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Design of allosteric stimulators of the HSP90 ATPase as novel anticancer leads

Ilda D'Annessa [a], Sara Sattin[b], Jiahui Tao[c], Marzia Pennati[d], Carlos Sànchez-Martìn[e], Elisabetta Moroni[f], Andrea Rasola[e], Nadia Zaffaroni[d], David A. Agard[c], Anna Bernardi[b] and Giorgio Colombo*[a]

Abstract: We rationally designed allosteric compounds that stimulate Hsp90 ATPase activity and show anticancer potencies in the low micromolar to nanomolar range. In parallel, we clarify their mode of action and developed a quantitative model that links the dynamic ligand-protein cross-talk to observed cellular and *in vitro* activities. Our results support the potential of using dynamics-based approaches to develop original mechanism-based cancer therapeutics.

Heat Shock Protein 90 (Hsp90) is a chaperone that controls the folding of more than 200 client proteins and constitutes a central node in many signaling pathways[1]. Overexpression and dysregulation of Hsp90 have been linked with cancer and neurodegeneration. It is thus not surprising that this chaperone has become an important drug-target: in principle its inhibition can result in the simultaneous degradation of multiple clients associated with different pathological hallmarks[2]. Inhibitors targeting the N-terminal ATP-binding site have been developed and some reached clinical trials [3]. However, they all showed problems due to the induction of the HSF1 mediated heat shock response and of Hsp70 overexpression, leading to drug resistance and toxicity[3a-c, 4].

A viable alternative to interfering with Hsp90 is represented by allosteric ligands, which perturb the chaperone by targeting sites alternative to the ATP-site. Novobiocin was shown to inhibit Hsp90 by binding the C-terminal region without inducing the heat shock response. Based on this observation, a number of new derivatives with promising activities against a variety of cancers were developed[5].

In this context, we have developed a method for the identification of allosteric pockets via the analysis of residue-pair distance fluctuations in the structural ensemble around the active state of the chaperone. Such analysis unveiled an allosteric pocket at 65Å from the active site, located at the MD:CTD border in a region in close proximity to the client binding site[6]. This facilitated the design of modulators showing promising anticancer activities and a novel molecular mechanism of perturbation of Hsp90 functions: the ligands in fact were synthesized and experimentally proved to be *activators* of closure kinetics and ATPase of the chaperone *in vitro*, to induce cancer cell death, and to interfere with client maturation[6b]. We developed a first Quantitative-Structure-Dynamics-Activity-Relationship (QSDAR) model correlating the structures of an initial set of modulators to observed activation effects[6c].

Here, based on this initial model, we report the rational design and synthesis of new allosteric ligands, reaching low micromolar to nanomolar anticancer activities, which support their potential in the development of anticancer therapeutics. On the computational side, we further develop a model to evaluate the potency of allosteric modulators by taking into account the dynamic cross-talk that exists between the protein and the ligand.

The conformational properties of the allosteric site were previously described [6-7]. Docking, MD refinement and analysis of the allosteric effects of the lead *O*-aryl rhamnoside benzofuran **1** revealed that the glycosidic moiety preferentially interacted with E477 and D503 of one protomer, while the propenyl substituent on the benzofuran could contact a hydrophobic pocket lined by R591 of the other protomer. This model led to redesigning **1** into **18** and **19** (Table 1) where the sugar moiety was replaced by an *N,N*-dimethyil-ethylamino or a propylamino group. Substitution of the propenyl group by a Cl atom was initially explored for synthetic convenience and proved to be beneficial in terms of solubility and activity,[6b] therefore it was maintained for **18** and **19**. Such modification resulted in an improved anticancer cytotoxicity of the ligands. A new round of MD simulations and allosteric coordination analysis supported the importance of interactions with E477 and D503 [6b, c]. Here, to establish structure-dynamics-activity relationships, in the absence of crystal structures difficult to obtain due to the flexibility of the system, we directed design efforts towards obtaining different amine substitutions on the phenyl-benzofuran scaffold. The structures of the compounds are reported in Table 1, representative examples of their best poses in the Hsp90 pocket are shown in Figure 1A, and the details of their synthesis are described in a previous report[8].

