N-terminal residues), a mature form of Smac effectively antagonizes XIAP, cIAP1 and cIAP2 proteins[22–26] promoting programmed cell death. Specifically, N-terminal tetrapeptide AVPI (Ala-Val-Pro-Ile), so called binding motif[22,23] of mature Smac, is responsible for proapoptotic effects of protein. In the case of XIAP, a homodimeric form of Smac is capable of binding to both BIR2 and BIR3 domains of the protein abrogating its inhibition of caspases-3, -7, and -9.[25,27] In the case of cIAP1 and cIAP2, only BIR3 domain is targeted by a single AVPI binding motif.[28]

Targeting IAP proteins represents a promising therapeutic approach in cancer treatment[14,29–31] and over the past 10 years a considerable amount of research was done in this particular field[32–35] including clinical trials.[35,36] Recently bivalent Smac analogues containing two AVPI mimics tethered with a linker and capable of binding to both BIR2 and BIR3 XIAP domains became the focus of researchers due to their high potency.[37–40] Available data[41] suggest that the overall hydrophobicity of the compounds may positively influence biological activity, most likely promoting cell permeability and increasing intracellular concentration of analogues resulting in more potent therapeutic effects. Therefore in the case of dimeric Smac mimics, we concluded that the overall therapeutic effect generally is dependent on 3 factors: (1) binding potency of the monomer(s), (2) length of the linker and (3) linker's hydrophobicity, with more hydrophobic compounds being generally more active due to increased cell permeability. Moreover, we theorized that cell permeability is a crucial limiting factor for Smacs' activity.

* Corresponding author. Tel.: +1 310 825 0133; fax: +1 310 206 8766.
E-mail address: pruchala@mednet.ucla.edu (P. Ruchala).
† Present address: Gilead Sciences, Inc., 4049 Avenida de la Plata, Oceanside, CA 92056, USA.

http://dx.doi.org/10.1016/j.bmcl.2014.02.024
0960-894X/

Subsequently, we decided to synthesize a small library of Smac analogues with varying hydrophobic residues (Xaa) in position 4, based on the modified structure of the previously described potent (K_d = 5 nM) monovalent compound, NMe-Ala-Tle-(4*S*)-4-phenoxy-Pro-(*R*)-tetrahydronaphth-1-yl amide[42] which was developed in Abbott Laboratories (NMeAla-(*N*-Methyl)alanine, Tle-*tert*-Leucine,). In our case we decided to use the following sequence: NMeAla-Tle-(4*R*)-4-Benzyl-Pro-Xaa–$NHCH_2CH_2$–SH, where Xaa stands for various hydrophobic residues and C-terminal cysteamide provides means for further multimerization based on thiol group reactivity (Fig. 1). This Letter describes synthesis and biological properties of these novel compounds.

All monovalent Smac analogs were synthesized[43] as C-terminal cysteamine-amides by the solid phase method using CEM Liberty automatic microwave peptide synthesizer (CEM Corporation Inc., Matthews, NC), applying 9-fluorenylmethyl-oxycarbonyl (Fmoc) chemistry[44] and standard, commercially available amino acid derivatives and reagents (EMD Biosciences, San Diego, CA and Chem-Impex International, Inc., Wood Dale, IL). Peptides were purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) to >90% homogeneity and their purity evaluated by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC.[45] Analytical data for obtained peptides as well as an example of MS-spectra and corresponding analytical RP-HPLC profile are presented in Supplementary material.

Since we assumed that hydrophobicity-dependent cell permeability is a crucial limiting factor for Smacs' bioactivity we decided to use exclusively cell-based assay for an initial evaluation of our compounds, namely cells' growth inhibition assay.[46] In our view, this simple method provides more reliable, data which take into account many factors like the compound's cell permeability, its binding potency, stability in the cell's microenvironment, etc., and in this particular case is better suited for such screening than pure biophysical method(s) for example, measurement of binding affinity to BIR2/BIR3 XIAP domains. For our in vitro studies we selected Smac-susceptible human non-metastatic breast cancer MCF-7 and metastatic MDA-MB-231 cell lines. An example of cell growth curves is presented in Figure 2. Initial screening of the monovalent Smac library (Table 1) against both human breast cancer cell lines suggested that for the best 'dual' activity against both MCF-7 and MDA-MB-231 cell lines, position 4 (Xaa) should be occupied either by Bip, 1Nal, 2Nal or Dpa, residues that possess fairly similar hydrophobic side chains. Interestingly, position 4 seems to also 'differentiate' between both tested cancer lines with some analogues being more potent against MCF-7 cells, that is, SMAC11 (Tic) and SMAC17 (Bip), and some being more potent against MDA-MB-231 cells, that is, SMAC6 (Chg) and SMAC14 (^{1}Nal).

