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SPION-Smac mimetic nano-conjugates: Putative pro-apoptotic agents in oncology

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ABSTRACT

Non-covalent (**NP-1/3**) and covalent (**NP-A-1/3**) pro-apoptotic SPION-Smac mimetic nano-conjugates antitumor agents are reported. The solution synthesis of key Smac mimetics, their support onto SPIONs through non-covalent adsorption (**NP-1/3**) or APTES-mediated covalent binding (**NP-A-1/3**), the analytical characterization of SPION-Smac mimetic conjugates, their target affinity in cell-free assays, and their cytotoxicity against tumor cells are thoroughly described.

Nanoparticles are popular tools in cancer detection and treatment, due to major advancements in nanosynthesis, bioengineering and imaging technology.[1] Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) are particularly attractive as diagnostics (e.g. biomarker-targeted magnetic resonance imaging/MRI contrast agents) and therapeutics (e.g. magnetic nanoparticle-enhanced hyperthermia).[2] Their large surface areas to conjugate targeting ligands and load therapeutic agents,[3] their unique magnetic properties,[4] their biocompatibility[5] and low toxicity[6] make them suitable technology platforms in oncology.

Anti-apoptotic Inhibition of Apoptosis Proteins (IAPs)[7] bind Cysteine ASPartic acid-specific proteASEs (caspases, CASP),[8] the major apoptotic effectors, through their baculovirus inhibitor repeat (BIR) domains. CASP-IAP interactions inactivate caspases and block apoptosis. The endogenous Smac protein[9] (Second Mitochondria-derived Activator of Caspases) binds to IAPs through its N-terminus AVPI sequence. The tetrapeptide binds to BIR3, the CASP-9 binding domain of IAPs, and prevents linker-BIR2-CASP-3/7 interactions through a weaker interaction.[10] Thus, Smac is a pro-apoptotic IAP ligand that restores caspase-dependent apoptosis in cancer cells.

We introduced 4-substituted, aza-bicyclo [5.3.0] decane (ABD)-based *N*-AVPI mimetics as potent, pro-apoptotic cytotoxic agents (see Fig. 1 for ABD structure and numbering). Monomers such as **1** (Smac136, Fig. 1) are potent, orally available BIR3 binders with moderate cytotoxicity.[11] Dimers such as **2** (Smac83, Fig. 1) bind IAPs on both BIR domains, resulting in a stronger cytotoxic activity, but possess a sub-optimal PK profile and cannot be administered orally.[12]

We reasoned that SPION-Smac mimetic nano-conjugates may take advantage of nanoparticle-specific access to cytosolic compartments (e.g. receptor-mediated endocytosis, macropinocytosis).[13] The spatial arrangement of monomeric Smac mimetics on the surface of SPIONs may allow, or even promote, the binding of two SPION-grafted Smac mimetics to a single IAP molecule (dimer-like binding). Finally, a single SPION-Smac mimetic nano-conjugate may bind several IAPs (multi-presentation mode). Thus, we decided to synthesize and biologically characterize a small library of SPION-Smac mimetic nano-conjugates.

4-CH_2OR and 4-$CH_2NR_1R_2$-substituted ABD Smac mimetics possess strong BIR/IAP affinity.[11] *N*-Boc-protected hydroxyacid **4** and *N*-Boc-protected aminoamide **5** were prepared from the tricyclic intermediate **3**[14] as previously reported[11a] (Scheme 1), although several reaction conditions were optimized. 4-Connected Smac-linker carboxylate constructs (**6**, ester connection, and **7**, amide connection) were obtained in good yields respectively from compounds **4** and **5** (Scheme 1).

