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Evaluation of Di-Sansalvamide A derivatives: Synthesis, structure-activity relationship, and mechanism of action

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Abstract

Described is the SAR of 18 Di-Sansalvamide A derivatives and the mechanism of action of the most potent compound. We show that this scaffold is a promising lead in the development of novel cancer therapeutics because it is: cytotoxic at nanomolar potency, inhibits a well-established oncogenic target (Hsp90), and does not share structural motifs with current drugs on the market.

Natural products often provide lead structures for the development of new drugs. These novel structures play critical roles in the development of new therapeutic small molecules that target unique biological pathways. Sansalvamide A (San A), a cyclic pentapeptide, is one such natural product. It was isolated from a marine fungus (*Fusarium ssp.*), and exhibits anti-tumor activity at micromolar potency.¹ Dimerization of active molecules to produce potent new structures is a common motif utilized both in nature as well as by medicinal chemists.^{2, 3} Herein we describe the synthesis of 18 new dimerized San A molecules, the structure-activity relationship (SAR) of 21 of these Di-Sansalvamide A (Di-San A) decapeptide derivatives (where 3 were reported previously) and we outline their proposed mode of action in cancer cells. Our earlier report describes the 1nM IC₅₀ that this scaffold exhibited against pancreatic cancer cell lines.⁴ Given that it is structurally unrelated to current drugs on the market, it was important to determine the SAR and mechanism of action of this molecule in order to evaluate its potential as a therapeutic. Through comparison of 21 compounds' cytotoxicity, we show that one compound is extremely potent. We then tested this potent scaffold in several mechanistic assays and found that it derives its cytotoxic behavior, at least in part, by inhibiting the interaction between heat shock protein 90 (Hsp90)¹ and a C-terminal domain client protein, inositol hexakisphosphate kinsase-2 (IP6K2). Given that Hsp90 is an established oncogenic target, inhibiting this interaction makes Di-San A an excellent potential lead as a chemotherapeutic.

Peptides are sometimes considered poor drugs due to their low solubility and rapid degradation inside the cell. Linear peptides require large numbers of amino acids in order to generate a defined 3-D structure (alpha helices, beta sheets, etc), which leads to large molecule weights (>1500) and makes them relatively insoluble. In contrast, cyclic peptides use only a few amino acids to generate a defined 3-D structure because the ring locks these residues into a rigid conformation. Thus with only a few residues, these cyclic peptides have an inflexible 3-D structure, leading to small molecular weights and relatively soluble molecules compared to a structurally defined linear peptide that would inhibit the same protein-protein interaction. In addition to having fewer solubility issues, cyclic peptides are usually degraded slower than

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Supporting Information Available: General experimental procedures, protocols, and NMR and mass spectral data for compounds are available free of charge on the Internet at <http://pubs.acs.org>.

linear peptides.⁵ Several key cyclic peptide-based drugs being used to treat disease include: Cyclosporin A (MW=1185), an 11 amino acid multibillion dollar drug used to suppress the immune system after organ transplants,⁶ Casopfungin (MW=1093), an antifungal compound, Vancomycin (MW=1431), an antibiotic, Fuzeon (MW= 4492), an anti-HIV molecule, and Aplidine (MW=1096), an 8 amino acid peptide-based anticancer agent.⁷ Thus, relatively large peptides are successfully used to treat diseases, setting excellent precedence for Di-San A drug development (MW= 1170).

Of the 18 compounds synthesized we used a solution phase method⁴ to generate six and a solid-phase method to produce twelve compounds (supplementary data Figure S1). The solution phase molecules were synthesized using the same process as described for the 5 initial derivatives.⁴ This is our first report where we use the solid phase approach, and it allowed for a faster synthesis of the linear decapeptide than the solution phase method. Starting with Phe chlorotrityl resin, sequentially coupling Fmoc protected amino acids using HOBt (3 equivs) and DIC (6 equivs) in 0.2M DMF and deprotecting the amine of the newly coupled amino acids using 20% piperidine and 80% DMF generated the resin bound linear decapeptide. Cleavage from the resin was accomplished using trifluoroethanol (TFE) and DCM in a 1:1 ratio for 24 hours. After complete removal of the TFE (it hinders cyclization) followed by confirmation of each linear decapeptide via LCMS, cyclization was accomplished using our standard cyclization conditions.⁸ These synthesis conditions generated a total of 12 compounds, all in reasonable yields (10–40%).