Based on these results, we selected two compounds, SMAC14 (^{1}Nal) and SMAC17 (Bip), for subsequent multimerization[47] based on S-alkylation principles. The multimerization of peptides is frequently used as means to increase an immunogenicity (MAP peptides), prolong serum half-life/stability or a way to increase affinity to the receptor by harnessing multivalency effects. As a convenient multimerization scaffold(s) we decided to use commercially available 1,4-bis(bromomethyl)benzene (dimerization) and 1,3,5-tris(bromomethyl)benzene (trimerization). Moreover, compounds resulting from dimerization of our monovalent analogues with the use of 1,4-bis(bromomethyl)benzene produce a bivalent Smacs that possess roughly the same length as optimal linkers previously reported[41] and have hydrophobic properties.

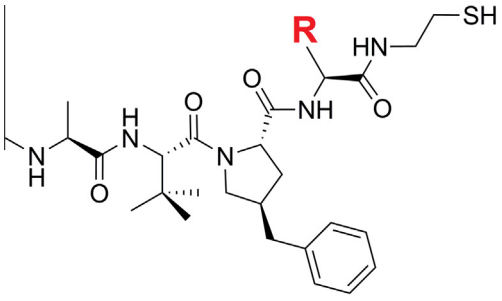


Figure 1. General structure of synthesized monovalent Smac compounds. R-various hydrophobic substituents (for list see Table 1).

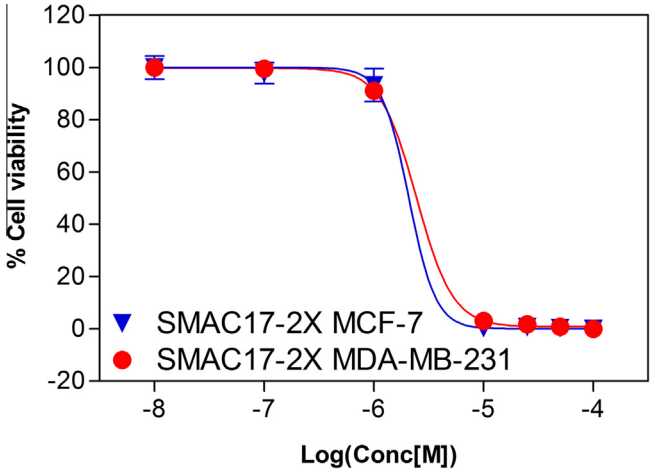


Figure 2. An example of cell viability curves obtained for MCF-7 and MDA-MB-231 human breast cancer cell lines treated with bivalent analogue SMAC17-2X.

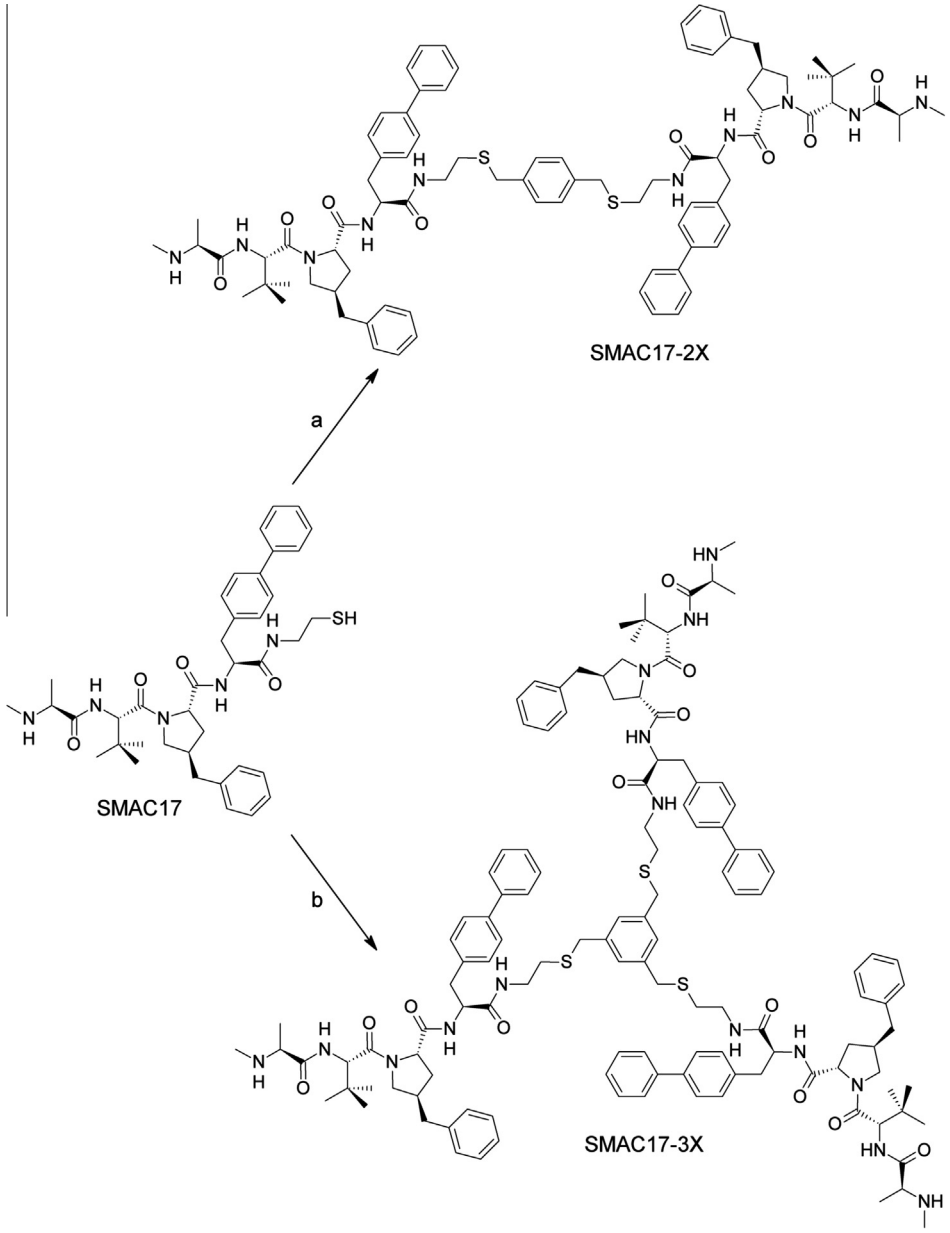
Table 1
Smac induced cell growth inhibition of MCF-7 and MDA-MB-231 human breast cancer cells