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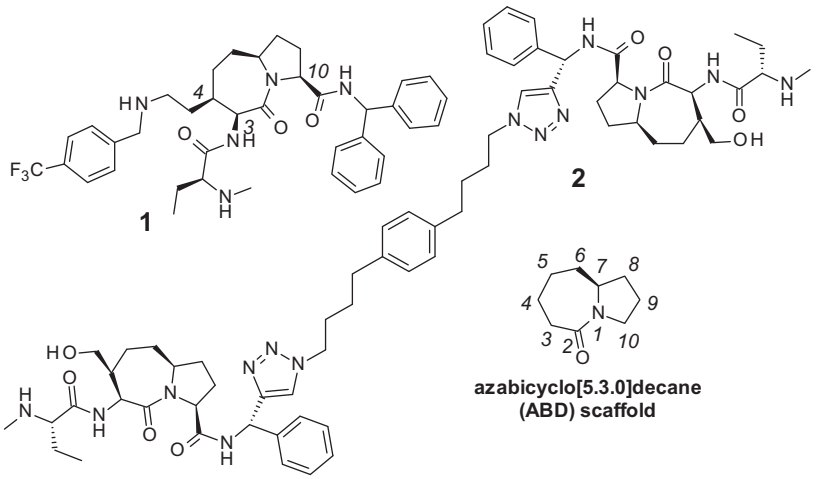
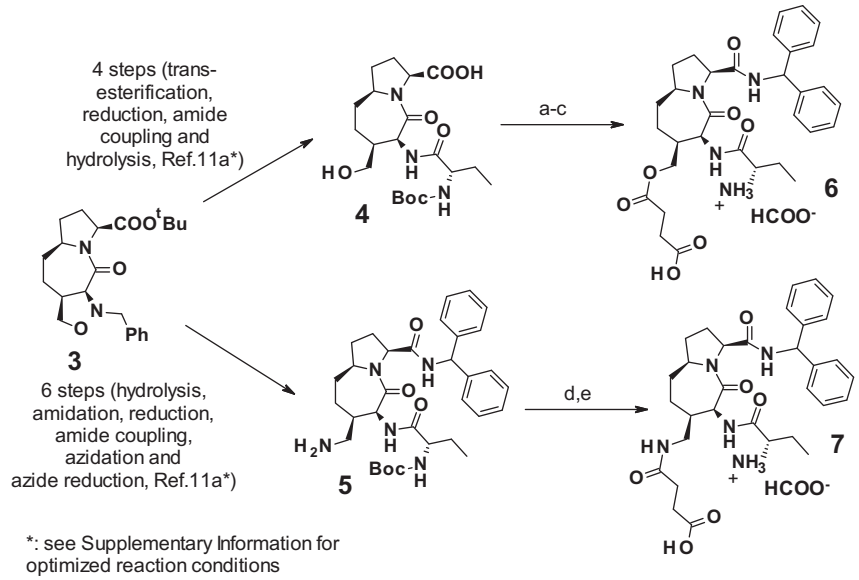
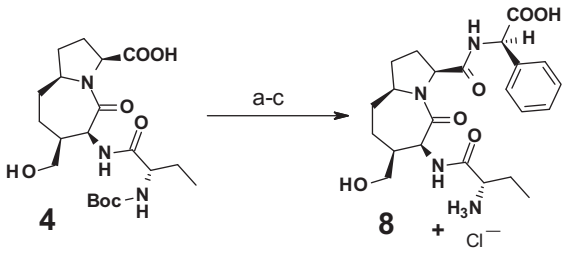


Figure 1. Monomeric and dimeric Smac mimetics.



a) Ph_2CHNH_2.HCl, EDC.HCl, HOBt, DIPEA, dry CH_2Cl_2, N_2, rt, 24hrs, 51%; b) succinic anhydride, cat. DMAP, DIPEA, dry CH_2Cl_2, N_2, rt, 20hrs, 85%; c) neat HCOOH, rt, 3hrs, 75%; d) as in a), 53%; e) as in c), quantitative.

Scheme 1. Synthesis of 4-connected Smac-linker carboxylate constructs **6** and **7**.



