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Biomolecular mechanism of San A-amide: an Allosteric Modulator of Hsp90

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Abstract: Here in we show that San A-amide is a structurally unique molecule that appears to allosterically modulate C-terminal client proteins of Hsp90, while binding to the N-M domain. We show that San A-amide specifically inhibits client proteins that bind to the C-terminus, while having no effect on a client protein that binds to the middle domain. Furthermore the cytotoxicity of San A-amide appears to be diminish upon Hsp90 up regulation. Thus, San A-amide influences a particular subset of cancer-related pathways by appearing to allosterically disrupt binding of Hsp90 to C-terminal client proteins, while binding at the interface of the N-M domain. This unique mechanism suggests that San A-amide is a promising lead in the development of new cancer therapies.

Keywords: San A-amide, Hsp90, Allosteric, cancer therapy

1. Introduction

There are serious limitations on molecules currently available to treat drug-resistant colon cancer. The natural product Sansalvamide A and Sansalvamide A peptide (San A-amide) derivatives (Figure 1a) possess unusual structures that share no homology to known anti-cancer agents and they inhibit growth of drug-resistant colon (HCT-116) cancer cell lines.[1,2] Our work has focused on designing new San A-amide derivatives that inhibit growth of drug resistant cancers. These scaffolds represent innovative structures that are structurally unique from current drugs on the market. To date, no mechanistic studies have explained the cytotoxicity of San A-amide in mammalian cells.[3] *In vitro* binding assays against viral encoded topoisomerase 1 (topo 1) suggested this may be a possible mechanism of San A-amide, however, studies using mammalian topo 1 indicated that this was not the mechanism of action. Herein we show that San A amide binds to heat shock protein 90 (Hsp90) and in doing so, it blocks the binding of Hsp90 to a client protein inositol hexakisphosphate kinase-2 (IP6K2). In addition based upon computation using the program Serial Analysis of Gene Expression (SAGE) additional relevant Hsp90 client proteins were discovered, one of which, FKBP52, was also shown to be inhibited from binding to Hsp90 in the presence of San A-amide. Therefore, San A amide inhibits the binding of multiple client proteins of Hsp90, and this is likely the primary mechanism for San A-amide's ability to induce programmed cell death.

1.1 Determining Target of San A-amide

Employing pull-down assays with a biotinylated derivative of San A-amide, allowed us to indentify potential oncogenic protein target(s) that bound to this molecule. Our SAR data indicated that position 4 is not critical for cytotoxicity,[1,2] and thus it represented a logical location for the incorporation of a biotinylated linker into these molecules. San A-amide-Biotin was synthesized and used in pull-down assays with HCT-116 colon cancer cells (Figure1b; Figure 2). The biotinylated compound was incubated with cancer cell lysate, whereupon neutravidin bound agarose beads were added to immobilize the compound along with bound

target protein(s). The beads were washed to remove non-specifically bound proteins followed by elution of San A-amide-Biotin with SDS-PAGE sample buffer.

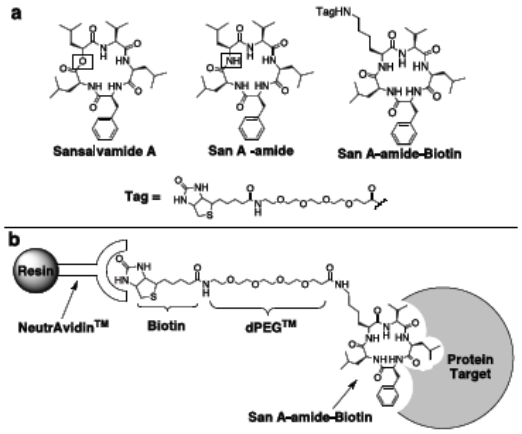

Figure 1. (a) The natural product Sansalvamide A and the derivative Sansalvamide A-amide (San A-amide) compound (1),and biotinylated San A-amide (San A-amide-Biotin). (b) Biotin pull-down assay method.

The eluted proteins were run on an SDS-PAGE gel and visualized with Coomassie blue. Subsequent sequencing of the most prominent band provided evidence that one band at 90 kDa is the primary protein isolated in this cell line. This protein was identified by nano-LC/MS/MS and data was searched against the NCBInr Eukaryotic database. The primary band on the gel was identified as the molecular chaperone Hsp90 (Figure 2). Comparison of the band at 90 kDa in lane 2 to the negative control (lane 3, the PEGylated biotin linker alone) showed that we specifically pulled down Hsp90 using San A-amide. Thus, San A-amide binds to a well-established oncogenic protein, Hsp90.

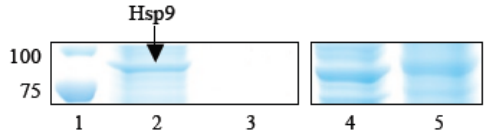

Figure 2. HCT-116 colon cancer cells, 10% SDS-page gel of bands isolated during pull-down assays. Lane 1 = MW marker 100 and 75kDa, 2 = San A-amide-Biotin, 3 = negative control (PEGlayted biotin linker), 4 and 5 = protein input for lanes 2 and 3 respectively.

1.2 Hsp90 is an Important Cancer Target

Hsp90 has over 100 identified client proteins, most of which are involved in signaling[4,5] and are up-regulated in the majority of cancers.[4,6] Inhibiting the function of Hsp90 affects multiple oncogenic substrates including those involved in cancer cell growth.[4,6] 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, inhibiting a protein that affects numerous cancer-related pathways, such as Hsp90, can be an effective and efficient means of treating drug-resistant cancers.[7]

There are currently two classes of inhibitors of Hsp90: the N-terminal inhibitors [e.g. 17-allylamino, 17 demethoxy geldanamycin (17-AAG)] which all target the ATP pocket, and a relatively weak C-terminal inhibitor, novobiocin.[7,8,9] The N-terminal domain is the binding site for all Hsp90 compounds currently in clinical trials. One client protein that binds to the C-terminus of Hsp90 is apoptosis inducing inositol hexakisphosphate kinase-2(IP6K2).[8] It has been shown that inhibiting the interaction between IP6K2 and Hsp90 elicits programmed cell death;[8] thus, disrupting this interaction may provide an approach to new chemotherapeutics.

1.3 IP6K2 Client of Hsp90 is a Potential Target for Chemotherapeutics

IP6K2's over-expression sensitizes cells to apoptotic agents, while depletion of IP6K2 by RNA interference prevents the apoptotic effects of several drugs.[8] Recently Snyder and co-workers demonstrated Hsp90 binds discriminatingly to IP6K2 and inhibits its catalytic activity.[8] Deletion mapping of Hsp90 revealed that IP6K2 binds preferentially to the C-terminus. Ablation of Hsp90 by RNA interference increases IP6K2 catalytic activity leading to apoptotic cell death. Novobiocin disrupts Hsp90-IP6K2 binding, whereupon free IP6K2 induces apoptosis in cells. In contrast, 17-allylamino, 17-demethoxygeldanamycin (17 AAG), a known N-terminal Hsp90 binder, has no inhibitory affect on the C-terminal client protein IP6K2 binding to Hsp90. Thus, IP6K2 mediates, at least in part, the cytotoxic actions of Novobiocin but not 17-AAG.

1.4 Ability of San A-amide to Disrupt Hsp90 IP6K2 Binding

To test the possibility that San A-amide influences Hsp90's C-terminal binding to client proteins, we performed an *in vitro* binding assay using recombinant 6x-histidine tagged IP6K2 (His-IP6K2) and native Hsp90 purified from HeLa cells. Our data (Figure 3a) shows that San A-amide disrupts the binding between IP6K2 and Hsp90 with an EC50 of ~1.4 uM. Thus, San A-amide binds to Hsp90 and inhibits the interaction between Hsp90 and IP6K2, and it does so at a significantly lower concentration than novobiocin (EC50 = ~100μM).[8] Furthermore, a similar binding assay involving Hsp90 and middle domain client protein Her2 in the presence of increasing concentrations of San A-amide showed no effect upon binding, suggesting that San A-amide is selective in client protein disruption (supporting information). In addition to biochemical assays, we performed cell based-assays and compared the effect of cell survival in the presence of San A-amide in HEK 293 cell lines (control) and IP6K2 over expressed (K2-O/E). Tet-inducible Myc-IP6K2 constructs were created for IP6K2 over expression. The control and K2-O/E were treated with San A-amide for 48 hours at 25 uM and viability was determined using a CCK-8 assay. When no drug is present, IP6K2 over-expression only moderately affects cell survival, (Figure 3b, 3% change, compare white bar set at 100% to grey bar, 97%). However, in the presence of 25 μM San A-amide, cell survival is substantially decreased in cells that over-express IP6K2 compared to wild type cells (~ 9 fold drop, 3% versus 27% change). Thus, San A-amide induces cell death, at least in part, through the Hsp90-IP6K2 pathway.