Peptide	R	EC_{50} (μM) MDA-MB-231	EC_{50} (μM) MCF-7
SMAC1	Phg	17.2 ± 6.4	31.2 ± 1.4
SMAC2	NMePhg	23.8 ± 4.5	43.6 ± 1.6
SMAC3	Amp	14.3 ± 3.2	49.1 ± 2.6
SMAC4	DISC	47.1 ± 15.9	41.6 ± 3.7
SMAC5	Idc	68.3 ± 4.0	476.7 ± 48.4
SMAC6	Chg	9.8 ± 3.1	31.2 ± 2.1
SMAC7	Amc	91.8 ± 16.3	86.3 ± 10.5
SMAC8	Phe	34.3 ± 6.7	33.3 ± 1.5
SMAC9	$PheF_5$	27.0 ± 3.2	19.8 ± 3.0
SMAC10	bhPhe	29.7 ± 3.3	23.9 ± 2.1
SMAC11	Tic	13.3 ± 1.3	5.3 ± 0.6
SMAC12	Cha	10.1 ± 2.6	21.2 ± 2.4
SMAC13	bhNalGly	21.7 ± 1.3	12.3 ± 0.5
SMAC14	^{1}Nal	4.5 ± 1.1	13.4 ± 0.5
SMAC14-2X	^{1}Nal	2.8 ± 0.1	3.5 ± 1.6
SMAC14-3X	^{1}Nal	7.2 ± 1.5	120.4 ± 8.4
SMAC15	^{2}Nal	9.4 ± 0.5	10.5 ± 0.6
SMAC16	Dpa	9.4 ± 0.6	11.3 ± 1.8
SMAC17	Bip	10.6 ± 0.9	5.7 ± 0.9
SMAC17-2X	Bip	2.4 ± 0.3	1.7 ± 0.4
SMAC17-3X	Bip	NA	NA
SMAC18	Ant	17.3 ± 1.0	15.1 ± 0.9
SMAC19	Trp	36.7 ± 3.2	27.6 ± 1.8

Abbreviations: Amc—*trans*-4-(aminomethyl)cyclohexane carboxylic acid, Amp—4-(aminomethyl)phenylacetic acid, Ant—3-(9-anthryl)alanine, Bip—biphenyl-alanine, Cha—cyclohexylalanine, Chg—cyclohexylglycine, bhPhe—β-homophenylalanine, bhNalGly—(*R*,*S*)-3-amino-3-(1-naphthyl)propionic acid, DISC—(*R*,*S*)-1,3-dihydro-2*H*-isoindole carboxylic acid, Dpa—diphenylalanine, Idc—(*S*)-indoline-2-carboxylic acid, ^{1}Nal—1-naphthylalanine, ^{2}Nal—2-naphthylalanine, NMePhg—(*N*-methyl)-phenylglycine, Phg—phenylglycine, $PheF_5$—pentafluorophenylalanine, Tic—(3*S*)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, NA—not active, 2X-dimer, 3X-trimer.