All compounds resulted in *activation* of ATP hydrolysis, with the activity depending on the nature of the substituent linked to the *O*-aryl moiety (Table 1). Compounds **20**-**23**, characterized by the presence of a bulky substituent on the amino group, display the lowest activity. The compounds **22** and **23** with an *N*-methyl piperazine group on the phenol side chain, stimulate ATPase to a degree comparable to that induced by the glycosidic

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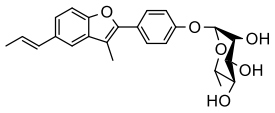
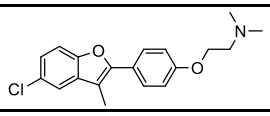
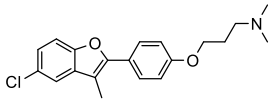
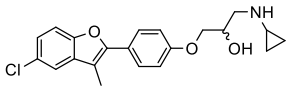
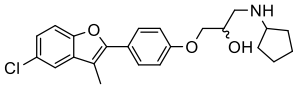
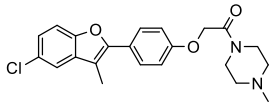
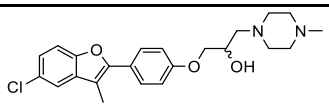
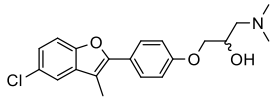
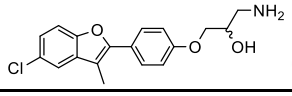
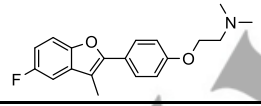
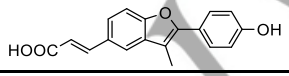
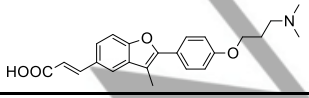
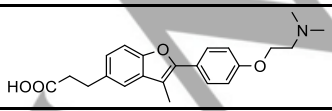
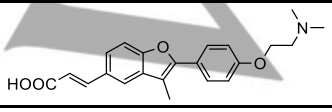
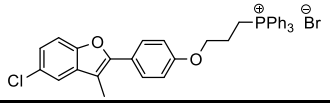
derivatives previously tested[6b,c], suggesting that the 6-membered ring actually mimics sugar substituents. Maximal stimulatory effects were observed for **24**, **25** and **26** indicating that minimizing the steric hindrance on the amine-pharmacophore, while maintaining its alkylation, is beneficial to ATPase stimulation, resulting in a 4-5 fold increase [6-7].

Table 1. Structures, stimulatory potencies and cytotoxic activities of designed compounds. ATPase stimulation and cytotoxicity were measured as described in Materials and Methods in Supporting Information.

	Compound	Normalized ATPase rate	Toxicity in STO (IC_{50} µM)
1		1.1[a]	57.0 ± 2.1[a]
18		4.8[a]	8.9 ± 1.1[a]
19		6[a]	9.9 ± 1.1[a]
20		1.5	16 ± 1
21		2.2	8.2 ± 2.7
22		1.9	11.7 ± 2.4
23		2.4	3.9 ± 1.8
24		4.1	8.6 ±2.1
25		4.5	22.1 ± 1.1
26		4.2	4.1 ± 0.4
27		1.4	not soluble
28		1.3	36 ± 6
29		1.17	> 75
30		0.98	> 100
31		-	0.57±0.04

[a] [a] Data reported in [6b]

Finally, compounds **28-30**, that carry a carboxylate group tethered to the benzofuran moiety, while conserving the *N,N*-dimethyl amino pharmacophore on the phenol ring showed a decreased stimulation compared to **24-26**. The structural reason for this effect is to be found in the fact that the hydrophobic region on protomer A occupied originally by the propenyl or chloride groups is lined by negatively charged residues E477 and D503 (blue in Figure 1): this determines unfavorable repulsive interactions with the carboxylates, which are detrimental to activity.