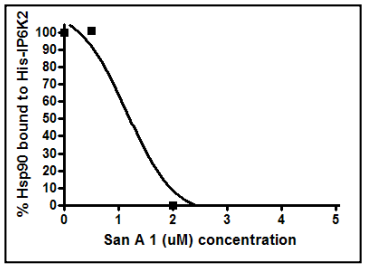

Figure 3a. *In-vitro* binding assay. His-IP6K2 immobilized on streptavidin resin incubated with native Hsp90 purified from HeLa cells. San A-amide inhibits the binding at and EC_{50} ~1.4uM.

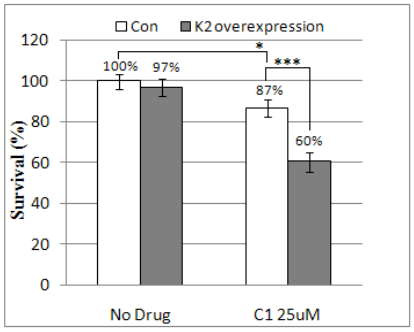

Figure 3b. Cytotoxicity of San-A amide in IP6K2 over expressed cell line (K2-O/E) compared to WT control (Con).

1.4 Using Serial Analysis of Gene Expression (SAGE) to identify additional clients of Hsp90

There are over 100 known clients proteins that interact with Hsp90 [4,5]. Thus in order to narrow down which of these client proteins may be relevant to the cytotoxicity caused by San A-amide in HCT116 colon cancer cells, the program Serial Analysis of Gene Expression (SAGE) was utilized. SAGE is a program that links to a database cataloging the mRNA expression profile of several different cancerous and normal cell lines[10]. Thus, through mRNA profiling, relative protein expression may be determined. Using SAGE, the mRNA profile of HCT116 colon cancer cells were directly compared to the mRNA profile of NC2 normal colon epithelial cells. The profiles were then filtered to determine which proteins were at least 2 fold higher in the HCT116 colon cancer cells versus the normal colon cells. Out of the 2 fold higher pool, proteins were filtered to identify which ones were either direct (D) or indirect (I) binders of Hsp90 (Figure 4). The purpose of these filters was to identify which client proteins of Hsp90 were selectively up regulated in colon cancer cells versus normal colon cells to identify selective cancer cell targets. One of the targets identified was the FK506 binding protein 4 (FKBP52). FKBP52 is a tetratricopeptide (TPR) motif containing protein that specifically binds the C-terminal MEEVD sequence of Hsp90. Thus, similar to IP6K2, FKBP52 is also a C-terminal client of Hsp90. In addition, inhibition of FKBP's binding to Hsp90 by perturbing TPR interactions has been linked to programmed cell death[11]. Indeed, it is thought that several FKBPs may be involved in multiple cell death mechanisms, one of which acts via the Bcl-2 pathway[12]. In order to test if San A-amide inhibited the binding between Hsp90 and FKPB52 a similar *in-vitro* binding assay was employed that elucidated the ability of

San A-amide to inhibit the binding between Hsp90 and IP6K2 (Figure 4b). San A-amide did indeed inhibit the binding between Hsp90 and FKPB52 at a similar concentration, EC_{50} ~1.0uM.

Cell Line	CL_Hct116; colon, WT p53, normal O2, carcinoma	
	vs.	
	B_NC2; colon, eipithelium, normal	
		D / I
Protein O / E	Hsp90A	D
in carcinom	AHSA1 Activator of Hsp90	D
	Hsp70 protein 8	D/I
	Hsp70 interacitive protein	D/I
	Ribosomal protein L30	I
	Ribosomal protein S11	I
	FK506 binding protein 4	I

Figure 4a. Table of data from SAGE listing proteins that are ≥ 2 fold over expressed in HCT116 colon cancer cells versus normal colon cells. (D) is direct interaction with Hsp90, (I) represents an indirect interaction with Hsp90.

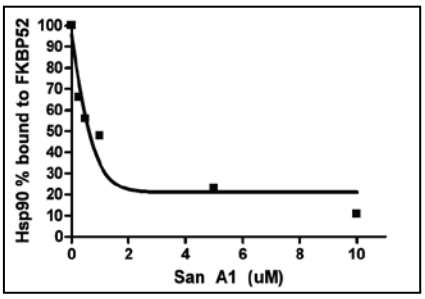

Figure 4b. *In-vitro* binding assay. GST-FKBP52 immobilized on glutathione resin incubated with native Hsp90 purified from HeLa cells. San A-amide inhibits the binding at and EC_{50} ~1.0uM.

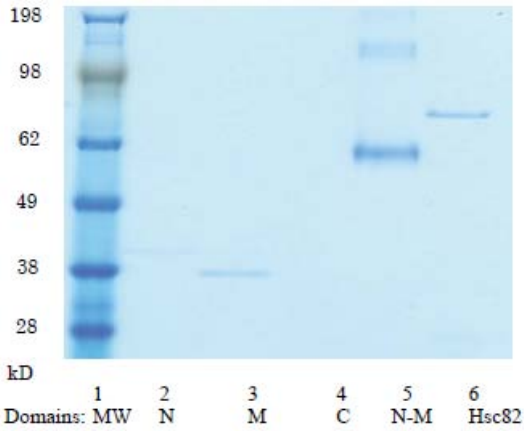

Figure 5: San A-amide pull down of Hsc82 full length and domains.

1.5 Domain of Hsp90 to which San A -amide Binds

To determine the binding site of San A-amide, pull downs were performed against the N-terminal, middle, C-terminal and N-middle domains of the yeast variant of Hsp90 (Hsc82, Figure 5). The Hsp90 domains were expressed in His-fusion vectors and purified. Pull-down assays with San A-amide-Biotin were run, and not surprisingly, San A-amide has a high affinity to full length Hsp90 (figure 4, lane 6). Interestingly, San A-amide has no affinity for the C-terminal domain (lane 4), but has optimal affinity for the N-middle construct (lane 5) and partial affinity for the middle domain.

1.6 Hsp90 up regulation experiments

In order to further explore the interaction of San A-amide with Hsp90 *in-vivo*, Hsp90 was up regulated in HCT116 cells followed by treatment with San A-amide to determine the effect upon cytotoxicity. As expected up regulation of Hsp90 ablated the cytotoxicity of San A-amide, suggesting that San A-amide does indeed bind to Hsp90 *in-vivo*.

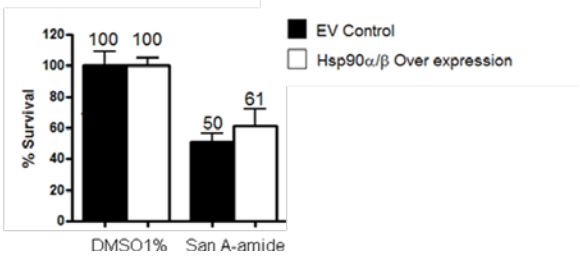

Figure 6: Myc-Hsp90 up regulation in HCT116 colon cancer cells followed by treatment with San A-amide at 50uM.

Conclusion

In summary, San A-amide is not only a valuable tool to study Hsp90 IP6K2 and FKBP52 interactions; it is a structurally unique molecule that allosterically modulates C-terminal client proteins of Hsp90 presumably by altering the Hsp90 conformational equilibrium. This unique mechanism suggests that San A-amide may influence a unique subset of other cancer-related pathways by allosterically or directly

disrupting binding of additional client proteins of Hsp90, thus providing a potentially efficient means of treating drug resistant cancers. Further, our data supports the development of a new cancer therapy approach: targeting a chaperone protein and modulating its oncogenic client proteins via a remote pathway. San A-amide's ability to inhibit Hsp90 IP6K2 and FKBP52 interactions within cells, along with its ability to interrupt other Hsp90 client proteins is under investigation.

Supporting Information: Full experimental procedures are available free of charge via the internet at http://pubs .acs.org.

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