For S-alkylation based multimerization, as a method of choice, we decided to use a modified protocol previously described by Salvatore et al.[48] that we adapted to peptides (Scheme 1). Our protocol allows for synthesis in relatively high concentrations of peptides (~5 mg/ml) resulting in solution(s) that may be directly loaded on the HPLC columns simplifying post-synthetic procedures. Analytical data for synthesized mono-, bi-, and trivalent analogues are presented in Supplementary material. Obtained compounds (dimers and trimmers) were tested in vitro yielding the most potent bivalent analog in this series, SMAC17-2X which shows EC_{50} values of 1.7 ± 0.4 and 2.4 ± 0.3 μM for MCF-7 and MDA-MB-231, respectively. Notably, trivalent compound SMAC14-3X shows significantly lower activity than respective bivalent analog SMAC14-2X. In the case of SMAC17-3X, no anticancer activity was observed. According to published results[37,40,41,49,50] and our in vitro data multimerization, specifically dimerization, seems to be beneficial for overall anticancer activity of Smac-peptides. However promising, dimerization is also associated with notable problems: (1) synthesis of dimers requires at least one additional synthetic step in the late stage of synthesis that lowers overall yield of the final product, (2) dimerization increases significantly molecular weight (at least doubles) of the bioactive compound(s) effectively multiplying cost of production, (3) in the case of our analogues, multimerization is also associated with decrease in solubility that can influence delivery, distribution and pharmacokinetics of the drug. Obviously, such factors are of crucial importance for the drug development and may heavily influence the fate of dimeric Smac-derivatives.

To test whether our approach may yield compounds with therapeutic potential we performed animal studies using subcutaneous engraftment mouse model and a human metastatic breast cancer line, MDA-MB-23.[51] Treatment of the experimental, cancer bearing animals with bivalent compound SMAC17-2X resulted in potent



Scheme 1. General synthetic route for the synthesis of bivalent (SMAC17-2X) and trivalent (SMAC17-3X) analogs. Reagents and conditions: (a) 1,4-bis(bromomethyl)benzene, Cs_2CO_3, TBAI, 50% DMSO in DMF (b) 1,3,5-Tris(bromomethyl) benzene, Cs_2CO_3, TBAI, 50% DMSO in DMF. Analogues SMAC14-2X and SMAC14-3X were synthesized in similar manner.

anticancer effects (Fig. 3). Animals treated with 10 doses of the compound at the concentration of 2.5 mg/kg showed ~10.2 days delay in tumor growth and treatment of animals with 3 times higher dose (7.5 mg/kg) resulted in ~23.4 days of tumor growth delay. Notably, no adverse effects were observed during animal experiments.

To confirm that newly synthesized peptides indeed promote apoptosis, we measured enzymatic activity of caspases-3/7 and -9 in a metastatic breast cancer cell line, MDA-MB-231 that was treated with selected SMAC peptides.[52] Comparison of SMAC14 and SMAC17 with their respective dimers (SMAC14-2X and SMAC17-2X) at 10 μM concentration resulted in significant increase in caspases' enzymatic activity (~2.1 ÷ 12.8 fold increase, Fig. 4). In both cases caspase-3/7 and caspase-9, dimers were more active than respective monomers. In agreement with cell viability data, SMAC17-2X showed the most potent effects causing ~12.8 fold increase in caspase-3/7 activity and ~6.1 fold increase in caspase-9 activity, and observed effects are dose dependent (Fig. 5).

To examine whether oligomerization of monovalent SMAC17 peptide induces a higher order structural features in multivalent counterparts we performed circular dichroism (CD) experiments[53] which are summarized in Table 2. Data suggest that oligomerization has no immediate effect on secondary structure of unbound analogs in experimental conditions.

The binding affinity of SMAC17-2X analog to the rhXIAP and its domains was assessed using surface plasmon resonance (SPR) experiments.[54] The peptide showed moderate affinity to the full-length rhXIAP (K_d = 317 nM), a low affinity to its BIR3 domain (K_d = 2.4 μM) and has no binding affinity to BIR2 domain (binding undetectable in experimental conditions).

Direct comparison of our in vitro and in vivo results with published data is somewhat difficult due to the differences in experimental conditions.[37,40,41,49,50] Nonetheless, reported results for cell growth inhibition assay in MDA-MB-231 cell line show activity in low nanomolar range[37,41,49,50] (i.e. compound **24**:IC_{50} = 1.2 ± 0.3 nM,[41] compound **13**:IC_{50} = 3.4 ± 0.6 nM,[49] compound **16**: IC_{50} = 0.9 ± 0.2 nM[50]) versus low micromolar range in the case of our compounds (Table 1). Notably, in the case of this particular assay the time of cells' incubation with Smac-compounds was in our case 48 h versus 96 h for abovementioned analogs **13**, **16** and **24**. Interestingly, a comparison of in vivo data for our most active bivalent analog, SMAC17-2X with previously reported compounds is more favorable. For example, SM-164[37] shows very similar tumor growth profile in the shown experimental timeframe (<50 days). Similar results were also reported for compound **27**[41] with slightly better bioactivity (**27** was tested in lower dosage with

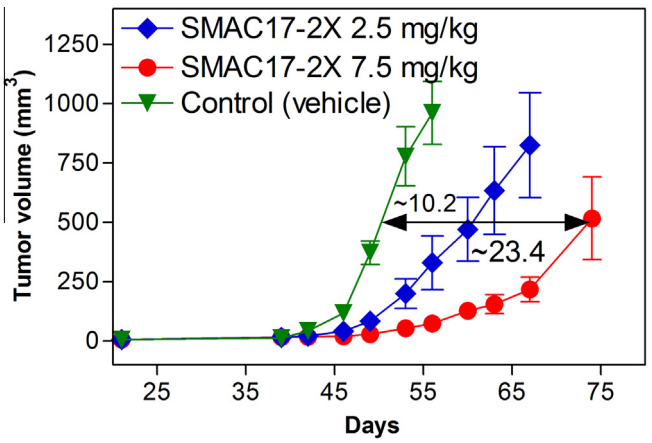


Figure 3. Anticancer effects of SMAC17-2X treatment in xenograft mouse model.