Given the importance of Hsp90 in determining cancer maintenance and development, we tested the antiproliferative activity of the new compounds in an experimental model of diffuse malignant peritoneal mesothelioma (STO), an uncommon and locally aggressive tumor, poorly responsive to conventional therapies [9].

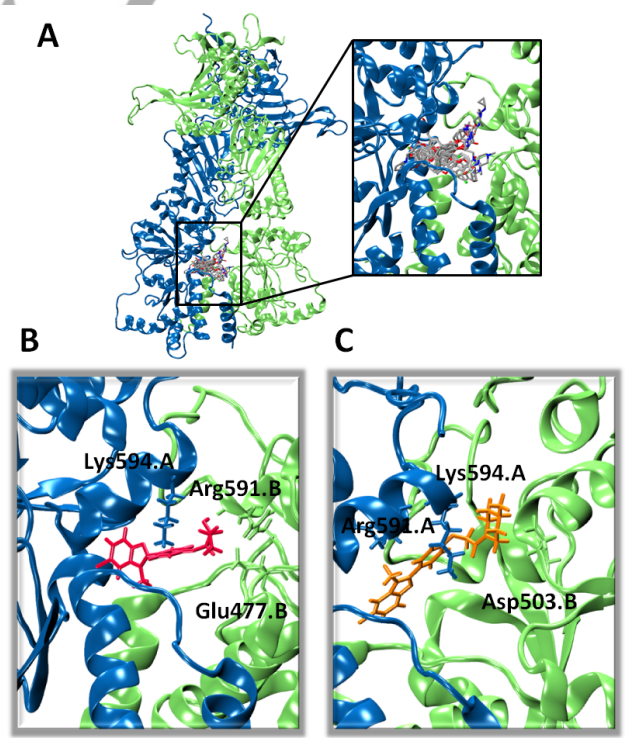


Figure 1. (**A**) Overall view and detailed superposition of the best pose for each compound in the allosteric site. Color code: Protomers A and B, light blue and green respectively; compounds: C=grey, O=red, N=blue, Cl=green, F=dark yellow. (**B**) Best pose obtained for compound **25**, in red, showing the highest ATPase stimulation. The side chains of residues interacting with **25** are shown with the color code reported in (**A**). (**C**) Same as (**B**), reporting compound **22** in orange, displaying lowest stimulation.

Cells were exposed to increasing concentrations (0.01-100 µM) of each compound for 72 hours, and the effect on cell proliferation was assessed by MTS assay. STO cells were highly responsive to compounds **23**, **24**, and **26**, as indicated by the IC_{50} of 3.9±1.8, 8.6±2.1, and 4.1±0.4µM, respectively. A slightly less pronounced effect was observed after exposure of STO

cells to compounds **22** and **25**, highlighting the relevance of alkyl substitutions on the amino groups to increase the antiproliferative activity. By contrast, the presence of the negatively charged carboxylate group led to a decreased (**28**) or null (**29** and **30**) activity (Table 1).

Interestingly, a remarkable antiproliferative effect (0.57±0.04μM) was observed for compound **31** (Table 1), obtained by replacing the *N,N*-dimethylamino group of **19** with a triphenylphosphonium group. This substituent is expected to improve cell and mitochondrial penetration [10]. For this molecule, we also analyzed the ability to modulate the Hsp90 mitochondrial homologue TRAP, by measuring the activity of succinate dehydrogenase in human cervix carcinoma HeLa cells, which is inhibited by TRAP1. Indeed, compound **31** decreases the activity of the enzyme in a dose-dependent way (Figure 2) as expected for a potential TRAP1 activator [11]. As a possible caveat, it is worth noting that we are assuming that TRAP1 is targeted by the compound in a similar way to Hsp90.