Our initial lead, **1**, was comprised of 10 amino acids with hydrophobic side chains and had a total of 4 D-amino acids (D-aas), located at positions II and III in a C-2 symmetrical fashion (Figure 1). Further, it has been shown that in order to ensure entry into cells, molecules need to maintain their hydrophobic character, which is one reason Cyclosporin A is so successful. Thus, we maintained the hydrophobic residues, while making substitutions that would allow us to investigate the SAR for these compounds. To this end, 18 compounds were synthesized with substitution of D- and L-amino acids and/or side chains were modified at specific positions designed to explore their effect on cytotoxicity. In order to explore how the rotation of 2 successive D-aas at other positions around the peptide backbone affected the cytotoxicity of the molecule, we synthesized seven compounds, **2**, **3**, **4**, **5**, **6**, **7**, and **8**, where only one of these has been previously reported (compound **5**). These seven compounds are all C-2 symmetrical and contain consecutive D-aas that are rotated around the ring. Rotation of a single D-amino acid around the macrocycle generated compounds **9**, **10**, **11**, and **12**, where only compound **11** has been previously reported. Compounds **13**, **14**, and **15** were synthesized as C-2 symmetrical molecules where more than 2 positions incorporated D-aas. Finally, we produced six new non-C-2 symmetrical derivatives where 5–7 D-aas were placed around the ring to yield compounds **16**, **17**, **18**, **19**, **20**, and **21**. Thus, this hydrophobic yet diverse series of structures allowed us to explore how the specific placement of the key amino acids from compound **1** affected potency when placed in other positions. All 21 compounds were tested against 2 cancer cell lines: HCT-116 colon cancer and PL45 pancreatic cancer (Figure 1). At 5 μ M, only **1** showed significant potency in both cell lines, with 99% growth inhibition values. The remaining structural variations did not show the same level of cytotoxicity observed with **1** indicating that potency is unique to this compound.⁹

Given that **1** is extremely potent (IC₅₀ of 1–20 nM against pancreatic cancer cells)⁴ it was important to discover its mechanism of action. We synthesized compound **1** with a biotinylated tag (Figure 2a, compound **1-IV tag**). The biotinylated compound was incubated with HCT-116 cell lysate, whereupon neutravidin-bound agarose beads were added to immobilize the compound along with the bound target protein(s). The beads were washed ten times to remove non-specifically bound proteins, followed by elution of proteins using sample buffer. The eluted proteins were run on an SDS-PAGE gel and visualized with silver stain (Figure 2b).

Protein sequencing of the entire lane 2 was performed using a nano-LC/MS/MS followed by searching the NCBI Eukaryotic database and peptide identification using fingerprinting software. Silver stain revealed nine protein bands in lane 2, and upon sequencing the entire lane, only 2 proteins were found that are associated with oncogenic pathways (supplementary data shows full gel, Figure S2). The most prominent protein pulled down was visualized as two bands at 90–95 kDa: Hsp90 (alpha and beta). Seven other major bands appeared between 40 and 250 kDa. Three bands were myosin, one band was lamin, another was Hsp70, two were alpha and beta tubulin, and the last was actin. It is well-established that myosin, tubulin, and actin are commonly pulled down due to their hydrophobicity. Further, lamins are proteins that are a large component of the nuclear lamina. The only two proteins that are connected to an oncogenic pathway are Hsp70 and Hsp90. Thus it appears that these two proteins are involved in the cytotoxic mechanism of compound **1**. Further, comparison of the two bands (alpha and beta Hsp90) at 90–95 kDa in lane 2 to the negative control (lane 3, the PEGylated biotin linker alone or lane 4, DMSO) suggests that the interaction between Hsp90 and Di-San A is specific. This is also true for the Hsp70 band. Given that Hsp90 and Hsp70 are the only proteins pulled down that are associated with an oncogenic pathway, we examined the interaction between Di-San A, Hsp90, and Hsp70 more closely.

Hsp90 plays an important role in cancer cell growth because it functions as a molecular chaperone for folding, assembling, and stabilizing oncogenic proteins.¹⁰ It has 3 distinct regions: the N-terminal, the middle, and the C-terminal domains. It exists as a dimer in which the subunits are constitutively associated via the C-terminal domain.¹¹ It has over 100 identified client proteins, many of which are involved in cell signaling.¹⁰ Interestingly, Hsp90 is up-regulated in the majority of cancers,¹² and inhibiting the function of Hsp90 affects multiple oncogenic pathways that are involved in cancer cell growth and programmed cell death.^{13–15} Given that the efficacy of target-specific anti-cancer drugs may decrease or even be lost over time due to the high epigenetic variation within cancer cells, blocking a protein that affects numerous cancer-related pathways, such as Hsp90, can be an effective and efficient means of treating drug-resistant cancers.^{13–15} Less is known about Hsp70's involvement in oncogenic pathways, however, Hsp70 is known to facilitate Hsp90's protein folding mechanism (Figure 5). Thus, additional mechanistic insights are needed before a conclusion can be drawn regarding the role of Hsp70/Hsp90 and Di-San A.