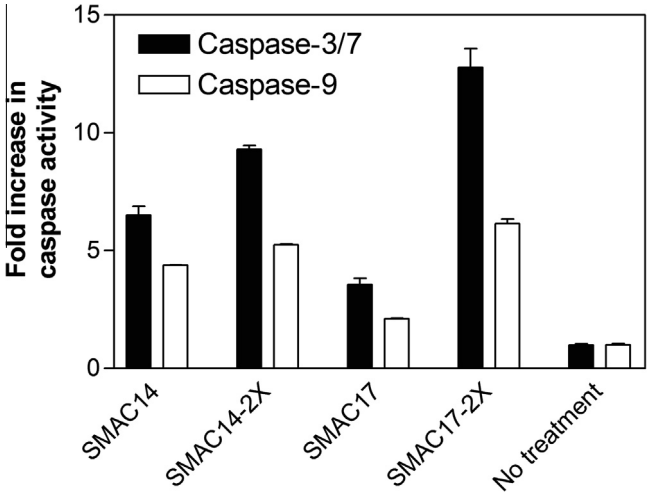


Figure 4. Increase in enzymatic activity of caspases-3/7 and -9 in MDA-MB-231 cells treated with SMAC peptides.

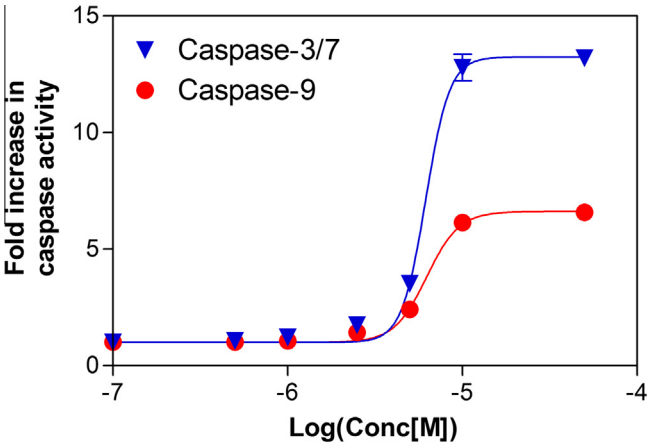


Figure 5. Increase in enzymatic activity of caspases-3/7 and -9 in MDA-MB-231 cells treated with various concentrations of SMAC17-2X.

Table 2
Proportions of different components of secondary structure for selected SMAC peptides in TFE-buffer based on circular dichroic spectroscopic analysis

Peptide*	% Conformation			
	α-Helix	Turns	β-Sheet	Disordered
SMAC17	4.0	27.0	43.0	26.0
SMAC17-2X	2.0	25.0	45.0	28.0
SMAC17-3X	2.0	29.0	44.0	25.0

* Peptides (100 μM) in TFE:10 mM HEPES buffer pH = 7.4, 4:6 (v/v) were analyzed for secondary conformation based on secondary structural analysis using SELCON.

similar results in the reported experimental range <50 days). Notably, recently reported results for SM-1200[50] show markedly improved activity comparing to all previously reported Smac-analogs. This potent bivalent Smac mimetic appears to promote complete and durable tumor regression in mouse model (<75 days).

In conclusion, a new family of anticancer Smac peptides was synthesized, characterized and screened for anticancer activity against human breast cancer cell lines, MCF-7 (non-metastatic) and MDA-MB-231 (metastatic). Selected analogues were oligomerized resulting in bivalent and trivalent Smac compounds. The most

active analogue (SMAC17-2X) was tested in vivo showing dose dependent, potent anticancer activity and appears to be a suitable template for the development of new anticancer Smac therapeutics.

Acknowledgments

This project was partially supported by funds from the Adams and Burnham endowments provided by the Dean's Office of the David Geffen School of Medicine at UCLA (P.R.).

Supplementary data

Supplementary data (analytical data for Smac analogs, representative analytical RP-HPLC profile and corresponding MALDI-MS spectra, and circular dichroism spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.02.024.