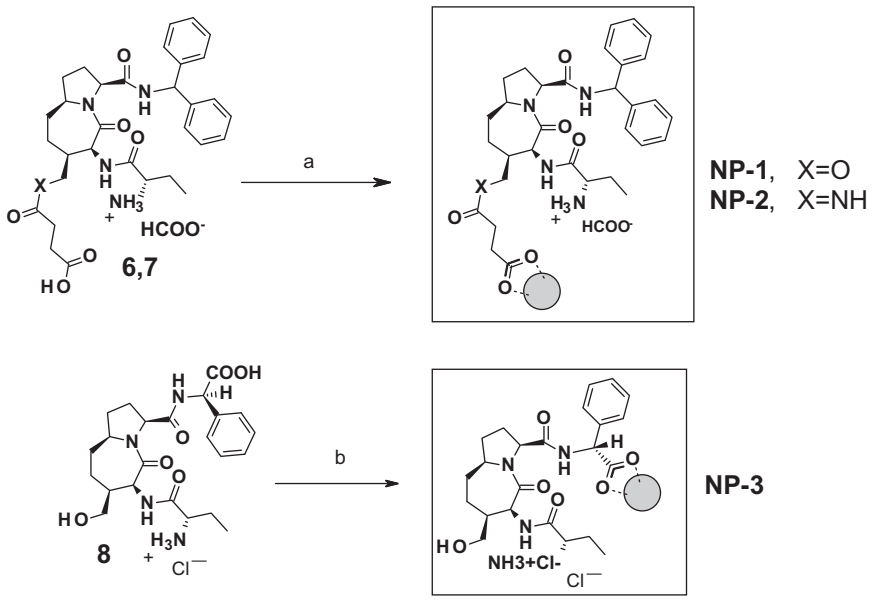
a) (R)-PheGlyOMe, EDC.HCl, HOBt, DIPEA, dry CH_2Cl_2, N_2, rt, 24hrs, 48%; b) 3N HCl in MeOH, rt, 20hrs, 95%; c) LiOH, H2O/1,4-dioxane, rt, 3hrs, then 2N aq. HCl, quantitative.

Scheme 2. Synthesis of C terminus-connected Smac-linker carboxylate construct **8**.

BIR3 affinity is also preserved when the C terminus diphenylmethyl amide of Smac mimetic **1** is replaced by an (R)-phenylglycine amide. N-protected hydroxyacid **4** was used to prepare in good yields the C terminus-connected Smac-linker carboxylate construct **8** (Scheme 2).

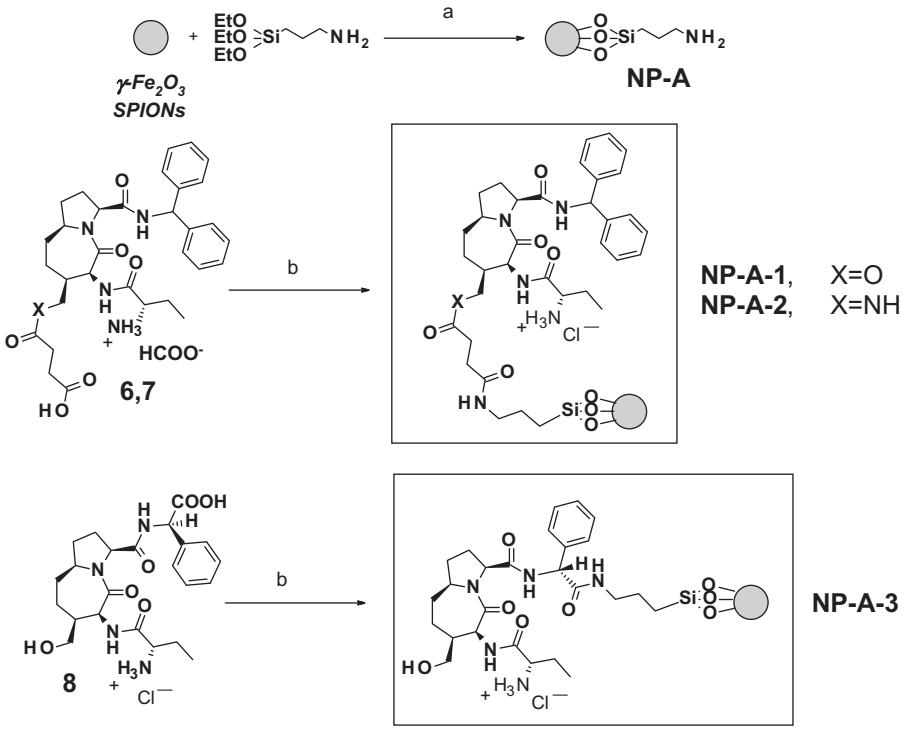
Smac-linker constructs **6**–**8** and all previously unreported intermediates were analytically and spectroscopically characterized (LC–MS, ^1H and ^{13}C NMR).