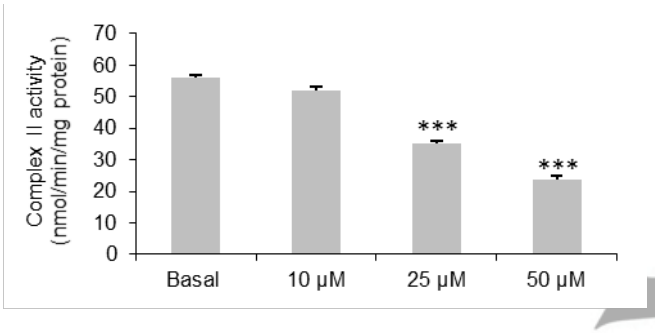


Figure 2. Analysis of the Sulfide-Quinone Reductase (SQR) enzymatic activity of succinate dehydrogenase in human cervix carcinoma HeLa cells pretreated for 30 minutes with compound **31** at the reported concentrations. Data are reported as mean ± SD values (n = 3); asterisks indicate significant differences (*** for a p<0.01 with a Student's *t* test analysis).

Because the designed compounds target a dynamic allosteric site on Hsp90 that does not overlap with and is far removed from the ATP binding site, it is expectedly hard to obtain correlations between computationally determined affinities and effects on chaperone functions or cellular activities [12].

To define a quantitative relationship between the ligands' structures, dynamics cross-talk with the protein and observed activities, we made use of an ensemble approach[6c]: we docked each of the ligands into the ten most representative structures extracted upon clusterization of Hsp90 MD trajectories (see Supplementary Methods). For each ligand, we ranked the best pose from the 10 runs, and for each run the pose with the best docking score was selected for further calculations. This method allows to describe the recognition event in terms of an esnamble of ligand poses interacting with an ensemble of (dynamic) protein structures. Superposition of the best pose selected for each ligand is reported in Figure 1A. All the compounds are characterized by interactions with the same set of residues: E477 interacts with the amine in all compounds, but it also binds the OH group of the phenol ether side-chain in compounds **24** (not shown) and **25** (Figure 1B). The *O*-aryl moiety contacts R591 of the same protomer. Compounds **22** (Figure 1C) and **23** (data not shown) interact with D503 (Figure 1C), as observed previously for *O*-aryl-sugars [6b, c]. The benzofuran moiety mainly establishes pi-cation interactions with K594 from the other protomer in all the ligands.

Next, we calculated Dynamic Ligand Efficiency (DLE). i.e. the Ligand Efficiency in a multiconformational protein ensemble[6c], see Supp. Information, to correlate predicted docking scores to ATPase stimulation and cellular effects. **31** was not included in this list, as its fluorescence prevented measuring ATPase. In Supplementary Figure 1 (SF1), we report the correlation between the DLE of the set of newly designed compounds together with the ones previously described in [6c]. The model provides a good correlation between DLEs and measured ATPase stimulations (R = -0.66 considering all compounds discussed here and in [6c]; R = -0.71 when considering only co-generic amino-derivatives). This finding supports the validity of our model for the design of allosteric activators of Hsp90. Next, we assessed the capacity of our new DLE descriptor to evaluate the potency of the designed compounds in antiproliferative assays. Importantly, the calculated DLE shows a significant correlation with measured cytotoxicities against the cancer STO cell line, with correlation value of 0.62 when considering the whole series, which raises to 0.67 when considering only amines (see Table 1; SF1) indicating the ability of this very simple model to quantitatively capture the main determinants of cytotoxic activities.

