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Lipid-conjugated Smac analogues

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ABSTRACT

A small library of monovalent and bivalent Smac mimics was synthesized based on 2 types of monomers, with general structure NMeAla-Xaa-Pro-BHA (Xaa = Cys or Lys). Position 2 of the compounds was utilized to dimerize both types of monomers employing various bis-reactive linkers, as well as to modify selected compounds with lipids. The resulting library was screened in vitro against metastatic human breast cancer cell line MDA-MB-231, and the two most active compounds selected for in vivo studies. The most active lipid-conjugated analogue **M11**, showed in vivo activity while administered both subcutaneously and orally. Collectively, our findings suggest that lipidation may be a viable approach in the development of new Smac-based therapeutic leads.

Apoptosis, also called programmed cell death (PCD) is an important mechanism controlling a variety of physiological processes including: host defense, development, homeostasis, and suppression of oncogenesis with implications in human pathologies spanning from cancer[1–5] to inflammation[6,7] and neurodegeneration.[8,9] Regulation of apoptosis depends on Inhibitors of Apoptosis Proteins (IAPs).[10–12] Structurally, IAPs contain one or more of Baculovirus IAP Repeat (BIR) domains,[12,13] which are capable of binding to and inhibition of various caspases, enzymes belonging to cysteine–aspartyl proteases family, which are crucial for apoptotic process.[14] To date, eight mammalian IAPs have been identified: neuronal IAP (NIAP), cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), X chromosome-linked IAP (XIAP), survivin, ubiquitin-conjugating BIR domain enzyme apollon, melanoma IAP (ML-IAP) and IAP-like protein 2, with the most potent caspase inhibitor family member being XIAP,[15,16] which simultaneously inhibits caspases-3, -7, and -9.[17–20] Except XIAP, only cIAP1, cIAP2, and ML-IAP, were shown to play a direct role in the regulation of apoptosis by inhibiting caspases' activity or their activation.[12] Anti-apoptotic activity of IAPs is in turn regulated by the second mitochondria derived activator of caspases (Smac), also called direct IAP binding protein with low pI (DIABLO),[21,22] which acts as their endogenous pro-apoptotic antagonist promoting programmed cell death.[21–25] Structurally, N-terminal tetrapeptide AVPI (Ala-Val-Pro-Ile), so called binding motif,[21,22] is responsible for pro-apoptotic effects of mature Smac. In the case of XIAP, 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.[24,26] For cIAP1 and cIAP2 only BIR3 domain is targeted by a single AVPI binding motif.[27]

Over the past decade Smac mimics have become a promising therapeutic modalities in anti-cancer treatment[28–47] with several compounds advancing into clinical trials.[32,41,42,46,48–50] Among these 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 investigation due to their high potency.[35–37,39,43,44,47] On the other hand, monovalent Smac mimics are also desirable due to their favorable pharmaceutical properties: low molecular weight, favorable pharmacokinetics and potential oral bioavailability.[41,46] Nonetheless, review of available literature revealed that lipid-derivatized Smac mimics were not synthesized to date which prompted our investigation.

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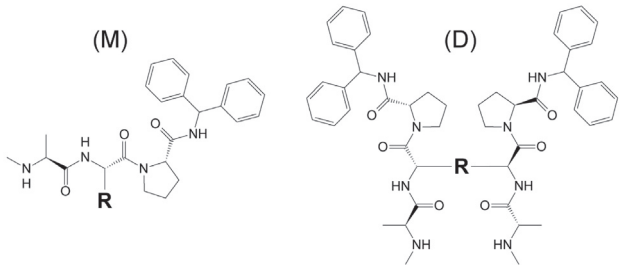
Lipidation is extensively used in the drug development, including therapeutic peptides.[51–53] Its application usually leads to new therapeutic entities with significantly changed physicochemical and pharmacological properties. In the case of peptides, such modification(s) may result in increased peptide stability, permeability and intestinal absorption.[54–63] An increased oral availability was also described.[64,65] Mechanistically, observed beneficial effects of lipidation are usually associated with binding of therapeutic entity to circulating albumin,[54,66–68] and/or targeted excretion by the liver rather than by the kidney.[54,63,65] An alternative mechanism employing high-density lipoprotein (HDL), low-density lipoprotein (LDL) and their receptors was also proposed.[69]