The carboxy group of Smac-linker constructs **6**–**8** is used to non-covalently adsorb the compounds onto surface-exposed OH



a) Naked SPIONs, H_2O, sonication, 60°C, 4hrs, loading 0.272 mmol/g_{SPION} (**NP-1**), 0.485 mmol/g_{SPION} (**NP-2**);
b) Naked SPIONs, dry toluene, sonication, 60°C, 4hrs, loading 0.168 mmol/g_{SPION}.

Scheme 3. Synthesis of non-covalent SPION-Smac mimetic nano-conjugates **NP-1/3**.



a) Naked SPIONs, dry toluene, N_2, sonication, 60°C, 4hrs, loading 0.836 mmol/g_{SPION} ; b) **NP-A**, EDC.HCl, dry CH_3CN, N_2, sonication, 60°C, 4hrs, loading 0.167 mmol/g_{SPION}(**NP-A-1**), 0.308 mmol/g_{SPION}(**NP-A-2**), 0.718 mmol/g_{SPION}(**NP-A-3**).

Scheme 4. Synthesis of covalent SPION-Smac mimetic nano-conjugates **NP-A-1/3**.

groups of commercial SPIONs. Non-covalent nano-conjugates **NP-1/3** are obtained with moderate to good Smac mimetic loadings (Scheme 3), as judged by elemental analysis.

Commercially available- maghemite γ-Fe_2O_3 SPIONs may be reacted with 3-aminopropyl triethoxysilane (APTES) to give the high loading SPION-APTES-NH_2 construct **NP-A** (Scheme 4). **NP-A** is then covalently coupled with the carboxy group of Smac-linker constructs **6**–**8**. Covalent nano-conjugates **NP-A-1/3** are obtained with moderate to good Smac loadings (Scheme 4) in unoptimized reaction conditions.

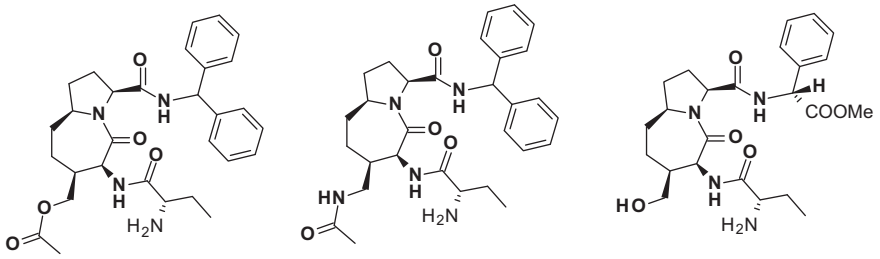


Figure 2. Reference Smac mimetics.

Non-covalent and covalent SPION-Smac mimetic nano-conjugates **NP-1/3** and **NP-A-1/3** were analytically and spectroscopically characterized (FT-IR, elemental analysis). Single reaction runs for each nanoconjugate often lead to good loadings (0.272–0.718 mmol/g_{SPION}; **NP-1**, **NP-2**, **NP-A-2**, **NP-A-3**). Observed moderate loadings (<0.200 mmol/g_{SPION}) could be improved by repeating the synthetic procedure for **NP-3** and **NP-A-1**.

The affinity of the six SPION-Smac mimetic nano-conjugates for mono-functional (BIR3 domains from X-linked IAP/XIAP, and from cellular IAPs/cIAP1 and cIAP2) and bi-functional IAP constructs (linker-BIR2-BIR3 multi-domain region from XIAP) was measured in a *cell-free* assay format. Standard reference compounds 4-acetoxymethyl/**9**, 4-acetamidomethyl/**10** and C terminus (*R*)-phenylglycinamide methyl ester/**11** (Fig. 2) were prepared in solution as previously described.[11a] They were biologically tested as soluble analogues respectively of non covalent/covalent SPION-Smac mimetic nano-conjugate pairs **NP-1/A-1** (**9**), **NP-2/A-2** (**10**) and **NP-3/A-3** (**11**).