To begin to map the binding site of Di-San A on Hsp90, pull downs were performed against the N-terminal, middle, C-terminal and middle-C domains of the mammalian variant of Hsp90 (Figure 3). The Hsp90 domain variances were expressed in GST-fusion vectors, purified, and used in pull-down assays with **I-IV tag** (Figure 2a). **I-IV tag** not surprisingly bound to full length Hsp90 (Figure 3, lane 5). There was no marginally detectable interaction between **I-IV tag** and the N-terminal, middle, or C-terminal domains (lanes 1–3), however, there was an optimal affinity for the middle-C construct (lane 4). These data indicate that compound **1** binds preferentially to the middle-C domain of Hsp90.

There are currently considered to be two classes of Hsp90 inhibitors: the N-terminal inhibitors [e.g. 17-allylamino, 17-demethoxygeldanamycin (17-AAG)] that target the ATP pocket, and a relatively weak C-terminal inhibitor, novobiocin.¹⁶ 17-AAG is currently in phase I and II clinical trials.^{13, 18, 19} There are currently no clinical trials involving molecules that inhibit the C-terminal binding proteins. Anticipating that the cytotoxic effect of Di-San A might be a direct result of its ability to inhibit the interaction between Hsp90 and cell signaling events at the C-terminus, we directly probed the effect of our small molecule on the binding interaction between Hsp90 and IP6K2, a C-terminal binding protein.¹⁶

We monitored the binding between IP6K2 and Hsp90 via an in vitro binding assay (figure 4a). Binding was monitored using Native Hsp90, purified from HeLa cells (Stressgen), along with

recombinant GST-tagged IP6K2 (GST-IP6K2) in the presence of increasing concentrations of Di-San A (0–100 nM) in separate reactions. Immobilized Glutathione agarose beads were added to each reaction, followed by three washes of the beads to eliminate nonspecific binding. The percent of Hsp90 bound to GST-IP6K2 was analyzed using western blots. We found that **1** inhibits the binding of the C-terminal interacting protein, IP6K2. In contrast, 17-AAG does not affect the binding of IP6K2.¹⁶ To determine if the effect of **1** could also be seen *in-vivo* we performed cell based-assays. We compared the effect of cell survival in the presence of **1** in wild type (control) and IP6K2 over-expressed (K2 O/E) cells using stable tetracycline inducible Myc-IP6K2 HEK293 constructs. The K2 O/E cells were treated with 1 µg/ml of tetracycline for 24hr whereas the control was incubated with vehicle alone. After tetracycline induction and vehicle treatment of the K2 O/E and control cells respectively, cells were treated with DMSO (no drug) or 25 nM of **1** for 48 hrs, and viability was determined using a CCK-8 assay. When no drug is present, IP6K2 over-expression only moderately affects cell survival, (Figure 4b, 3% change when comparing control, white bar, set at 100% to K2-O/E, grey bar, 97%). However, in the presence of **1**, cell survival is substantially decreased in cells that over-express IP6K2 compared to wild type cells (~ 9 fold decrease, 3% versus 27% change). Thus, Di-San A induces cell death, at least in part, through the Hsp90-IP6K2 pathway. By disrupting this Hsp90-IP6K2 interaction, Di-San A provides a new approach to chemotherapeutics.

Thus, these data show that Di-San A is a molecule that has a mechanism of action unique from the most universal Hsp90 inhibitor, 17-AAG. It is 50,000 fold more potent than the current molecule of choice used to inhibit C-terminal client proteins (novobiocin) where the EC₅₀s are ~100 µM¹⁶ and ~2 nM for novobiocin and **1**, respectively. Although it is too early to make a conclusion about the mechanism of action, our data is consistent with our molecule inhibiting Hsp90's protein folding cycle at a unique and earlier stage than 17-AAG (Figure 5).

In summary, we outline the synthesis of 18 new compounds, explore the SAR of these derivatives, and determine compound **1**'s mechanism of action. We conclude that **1**'s potency is due, at least in part, to its inhibition of Hsp90. It binds selectively to the Middle-C domain of Hsp90, and inhibits the ability of IP6K2 to bind to Hsp90 at very low concentrations. Mechanistic studies are ongoing and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SAR	structure-activity relationship
Hsp90	heat shock protein 90
IP6K2	inositol hexakisphosphate kinase-2
TFE	trifluoroethanol
17-AAG	17-allylamino-17-demethoxygeldanamycin