To ascertain whether lipidation can be a useful approach in the development of Smac mimics, we decided to synthesize and test small library of analogues with structure(s) schematically shown in Figure 1. Available data[39,47] suggest that overall hydrophobicity of the Smac mimics may be important for their biological activity, most likely promoting cell permeability and increasing intracellular concentration of analogues, resulting in more potent therapeutic effects. In this context, lipidation seems to be viable modification approach.



Compound	R	EC_{50} [μM] MDA-MB-231
M1	Ser^{OH}	NA
M2	Cys^{SH}	NA
M3	$Cys^{S\text{-}StBu}$	49.9±0.7
M4	Lys	NA
M5	Lys^{Gu}	NA
M6	$Lys^{NH\text{-}DNFB}$	44.7±15.0
M7	$Lys^{NH\text{-}Fmoc}$	19.0±1.6
M8	$Lys^{NH\text{-}Pal}$	6.1±0.1
M9	$Lys^{NH\text{-}Lig}$	NA
M10	$Lys^{NH\text{-}Chol}$	9.7±0.5
M11	$Cys^{S\text{-}Ste}$	4.4±0.1
D1	$Lys^{NH\text{-}Urea}$	NA
D2	$Lys^{NH\text{-}Sub}$	NA
D3	$Lys^{NH\text{-}DFDNB}$	3.3±0.2
D4	$Lys^{NH\text{-}PDI}$	34.8±2.7
D5	$Lys^{NH\text{-}OPI}$	14.4±1.4
D6	$Lys^{NH\text{-}Ida}$	NA
D7	$Lys^{NH\text{-}Ida\text{-}Pal}$	4.2±0.1
D8	$Lys^{NH\text{-}Ida\text{-}N\text{-}EtO\text{-}Pal}$	4.3±0.1
D9	$Cys^{S\text{-}DVS}$	52.4±4.3
D10	$Cys^{S\text{-}CAEDA}$	NA
D11	$Cys^{S\text{-}pBMB}$	9.6±0.6
D12	$Cys^{S\text{-}mBMB}$	7.4±0.4
D13	$Cys^{S\text{-}Bip}$	4.0±0.1
D14	$Cys^{S\text{-}CMPB}$	18.4±1.1
D15	$Cys^{S\text{-}BMBB}$	NA
D16	$Cys^{S\text{-}mBMPB}$	11.3±0.6

Figure 1. General structure of synthesized monomeric (**M**) and dimeric (**D**) Smac derivatives and their respective in vitro activity in cell growth inhibition assay, using MDA-MB-231 human metastatic breast cancer cell line. *Abbreviations*: Bip, 4,4′-bis(bromomethyl)biphenyl; BMBB, 1-(bromo-methyl)-3-[3-(bromomethyl) benzyl]benzene; CAEDA, *N*,*N*′-bis(2-chloro-acetylo)ethylenediamine; CMPB, 1-(chloromethyl)-4-[4-(chloromethyl)-phenoxy]benzene; Chol, cholesterol; DFDNB, 1,5-difluoro-2,4-dinitroben-zene; DNFB, 1-fluoro-2,4-dinitrobenzene; Fmoc, fluorenylmethyloxy-carbo-nyl; Gu, guanidine; Ida, iminodiacetic acid; Lig, lignoceric acid; mBMB, 1,3-bis(bromomethyl)benzene; mBMPB, 3,5-bis(bromomethyl)-1-(methyl-*S*-palmityl)-benzene; OPI, 4,4′-oxybis(phenyl isocyanate); Pal, palmitic acid; pBMB, 1,4-bis(bromomethyl)benzene; PDI, 1,4-phenylene diisocyanate; StBu, *S*-tertbutylthio; Ste, stearyl; Sub, suberic acid; NA, not active. All peptides were synthesized as C-terminal benzhydrylamides. EC_{50} values higher than 100 μM are denoted as NA.