To the best of our knowledge, these results are the first that show the actual feasibility of pushing integrated knowledge of dynamic protein-ligand cross-talk into the design of new Hsp90 allosteric compounds, with novel functional impacts as well as improved antiproliferative activities. We have shown before [6b] that these compounds stimulate Hsp90 ATPase activity by accelerating the protein conformational cycle and favoring the catalytically active state: we hypothesize that this reverberates in a modification of the population of the chaperone structural ensembles and of the timing with which Hsp90 conformational families are presented to interaction with co-chaperones and clients. Consistent with recent findings based on mutational studies [6b, 13], this novel way of perturbing chaperone populations and kinetics can expectedly be detrimental to cell viability. It should be noted here that Hsp90 expression in tumor cells can reach up to the 4-7% of the expressed proteome. A marked increase in its chaperone activity might induce loss of homeostatic and feedback controls in a variety of oncogenic signal transduction pathways finely-tuned by Hsp90. Moreover, an excess Hsp90 activity could prompt proteostatic and bioenergetic stress in neoplastic cells by affecting the proteasomal degradation of several client proteins and by causing a harmful drop in ATP levels. These considerations, combined to further tests of the novel series of molecules, may open new perspectives for Hsp90 based therapeutics. In conclusion, our compounds may represent a new series of Hsp90 modulators as anticancer drugs with a novel mechanism of action based on the perturbation of the conformational population and kinetics of the Hsp90 machinery.

Experimental Section

Experimental Details are reported in the accompanying Supplementary Information

Acknowledgements

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Keywords: Hsp90 • Allostery • Molecular Dynamics • Drug Design • Modulators

[1] K. A. Krukenberg, T. O. Street, L. A. Lavery and D. A. Agard, *Q. Rev. Biophys.* **2011**, *44*, 229-255.

[2] a) L. Whitesell and S. L. Lindquist, *Nat. Rev. Cancer* **2005**, *5*, 761-772; b) M. Taipale, D. F. Jarosz and S. Lindquist, *Nat Rev Mol Cell Biol.* **2010**, *11*, 515-528.

[3] a) P. Workman, F. J. Burrows, L. Neckers and N. Rosen, *Ann. N.Y. Acad. Sci.* **2007**, *1113*, 202-216; b) J. B. Trepel, M. Mollapour, G. Giaccone and L. Neckers, *Nat. Rev. Cancer* **2010**, *10*, 537-549; c) L. Neckers and P. Workman, *Clin Cancer Res* **2013**, *18*; d) L. Neckers and J. B. Trepel, *Clin Cancer Res* **2014**, *20*, 275-277; e) A. Bracci, G. Colombo, F. Ronchetti and F. Compostella, *Eur. J. Org. Chem.* **2009**, 5913-5919.

[4] G. E. L. Brandt and B. S. J. Blagg, *Curr. Top. Med. Chem.* **2009**, *9*, 1447-1461.

[5] a) K. M. Byrd, C. Subramanian, J. Sanchez, H. F. Motiwala, W. Liu, M. S. Cohen, J. Holzbeierlein and B. S. J. Blagg, *Chem. Eur.J.* **2016**, *22*, 6921-6931; b) H. P. Zhao, A. C. Donnelly, B. R. Kusuma, G. E. L. Brandt, D. Brown, R. A. Rajewski, G. Vielhauer, J. Holzbeierlein, M. S. Cohen and B. S. J. Blagg, *J. Med. Chem.* **2011**, *54*, 3839-3853.

[6] a) G. Morra, M. A. C. Neves, C. J. Plescia, S. Tsutsumi, L. Neckers, G. Verkhivker, D. C. Altieri and G. Colombo, *J. Chem. Theory Comput.* **2010**, *6*, 2978-2989; b) S. Sattin, J. H. Tao, G. Vettoretti, E. Moroni, M. Pennati, A. Lopergolo, L. Morelli, A. Bugatti, A. Zuehlke, M. Moses, T. Prince, T. Kijima, K. Beebe, M. Rusnati, L. Neckers, N. Zaffaroni, D. A. Agard, A. Bernardi and G. Colombo, *Chem. Eur.J.* **2015**, *21*, 13598-13608; c) G. Vettoretti, E. Moroni, S. Sattin, J. Tao, D. Agard, A. Bernardi and G. Colombo, *Sci. Rep.* **2016**, *6*, 23830.