References

1. Belofsky GN, Jensen PR, Fenical W. Sansalvamide: A new cytotoxic cyclic depsipeptide produced by a marine fungus of the genus *Fusarium*. *Tetrahedron Lett* 1999;40:2913–2916.
2. Hadden MK, Blagg BSJ. Dimeric approaches to anti-cancer chemotherapeutics. *Anti-Cancer Agents in Med Chem* 2008;8:807–816.
3. Yang ST, Kim JI, Shin SY. Effect of dimerization of a β -turn antimicrobial peptide, PST13-RK, on antimicrobial activity and mammalian cell toxicity. *Biotech Lett* 2009;31:233–237.
4. Davis MR, Styers TJ, Rodriguez RA, Pan PS, Vasko RC, McAlpine SR. Synthesis and cytotoxicity of a new class of potent decapeptide macrocycles. *Org Lett* 2008;10:177–180. [PubMed: 18095697]
5. Singh EK, Sellers RP, Alexander LD, McAlpine SR. Conformational based design of macrocycles as antitumor agents. *Current opinion in Drug Discovery and Development* 2008;11:544–552.
6. Starzl TE, Klintmalm GB, Porter KA, Iwatsuki S, Schroter GP. Liver transplantation with use of cyclosporin A and prednisone. *New England Journal of Medicine* 1981;305:266–269. [PubMed: 7017414]
7. Le Tourneau C, Raymond E, Faivre S. Reports of Clinical Benefit of Plitidepsin (Aplidine), a New Marine-Derived Anticancer Agent, in Patients With Advanced Medullary Thyroid Carcinoma. *Curr Pharm Des* 2007;13:3427–3429. [PubMed: 18045196]
8. Styers TJ, Rodriguez RA, Pan PS, McAlpine SR. High-yielding macrocyclization conditions used in the synthesis of novel Sansalvamide A derivatives. *Tetrahedron Lett* 2006;47:515–517.
9. Heller M, Sukopp M, Tsomaia N, John M, Mierke DF, Reif B, Kessler H. The conformation of cyclo (-Dpro-ala-) as a model for cyclic pentapeptides of the DL type. *J Am Chem Soc* 2006;128:13806–13814. [PubMed: 17044709]
10. Neckers L. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol Med* 2002;8:S55–S61. [PubMed: 11927289]
11. Nemoto T, Ohara-Nemoto Y, Ota M, Takagi T, Yokoyama K. Mechanism of dimer formation of the 90-kDa heat shock protein. *Eur J Biochem* 1995;233:1–8. [PubMed: 7588731]
12. Matei D, Satpathy M, Cao L, Lai YK, Nakshatri H, Donner DB. The platelet-derived growth factor receptor alpha is destabilized by geldanamycins in cancer cells. *J Biol Chem* 2007;282:445–453. [PubMed: 17079230]
13. Usmani SZ, Bona R, Li ZH. 17 AAG for HSP90 Inhibition in Cancer - From Bench to Bedside. *Curr Mol Med* 2009;9:654–664. [PubMed: 19601813]
14. Koga F, Kihara K, Neckers L. Inhibition of Cancer Invasion and Metastasis by Targeting the Molecular Chaperone Heat-shock Protein 90. *Anticancer Res* 2009;29:797–807. [PubMed: 19414312]
15. Barginear MF, Van Poznak C, Rosen N, Miodi S, Hudis CA, Budman DR. The Heat Shock Protein 90 Chaperone Complex: An Evolving Therapeutic Target. *Curr Cancer Drug Targets* 2008;8:522–535. [PubMed: 18781898]
16. Chakraborty A, Koldobskiy MA, Sixt KM, Juluri K, Mustafa AK, Snowman AM, van Rossum DB, Patterson RL, Snyder SH. Hsp90 regulates cell survival via inositol hexakisphosphate kinase-2. *PNAS* 2008;105:1134–1139. [PubMed: 18195352]
17. Donnelly A, Blagg BSJ. Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. *Curr Med Chem* 2008;15:2702–2717. [PubMed: 18991631]
18. Heath EI, Hillman DW, Vaishampayan U, Sheng S, Sarkar F, Harper F, Gaskins M, Pitot HC, Tan W, Ivy SP, Pili R, Carducci MA, Erlichman C, Liu G. A phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer. *Clin Cancer Res* 2008;14:7940–7946. [PubMed: 19047126]
19. Solit DB, Osman I, Polsky D, Panageas KS, Daud A, Goydos JS, Teitcher J, Wolchok JD, Germino FJ, Krown SE, Coit D, Rosen N, Chapman PB. Phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with metastatic melanoma. *Clin Cancer Res* 2008;14:8302–8307. [PubMed: 19088048]

Figure 1.
Structures and cytotoxicity of 21 Di-San A molecules.

Figure 2.

a) 1 with biotinylated tag and b) Pull-down assay results showing Hsp90 and Hsp70 band.

Figure 3.
Pull down of mammalian Hsp90 domains.

Figure 4.

a) Binding assay with **1**, Hsp90 and IP6K2. **b)** cytotoxicity of **1** in IP6K2 over expressed cell line (K2-O/E, gray bar compared to wild-type control (Con, white bar). **, $p < 0.01$, ***, $p < 0.001$.

Figure 5.
Schematic of Hsp90 protein folding cycle.