References and notes

1. Cotter, T. G. *Nat. Rev. Cancer* **2009**, *9*, 501.
2. Fulda, S.; Debatin, K. M. *Ann. N.Y. Acad. Sci.* **2004**, *1028*, 150.
3. Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57.
4. Kim, R. *Cancer* **2005**, *103*, 1551.
5. Mehrotra, S.; Languino, L. R.; Raskett, C. M.; Mercurio, A. M.; Dohi, T.; Altieri, D. C. *Cancer Cell* **2010**, *17*, 53.
6. Damgaard, R. B.; Gyrd-Hansen, M. *Discovery Med.* **2011**, *11*, 221.
7. Nagata, S. *Ann. N.Y. Acad. Sci.* **2010**, *1209*, 10.
8. Baratchi, S.; Kanwar, R. K.; Kanwar, J. R. *Crit. Rev. Biochem. Mol. Biol.* **2010**, *45*, 535.
9. Okouchi, M.; Ekshyyan, O.; Maracine, M.; Aw, T. Y. *Antioxid. Redox Signaling* **2007**, *9*, 1059.
10. Altieri, D. C. *Biochem. J.* **2010**, *430*, 199.
11. Danson, S.; Dean, E.; Dive, C.; Ranson, M. *Curr. Cancer Drug Targets* **2007**, *7*, 785.
12. Salvesen, G. S.; Duckett, C. S. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 401.
13. Deveraux, Q. L.; Reed, J. C. *Genes Dev.* **1999**, *13*, 239.
14. Lacasse, E. C.; Mahoney, D. J.; Cheung, H. H.; Plenchette, S.; Baird, S.; Korneluk, R. G. *Oncogene* **2008**, *27*, 6252.
15. Pop, C.; Salvesen, G. S. *J. Biol. Chem.* **2009**, *284*, 21777.
16. Holcik, M.; Gibson, H.; Korneluk, R. G. *Apoptosis* **2001**, *6*, 253.
17. Schimmer, A. D.; Dalili, S.; Batey, R. A.; Riedl, S. J. *Cell Death Differ.* **2006**, *13*, 179.
18. Chai, J.; Shiozaki, E.; Srinivasula, S. M.; Wu, Q.; Datta, P.; Alnemri, E. S.; Shi, Y. *Cell* **2001**, *104*, 769.
19. Huang, Y.; Park, Y. C.; Rich, R. L.; Segal, D.; Myszka, D. G.; Wu, H. *Cell* **2001**, *104*, 781.
20. Riedl, S. J.; Renatus, M.; Schwarzenbacher, R.; Zhou, Q.; Sun, C.; Fesik, S. W.; Liddington, R. C.; Salvesen, G. S. *Cell* **2001**, *104*, 791.
21. Srinivasula, S. M.; Hegde, R.; Saleh, A.; Datta, P.; Shiozaki, E.; Chai, J.; Lee, R. A.; Robbins, P. D.; Fernandes-Alnemri, T.; Shi, Y.; Alnemri, E. S. *Nature* **2001**, *410*, 112.
22. Du, C.; Fang, M.; Li, Y.; Li, L.; Wang, X. *Cell* **2000**, *102*, 33.
23. Verhagen, A. M.; Ekert, P. G.; Pakusch, M.; Silke, J.; Connolly, L. M.; Reid, G. E.; Moritz, R. L.; Simpson, R. J.; Vaux, D. L. *Cell* **2000**, *102*, 43.
24. Liu, Z.; Sun, C.; Olejniczak, E. T.; Meadows, R. P.; Betz, S. F.; Oost, T.; Herrmann, J.; Wu, J. C.; Fesik, S. W. *Nature* **2000**, *408*, 1004.
25. Shiozaki, E. N.; Shi, Y. *Trends Biochem. Sci.* **2004**, *29*, 486.
26. Wu, G.; Chai, J.; Suber, T. L.; Wu, J. W.; Du, C.; Wang, X.; Shi, Y. *Nature* **2000**, *408*, 1008.
27. Huang, Y.; Rich, R. L.; Myszka, D. G.; Wu, H. *J. Biol. Chem.* **2003**, *278*, 49517.
28. Samuel, T.; Welsh, K.; Lober, T.; Togo, S. H.; Zapata, J. M.; Reed, J. C. *J. Biol. Chem.* **2006**, *281*, 1080.
29. Dean, E. J.; Ranson, M.; Blackhall, F.; Dive, C. *Expert Opin. Ther. Targets* **2007**, *11*, 1459.
30. Fulda, S. *Expert Rev. Anticancer Ther.* **2007**, *7*, 1255.
31. Vucic, D.; Fairbrother, W. J. *Clin. Cancer Res.* **2007**, *13*, 5995.
32. Bai, L.; Wang, S. *Annu. Rev. Med.* **2013**, *65*. 20.1.
33. Chen, D. J.; Huerta, S. *Anticancer Drugs* **2009**, *20*, 646.
34. Sun, H.; Nikolovska-Coleska, Z.; Yang, C. Y.; Qian, D.; Lu, J.; Qiu, S.; Bai, L.; Peng, Y.; Cai, Q.; Wang, S. *Acc. Chem. Res.* **2008**, *41*, 1264.
35. Wang, S. *Curr. Top. Microbiol. Immunol.* **2011**, *348*, 89.
36. Cai, Q.; Sun, H.; Peng, Y.; Lu, J.; Nikolovska-Coleska, Z.; McEachern, D.; Liu, L.; Qiu, S.; Yang, C. Y.; Miller, R.; Yi, H.; Zhang, T.; Sun, D.; Kang, S.; Guo, M.; Leopold, L.; Yang, D.; Wang, S. *J. Med. Chem.* **2011**, *54*, 2714.
37. Lu, J.; Bai, L.; Sun, H.; Nikolovska-Coleska, Z.; McEachern, D.; Qiu, S.; Miller, R. S.; Yi, H.; Shangary, S.; Sun, Y.; Meagher, J. L.; Stuckey, J. A.; Wang, S. *Cancer Res.* **2008**, *68*, 9384.
38. Nikolovska-Coleska, Z.; Meagher, J. L.; Jiang, S.; Yang, C. Y.; Qiu, S.; Roller, P. P.; Stuckey, J. A.; Wang, S. *Biochemistry* **2008**, *47*, 9811.
39. Sun, H.; Nikolovska-Coleska, Z.; Lu, J.; Qiu, S.; Yang, C. Y.; Gao, W.; Meagher, J.; Stuckey, J.; Wang, S. *J. Med. Chem.* **2006**, *49*, 7916.
40. Sun, H.; Nikolovska-Coleska, Z.; Lu, J.; Meagher, J. L.; Yang, C. Y.; Qiu, S.; Tomita, Y.; Ueda, Y.; Jiang, S.; Krajewski, K.; Roller, P. P.; Stuckey, J. A.; Wang, S. *J. Am. Chem. Soc.* **2007**, *129*, 15279.
41. Sun, H.; Liu, L.; Lu, J.; Bai, L.; Li, X.; Nikolovska-Coleska, Z.; McEachern, D.; Yang, C. Y.; Qiu, S.; Yi, H.; Sun, D.; Wang, S. *J. Med. Chem.* **2011**, *54*, 3306.
42. Oost, T. K.; Sun, C.; Armstrong, R. C.; Al-Assaad, A. S.; Betz, S. F.; Deckwerth, T. L.; Ding, H.; Elmore, S. W.; Meadows, R. P.; Olejniczak, E. T.; Oleksijew, A.; Oltersdorf, T.; Rosenberg, S. H.; Shoemaker, A. R.; Tomaselli, K. J.; Zou, H.; Fesik, S. W. *J. Med. Chem.* **2004**, *47*, 4417.
43. All peptides were synthesized as C-terminal cysteamine-amides by the solid phase method using CEM Liberty automatic microwave peptide synthesizer (CEM Corporation Inc., Matthews, NC), applying 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and commercially available amino acid derivatives and reagents (EMD Biosciences, San Diego, CA and Chem-Impex International Inc, Wood Dale, IL). Cysteamine 2-Chlorotrityl Resin (EMD Biosciences, San Diego, CA) was used as a solid support. Peptides were cleaved from resin using modified reagent K (TFA 94% (v/v); phenol, 2% (w/v); water, 2% (v/v); TIS, 1% (v/v); EDT, 1% (v/v); 2 h) and precipitated by addition of ice-cold diethyl ether. Reduced peptides were purified by preparative reverse-phase high performance liquid chromatography (RP-HPLC) to >90% homogeneity and their purity evaluated by matrix-assisted laser desorption ionization spectrometry (MALDI-MS) as well as analytical RP-HPLC.
44. Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161.
45. Analytical RP-HPLC was performed on a Varian ProStar 210 HPLC system equipped with ProStar 325 Dual Wavelength UV–Vis detector with the wavelengths set at 220 and 280 nm (Varian Inc., Palo Alto, CA). Mobile phases consisted of solvent A, 0.1% TFA in water, and solvent B, 0.1% TFA in acetonitrile. Analyses of peptides were performed with an analytical C18 Vydac 218TP54 column, 4.6 × 250 mm, 5 μm (Grace, Deerfield, IL) applying linear gradient of solvent B from 0% to 100% over 100 min (flow rate: 1 ml/min). Analytical data for synthesized peptides are presented in Supplementary material.
46. Experiments were carried out using PrestoBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Briefly, Smac-susceptible human non-metastatic breast cancer MCF-7 (or metastatic MDA-MB-231) cells were plated in a 96-well plate at a density of 5×10^3 cells/well in a total volume of 50 μl of culture media and treated with various concentrations of tested peptides (50 μl of 0–200 μM peptides in culture media). The cells' viability was assessed after 48 h by fluorescence measurement (E_x/E_m:560/590, incubation time 30 min) employing the SpectraMAX M2 microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were carried out in triplicate.
47. Dimerization and trimerization of peptides was performed in 50% solution of DMSO in DMF using modified S-alkylation protocol previously described by Salvatore and co-workers[48] that was adapted to peptides. Briefly, peptides (3 equiv) were dissolved at final concentration 5 mg/ml. For dimerization 1,4-bis(bromomethyl)benzene (1.5 equiv) and for trimerization 1,3,5-tris(bromomethyl)benzene (1 equiv) were used with addition of anhydrous cesium carbonate (Cs_2CO_3, 15 equiv) and tetrabutyl-ammonium iodide, (TBAI, 6 equiv). Solution was vigorously mixed on magnetic stirrer and progress of reaction monitored by analytical RP-HPLC. Subsequently peptides were purified and characterized as described in the peptide synthesis section.
48. Salvatore, R. N.; Smith, R. A.; Nischwitz, A. K.; Gavin, T. *Tetrahedron Lett.* **2005**, *46*, 8931.
49. Peng, Y.; Sun, H.; Lu, J.; Liu, L.; Cai, Q.; Shen, R.; Yang, C. Y.; Yi, H.; Wang, S. *J. Med. Chem.* **2012**, *55*, 106.
50. Sheng, R.; Sun, H.; Liu, L.; Lu, J.; McEachern, D.; Wang, G.; Wen, J.; Min, P.; Du, Z.; Lu, H.; Kang, S.; Guo, M.; Yang, D.; Wang, S. *J. Med. Chem.* **2013**, *56*, 3969.
51. All animal experiments were approved by the UCLA Animal Care and Use Committee (ARC#1999-173-23) and conformed to local and national guidelines. Presented in vivo results were calculated from at least 2 independent experiments. Each group consisted 8 experimental animals. For Subcutaneous engraftment model experiments BALB/SCID gnotobiotic mice (8 weeks old, females) were obtained from the UCLA AALAC-accredited Department of Radiation Oncology Facility and subcutaneously injected with 2.0×10^6 cells of human metastatic breast cancer line (MDA-MB-231, leg). After 3 weeks, palpable tumors of approximately 5 mm diameter appeared and treatment was initiated. In general, each animal received intraperitoneally a total of 10 doses of SMAC17-2X at 2.5 or 7.5 mg/kg on days 1–5 and 8–12 with 8 mice per group. The peptide was formulated in 2% Cremophor EL (Sigma–Aldrich, St Louis, MO) in phosphate-buffered saline (PBS, vol/vol). Control animals were injected with vehicle alone. Tumor size was assessed every two days and animals sacrificed as necessary according to the UCLA Animal Care guidelines.
52. Enzymatic activity of caspases-3/7 and -9 was measured using commercially available Caspase-Glo® 3/7 and Caspase-Glo® 9 assays (Promega Corp., Madison, WI) utilizing manufacturers protocols. Briefly, MDA-MB-231 cells were plated in a white-walled 96-well plate at a density of 5×10^3 cells/well in a total volume of 50 μl of culture media and treated with various concentrations of tested peptides (50 μl of 0–100 μM peptides in culture media) for 24 h. Subsequently, 100 μl of appropriate Caspase-Glo® reagent was added to each well and cells incubated for additional 60 min. Luminescence values were determined employing the SpectraMAX M2 microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were carried out in triplicate.

53. Circular dichroism (CD) spectra (185–260 nm) of SMAC peptides were measured in solution of trifluorethanol-buffer (TFE/HEPES 10 mM pH 7.4, 4:6, v:v) using a JASCO 715 spectropolarimeter (Jasco Inc., Easton, MD). The instrument was routinely calibrated for wavelength and optical rotation using 10-camphorsulphonic acid and samples were scanned using 0.01 cm pathlength cells at a rate of 20 nm per minute, a sample interval of 0.2 nm, and a temperature of 25 °C. Sample concentration was determined by FTIR quantitation. Sample spectra were baseline corrected by subtracting spectra of peptide-free solution and expressed as the Mean Residue Ellipticity $[\Theta]_{MRE}$. Quantitative estimates of the secondary structural contributions were made with SELCON using the spectral basis set for proteins implemented in the Olis Global Works™ software package (Olis Inc., Bogart, GA).
54. Binding studies were performed by surface plasmon resonance (SPR) on a Biacore 3000 system (Biacore AB, Piscataway, NJ). The full-length rhXIAP, rhXIAP BIR2 domain and rhXIAP BIR3 domain (R&D Systems Inc, Minneapolis, MN) were immobilized on a CM5 sensor chip using the amine coupling method. The chip was activated by mixing 400 mM *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide. Residual reactive groups on the chip surface were blocked using 1.0 M ethanolamine/HCl (pH = 8.5). The flow cell-1 chip, which served as a control, lacked immobilized protein but was treated with *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride, *N*-hydroxy-succinimide, and ethanolamine/HCl. Experiments were performed in HBS-EP running buffer (pH = 7.4) containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20. Binding signals were corrected for nonspecific binding by subtracting the flow cell-1 signal. To regenerate chip surfaces, bound ligands were removed with 10 mM HCl. Data were analyzed with BIAevaluation 4.1 software (Biacore, Piscataway, NJ).