[7] a) G. Morra, R. Potestio, C. Micheletti and G. Colombo, *Plos Comput. Biol.* **2012**, *8*, e1002433; b) E. Moroni, H. Zhao, B. S. Blagg and G. Colombo, *J Chem Inf Model* **2014**, *54*, 195-208.

[8] S. Sattin, M. Panza, F. Vasile, F. Berni, G. Goti, J. H. Tao, E. Moroni, D. Agard, G. Colombo and A. Bernardi, *Eur. J. Org. Chem.* **2016**, 3349-3364.

[9] a) N. Zaffaroni, A. Costa, M. Pennati, C. De Marco, E. Affini, M. Madeo, R. Erdas, A. Cabras, S. Kusamura, D. Baratti, M. Deraco and M. G. Daidone, *Cell Oncol.* **2007**, *29* 453-466; b) M. De Cesare, D. Cominetti, V. Doldi, A. Lopergolo, M. Deraco, P. Gandellini, S. Friedlander, Y. Landesman, M. G. Kauffman, S. Shacham, M. Pennati and N. Zaffaroni *Oncotarget* **2015**, *6*, 13119-13132.

[10] Lee, C.; Park, H. K.; Jeong, H.; Lim, J.; Lee, A. J.; Cheon, K. Y.; Kim, C. S.; Thomas, A. P.; Bae, B.; Kim, N. D.; Kim, S. H.; Suh, P. G.; Ryu, J. H.; Kang, B. H. *J Am Chem Soc* **2015**, *137*, 4358-4367

[11] M. Sciacovelli, G. Guzzo, V. Morello, C. Frezza, L. Zheng, N. Nannini, F. Calabrese, G. Laudiero, F. Esposito, M. Landriscina, P. Defilippi, P. Bernardi and A. Rasola, *Cell Metabolism* **2013**, *17*, 988-999.

[12] a) J. E. Gestwicki, G. R. Crabtree and I. A. Graef, *Science* **2004**, *306*, 865-869; b) X. Li, S. R. Srinivasan, J. Connarn, A. Ahmad, Z. T. Young, A. M. Kabza, E. R. Zuiderweg, D. Sun and J. E. Gestwicki, *ACS Med. Chem. Lett.* **2013**, *4*, 1042-1047; c) Y. Miyata, X. Li, H. F. Lee, U. K. Jinwal, S. R. Srinivasan, S. P. Seguin, Z. T. Young, J. L. Brodsky, C. A. Dickey, D. Sun and J. E. Gestwicki, *ACS Chem Neurosci* **2013**, *4*, 930-939.

[13] a) B. K. Zierer, M. Rubbelke, F. Tippel, T. Madl, F. H. Schopf, D. A. Rutz, K. Richter, M. Sattler and J. Buchner, *Nat Struct Mol Biol* **2016**, *23*, 1020-1028; b) A. Rehn, E. Moroni, B. K. Zierer, F. Tippel, G. Morra, C. John, K. Richter, G. Colombo and J. Buchner, *J. Mol. Biol.* **2016**, *428* 4559-4571.

Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

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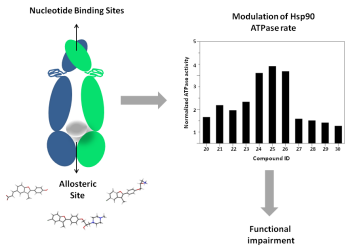
Text for Table of Contents

COMMUNICATION

Perturbation of Hsp90 kinetics. Development of Quantitative Structure-Dynamics-Activity Relationships facilitate the rational design of allosteric stimulators of the ATPase activity of the molecular chaperone Hsp90. The compounds modulate the functional cycle of the chaperone and represent promising anticancer drugs.



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Title
Design of allosteric stimulators of the HSP90 ATPase as novel anticancer leads

Layout 2: