A small molecule that preferentially binds the closed conformation of Hsp90

Leslie D. Alexander\textsuperscript{a, b}, James R. Partridge\textsuperscript{c}, David A. Agard\textsuperscript{c}, Shelli R. McAlpine\textsuperscript{a, *}

\textsuperscript{a}Department of Chemistry, University of New South Wales, Sydney NSW 2052, Australia
\textsuperscript{b}Department of Chemistry and Biochemistry, San Diego State University, 5500 Campanile Dr., San Diego, CA 92182, USA
\textsuperscript{c}Howard Hughes Medical Institute and the Department of Biochemistry and Biophysics, University of California, San Francisco, 600 16th Street, San Francisco, CA 94158, USA

A R T I C L E   I N F O

Article history:
Received 13 August 2011
Revised 22 September 2011
Accepted 22 September 2011
Available online 29 September 2011

Keywords:
Sansalvamide A
Hsp90
Fluorescein
Fluorescence polarization
Anticancer
Anisotropy

A B S T R A C T

Described is the synthesis of three different fluorescein-tagged derivatives of a macrocycle, and their binding affinity to heat shock protein 90 (Hsp90). Using fluorescence polarization anisotropy, we report the binding affinity of these fluorescein-labeled compounds to Hsp90 in its open state and ATP-dependent closed state. We show that the compounds demonstrate a conformation-dependent preference for binding to the closed state.

Published by Elsevier Ltd.

Molecules that bind to and inhibit the function of heat shock protein 90 (Hsp90) have proven to be excellent clinical candidates in oncogenic therapies.\textsuperscript{1-6} Hsp90 is an ATP-dependent chaperone protein that helps fold and regulate more than 200 substrate ‘client’ proteins that are involved in cell growth and signaling.\textsuperscript{4,7} Since pathway-specific inhibitors can be problematic in drug-resistant cancers, shutting down multiple pathways at once is a promising approach when developing new therapeutics. Given Hsp90’s ability to modulate many growth and cell signaling pathways simultaneously,\textsuperscript{5} this protein has become an attractive target in the field of cancer therapeutics.

Recently, we reported the mechanism of action of the cyclic pentapeptide Sansalvamide A-amide (Fig. 1, compound \textsuperscript{1}).\textsuperscript{9-11} The peptide structure is based on the natural product depsipeptide Sansalvamide A (San A) that was isolated by the Fenical group from a marine fungus of the genus Fusarium sp. and found to exhibit anticancer activity.\textsuperscript{12} We recently reported that San A-amide binds to and modulates Hsp90\textsuperscript{9-11} as does compound \textsuperscript{2}.\textsuperscript{10} Compound \textsuperscript{2} (Fig. 1) exhibited lower IC\textsubscript{50} values than compound \textsuperscript{1} and other San A-amide structures, and has been shown to modulate the binding between numerous client proteins and Hsp90.\textsuperscript{10} Thus, it was chosen as a tool for further investigation.

Hsp90 is a constitutive dimer that contains three domains per monomer: the N-terminal domain, which houses an ATP-binding site, the middle domain, and the C-terminal domain (known as N, M, and C, respectively).\textsuperscript{13} It functions in conjunction with several co-chaperones, progressing through a complex ATP-dependent conformational cycle (Fig. 2). Without ATP, Hsp90 maintains an open dimerized conformation (Fig. 2a). Upon ATP binding (2b), it converts to a stabilized closed and twisted conformation (2c), which is favorable for the subsequent hydrolysis of ATP. Upon hydrolysis of ATP Hsp90 forms a more compact ADP-bound state (2d). After ADP is released, Hsp90 reverts back to its open state (2a).\textsuperscript{14-16}

Prior to our work, two types of Hsp90 inhibitors had been reported: those that bind to the ATP-binding pocket in the N-domain, thus inhibiting Hsp90's function,\textsuperscript{17} and those that bind to the C-domain.\textsuperscript{18,19} All of the compounds currently in clinical trials bind to the N-domain and most are closely related to the natural product

\textsuperscript{9}Corresponding author. Tel.: +61 4 1672 8896; fax: +61 2 9385 6141.
E-mail address: s.mcalpine@unsw.edu.au (S.R. McAlpine).

\textsuperscript{19}0960-894X/$ - see front matter Published by Elsevier Ltd.

Figure 1. Sansalvamide A-amide and derivative.
Geldanamycin ($K_d \sim 1 \mu M$). Compounds that bind to the C-domain include the natural product antibiotic novobiocin and its derivatives. The advancement of novobiocin as a chemotherapeutic agent has been hampered by its high micromolar cytotoxic activity ($\sim 700 \mu M$) and poor binding affinity for Hsp90 (calculated $K_d$ in the millimolar range). Recent studies have reported novobiocin derivatives with low micromolar affinities for Hsp90, but to date no molecules that bind to the C-terminal site of Hsp90 have been tested in clinical trials. Unlike the current inhibitors, we have established that San A-amide and compound 2 bind to the N–M domain and allosterically modulate the C-domain client protein IP6K2, and co-chaperones FKBP52, and HOP.