Generally monomeric (**M**) analogues had a sequence NMeAla-Cys/Lys-Pro-BHA which is closely related to various potent analogues developed by Wang group.[35,37–39,43,44] Based on literature data,[70] position 2, which in our analogues is occupied by either (L)Lys or (L)Cys, was chosen as viable modification/dimerization point. Synthesis of monomers was carried out in solution according to reaction Schemes 1 and 2. Dimerization of monomers was carried out utilizing either bis-amine-reactive linkers (**D1**–**D8**, Fig. 4) or bis-thiol-reactive linkers (**D9**–**D16**, Fig. 5) listed in Figure 1. This report describes synthesis and biological properties of these novel compounds. Detailed experimental methods, analytical data for obtained peptides as well as an example of MS-spectra and corresponding analytical RP-HPLC profile are presented in Supplementary material.

The synthesis of monomers proceeded efficiently and due to simplicity of final products, was carried out with minimal purification of the intermediates. Four different lipids were used to modify **M** compounds in position 2: palmitic acid (Pal, C_{16}), lignoceric acid (Lig, C_{24}), cholesterol (Chol, C_{27}) and stearyl chain (Ste, C_{18}) afforded by 1-bromooctadecane, with first two using side chain amine group of Lys for modifications with palmitoyl chloride or lignoceroyl chloride, respectively. Cholesterol was introduced in similar manner using cholesteryl chloroformate giving urethane type connectivity (see Scheme 2). Analogue **M11** was synthesized using a previously described 1,1,3,3-tetramethylguanidine (TMG) driven alkylation of thiol(s) in organic solvents[71] that we adapted to peptides.[72] Notably, the same *S*-alkylation protocol was successfully employed in the synthesis of dimers **D9**–**D16**. Among all dimers synthesized for this study only three, **D7**, **D8** and **D16** underwent lipidation. **D7** and **D8** were modified with palmityl moiety using either physiologically stable amide bond (**D7**) or cleavable ester type (**D8**) connectivity. **D16** was modified with stearyl chain afforded by mBMPB-3,5-bis(bromomethyl)-1-(methyl-*S*-palmityl)-benzene however efficiency of the reaction was particularly low (<3%).

An initial evaluation of bioactivity of our Smac mimics was carried out in vitro using exclusively growth inhibition assay (PrestoBlue™, Invitrogen, Carlsbad, CA) and MDA-MB-231 metastatic human breast cancer cell line, which in our view provides more reliable data than pure biophysical method(s), for example, measurement of binding affinity to BIR2/BIR3 XIAP domain as it takes into account many factors like the compound's cell permeability, its binding potency, stability in the cell's microenvironment, etc. Notably, we had previously employed this approach in the synthesis of Smac mimics candidates with positive results.[47] Obtained results are summarized in Figure 1 and an example of cell growth curves is presented in Figure 2A.

Initial screening of monovalent Smac analogues (**M1**–**M11**) (Fig. 1) suggested that indeed optimal hydrophobicity plays an important role in overall bioactivity of position 2 modified compounds and observed activity gain can be significant in both ^{2}Cys (**M2** < **M3** < **M11**, EC_{50} (μM): NA < 49.9 ± 0.7 < 4.4 ± 0.1, respectively) and ^{2}Lys series (**M4** < **M6** < **M7** < **M8**, EC_{50} (μM): NA < 44.7 ± 15.0 < 19.0 ± 1.6 < 6.1 ± 0.1, respectively). Simultaneously exceedingly hydrophobic substituents (Lig, Chol) in position 2 seem to be undesirable as is the presence of hydrophilic/ionizable amine and guanidine moieties (**M4** and **M5**, respectively). The similar conclusions could be drawn from dimeric mimics as analogues utilizing hydrophilic linkers have shown low potency (**D1**, **D6**, **D9**, **D10**). Lipidation is also clearly beneficial, as