The IC_{50} values determined for ABD-based Smac mimetics **9**–**11** and SPION-Smac mimetic non-covalent (**NP-1/3**) and covalent (**NP-A-1/3**) nano-conjugates are listed in Table 1.

SPION-Smac mimetic nano-conjugates generally maintain the nanomolar binding affinity of reference compounds **9**–**11** for single BIR3 domains from IAPs. Both non-covalent adsorption and covalent drafting of Smac mimetics onto SPIONs are compatible with IAP binding (Table 1). For example, the non-covalent conjugate **NP-1** and the reference standard **9** show similar binding strength in presence of BIR3 from XIAP and L-BIR2-BIR3 from XIAP; and the same behavior is shown by covalent conjugate **NP-A-3** and reference standard **11** (Table 1). Loading may influence affinity, as **NP-3** and **NP-A-1** (loading <0.2 mmol/g_{SPION}) show extremely reduced affinity for IAP proteins. Unfortunately, there is no evidence of a dimer-like binding/increase of affinity (expected IC_{50} < 10 nM for dimer-like compounds) for any nano-conjugate against the bifunctional L-BIR2-BIR3 construct from XIAP.

The cytotoxicity of cell free-active **NP-1/2** and **NP-A-2/3** nano-conjugates was tested on three human tumor cell lines (breast cancer, MDA-MB-231, ovarian carcinoma, IGROV-1, cervical cancer, HeLa cells). The nano-conjugates were generally inactive against tumor cells, with slight signs of cytotoxicity at the highest concentrations (≈25 μM). **NP-1/2** and **NP-A-2/3** show monomer-like cell free binding to IAP constructs, and reference monomers **9**–**11** show moderate cytotoxicity (IC_{50} between 10 and 25 μM against MDA-MB-231 cells). Thus, the weak cytotoxicity of nano-conjugates is likely due to a surprising lack of multimeric behavior that prevents their expected, dimer-like interaction with multiple bifunctional L-BIR2-BIR3 construct from XIAP.

Table 1
Binding affinity on BIR domains, IC_{50} (nM)

Compd	BIR3 XIAP	BIR3 cIAP1	BIR3 cIAP2	L-BIR2-3 XIAP
9	400	NT[a]	NT	320
NP-1	360	38	78	300
NP-A-1	>2000	>2000	>2000	>2000
10	330	NT	NT	190
NP-2	560	150	62	240
NP-A-2	230	38	NT	120
11	760	160	180	190
NP-3	>2000	1400	>2000	410
NP-A-3	580	270	180	410

[a] Not tested.

In summary, we report here the synthesis, the spectroscopical and biological characterization of non-covalent (**NP-1/3**) and covalent (**NP-A-1/3**) SPION-Smac mimetic nano-conjugates as IAP-targeted pro-apoptotic agents. SPION-Smac mimetic nano-conjugates behave as their soluble monomer analogues, retaining monomer-like cell free binding affinity for IAP targets, while being almost inactive in cellular assays. Further efforts will aim to achieve cellular and in vivo activity for SPION-Smac mimetic nano-conjugates in suitable cellular apoptosis/oncology models. Namely, we will vary the connection between Smac mimetics and SPIONs (chemical bond, linker/spacer length, hydro/lipophilicity, etc.), and we will decorate SPIONs with more potent monomeric and dimeric Smac mimetics.

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Supplementary data

Supplementary data (experimental procedures for the synthesis in solution of Smac mimetics **6**–**8** and **11**. LC–MS and NMR characterization of Smac mimetics **6**–**8** and **11**. Experimental procedures for the preparation of non-covalent (**NP-1/3**) and covalent (**NP-A-1/3**) SPION-Smac mimetic nano-conjugates. Elemental analysis and FT-IR of **NP-1/3** and **NP-A-1/3**. Experimental procedures for cell-free testing/binding affinity determination of soluble Smac mimetics and SPION-Smac mimetic nano-conjugates in presence of BIR domains/constructs from IAPs. Experimental procedures for cellular cytotoxicity testing of SPION-Smac mimetic nano-conjugates against human tumor cell lines) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.048.

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