In an effort to further explore San A-amide's unique mechanism of action we synthesized three fluorescein-tagged derivatives of compound 2 (Fig. 1). Using fluorescence polarization anisotropy, we determined that the presence of an ADP group of the lysine was removed, and fluorescein was coupled to the macrocycle using activated NHS-fluorescein and 8 equiv of DIC. The final fluorescein-tagged compounds were purified by reverse-phase HPLC.

We placed the tags at positions I, III, and IV of compound 2 (Fig. 3). We have established that the presence of an N-methyl-d-phenylalanine is an important motif in our potent molecules, thus we did not place a tag at position II as that would require replacing this residue. In addition, our SAR has indicated that the Cbz-protected lysine at position V is also important for biological activity hence we did not tag that position.

The tagged compounds were synthesized following a solid-phase synthesis and tagging method (Scheme 1). Beginning with pre-loaded 2-chlorotrityl-leucine resin, Fmoc-protected amino acids were subsequently coupled and deprotected until the desired linear pentapeptide was reached. A Boc-protected lysine was used in place of the amino acid at positions I, III, and IV, respectively. The peptide was cleaved from the resin in 50% trifluoroethanol in methylene chloride followed by cyclization using optimized macrocyclization conditions. After purification, the Boc-protecting group of the lysine was removed, and fluorescein was coupled to the macrocycle using activated NHS-fluorescein and 8 equiv of DIPEA. The final fluorescein-tagged compounds were purified by reverse-phase HPLC.

Using fluorescence polarization anisotropy, we determined that all three tagged derivatives of compound 2 bind effectively to Hsc82, the full-length yeast homolog of Hsp90, where their micromolar $K_d$S were reasonably similar to our cytotoxicity data for the non-tagged compounds (Fig. 4, and Table 1). In addition, we found that the placement of the tag was very important, where 2-T-III had a $K_d$ of 57 $\mu M$ for the open apo state, 2-T-I and 2-T-IV had $K_d$s almost 4- and 2-fold higher, respectively (Table 1).

In addition to testing the affinity of compound 2 for Hsc82 in the absence of nucleotide, we also examined compound binding to Hsc82 pre-incubated with the non-hydrolyzable ATP analog, AMPPNP. While AMPPNP only subtly shifts the equilibrium of human Hsp90 towards the closed state, the yeast homolog (Hsc82) is quantitatively shifted towards the closed conformation (Fig. 5c). Thus, using Hsc82 allows us to probe conformational discrimination of our molecules. All three compounds, 2-T-I, 2-T-III, and 2-T-IV prefer the closed conformation (conformation 5c) of Hsc82 over the open conformation (conformation 5a and Table 1). Previous data showed that compound 2 binds to a site on the N–M domain of Hsp90. The interface between N and M domains changes...
dramatically during the ATPase cycle. We had also already reported that allostERIC modulation of Hsp90 by San A-amide and compound 2 effectively inhibit the interactions between Hsp90 and C-terminal-interacting co-chaperones and client proteins. The observed preference of compound 2 for the closed conformation is consistent with compound 2’s binding to the N–M domain, and to its allostERIC inhibition of C-terminal client proteins and co-chaperones. That is, binding to a site that is sensitive to conformational rearrangements during the ATPase cycle of Hsp90, and having a preference for a particular Hsp90 conformation, may block client protein access to Hsp90’s C-terminus (Fig. 5). Although only 2-fold preference, this modification could be optimized by SAR, constituting an approach that is unique among known Hsp90 inhibitors, and making compound 2 a useful tool in exploring Hsp90 pathways.

In summary, we report the synthesis of fluorescein-tagged derivatives of a San A macrocycle, compound 2, which binds to Hsp90 at the N–M domain. We used these compounds in fluorescent assays to determine their preference for binding to Hsp90 in its open versus ATP-induced closed state and we show that compound 2 binds preferentially to the closed state. This is the first observation of preference of a non-nucleotide mimetic for the closed state. Based on these findings and our previous reports, compound 2 has an inhibitory profile unlike that of any Hsp90 inhibitor reported to date. It’s ability to bind to the N–M domain of Hsp90, allostERICally modulate C-terminal client proteins, and a preference for Hsp90’s ATP-driven closed state will expand the range of mechanistic tools useful in exploring Hsp90’s protein folding cycle. Further studies are currently underway to gain a deeper understanding of this interaction.

Acknowledgments

We thank the University of New South Wales Sydney for support of S.R.M., the Frasch Foundation (658-HF07) (support of D.A.A.), NIH 1R01CA137873 (support of S.R.M. and L.D.A.), NIH MIRT (support of L.D.A.). We thank HHMI in support of J.R.P. and D.A.A.

Supplementary data

Supplementary data (spectral data and experimental details for compounds and fluorescence polarization methods) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.096.

References and notes

22. See Supplementary data for experimental details.