Allosteric modulators of Hsp90 and Hsp70:

Dynamics Meets Function through Structure-Based Drug Design.

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Abstract

Molecular chaperones Hsp90 and Hsp70 are essential regulators of the folding and activation of a disparate ensemble of client proteins. They function through ATP hydrolysis and the assembly of multiprotein complexes with co-chaperones and clients. While their therapeutic relevance is recognized, important details underlying the links between ATP-dependent conformational dynamics and clients/co-chaperones recruitment remain elusive. Allosteric modulators represent fundamental tools to obtain molecular insights into functional regulation. By selectively perturbing different aspect of Hsp90/Hsp70 activities, allosteric drugs can tune rather than completely inhibit signaling cascades, providing information on the relationships between structure-dynamics and function.

Herein, we review advances in the design of Hsp90 and Hsp70 allosteric modulators. We consider inhibitors and activators in different biochemical and disease models. We discuss these compounds as probes to decipher the complexity of the chaperone machinery that at the same time represent starting leads for the development of drugs against cancer and neurodegeneration.

Introduction

Molecular chaperones are essential nodal proteins that integrate multiple biochemical networks fundamental for cell survival, proliferation, adaptation and migration. In this framework, they act as the main regulators of proteostasis, supervising protein folding, activation, aggregation suppression and degradation. All these tasks depend on enzymatic hydrolysis of ATP, fine-tuned structural dynamics, and the formation of multimolecular complexes with co-chaperones and cofactors. Dysregulation of these functions {Whitesell, 2003 #12367; Hartl, 2011 #12935; Young, 2004 #9997} has been associated to diverse kinds of human diseases, such as cancer, neurodegeneration, and inflammatory and infectious diseases.

Because of their roles at the crossroads of multiple cellular functions and the likelihood that manipulation of their activities may impact the treatment of diverse pathologies, efforts to target chaperone pathways with small molecules have been actively pursued for both fundamental and practical reasons. From the fundamental point of view, chemical tools that probe the intricate mechanistic aspects of chaperone function would represent important instruments to improve our knowledge of how complex biological systems are regulated. From the practical point of view, active small molecules could be engineered into new drug candidates targeting a number of diseases with novel modes of action. Indeed, some designed lead compounds targeting chaperones recently moved to clinical testing {Do, 2015 #13540}.

Hsps include biomolecules such as Hsp90, Hsp70, Hsp60, Hsp47, Hsp40 and Hsp27, classified according to their respective molecular weights {Brehme, 2014 #13888; Finka, 2013 #13889}. These proteins are highly abundant in the cell and amount to *circa* 10% of the cellular mass. Hsp90s and Hsp70s alone account for more than half of that mass {Shrestha, 2016 #13020}. In

general, Hsps show a multipartite structural organization that consists of different domains endowed with distinct functions {Hartl, 2011 #12935}: in a simple schematic representation, a specific domain is dedicated to binding and processing the nucleotide, while different domains are dedicated to binding and processing substrate proteins, also known as “clients”. Conformational signals encoded by the nucleotide underlie communication among domains and the regulation of functionally-oriented structural dynamics. In most cases, various co-chaperones and effectors contribute additional levels of functional modulation by selecting different pools of chaperone conformations, binding and/or covalently modifying them {Johnson, 2012 #12674; Fernandez-Fernandez, 2017 #13928}. From the points of view of chemical biology and drug design, the members of the Hsp70 and Hsp90 families have been the focus of most of the attention (FIGURE 1, CHAPERONE FOLDING SCHEME, HSP70 AND 90 TOGETHER).

The initial interest in targeting these systems was based on the observation that, in cancer, key client oncoproteins depend on chaperone activity for functional stabilization. Indeed, Hsp90 is a central member of the stress response machinery, and cancer cells have shown enhanced dependence on this protein for the efficient maintenance of intracellular proteostasis {Travers, 2012 #12672}. Inhibition of Hsp90 in cancer has proven to effectively exploit this biological vulnerability; by hitting key oncogenic networks at multiple points and blocking diverse client proteins, Hsp90 inhibitors have also proved to minimize the development of drug resistance, e.g., through kinase mutation or pathway switching{Neckers, 2014 #12673}. In this context, it has also been observed that Hsp70 over-expression {Nylandsted, 2000 #13902; Kaul, 2003 #13904} correlated with unfavorable prognosis and inhibition of cell apoptosis and senescence {Murphy, 2013 #13892}, facilitating the survival of transformed cells in otherwise adverse stress conditions{Garrido, 2006 #13905}.

In neurodegenerative diseases {Shelton, 2017 #13890}, the Hsp90-Hsp70 system regulates the

levels and cellular processing of the aberrant forms of the protein tau, whose aggregation has been linked to the formation of cytotoxic species in Alzheimer's and other protein misfolding diseases {Wang, 2013 #13893;Wang, 2013 #13893}.

At the molecular level, Hsp90 and Hsp70 form multiprotein complexes {Pratt, 2003 #2928;Rodina, 2016 #13528;Kirschke, 2014 #12762} with co-chaperones to assist and facilitate the maturation of their client proteins (FIGURE 1, CHAPERONE FOLDING SCHEME, Hsp70 AND 90 TOGETHER). In this scenario, assemblies and networks of Hsp90 and Hsp70 are ideal candidates to develop and test new chemical approaches and ideas aimed to mine structural and functional information on proteins and eventually translate it into novel effector molecules.

At a more general level, this process would provide the prospect of overcoming current limitations in drug development by taking into account the molecular complexities associated to the structural organization of biochemical networks: in post-genomic chemical biology and medicinal chemistry, what has become clear is that, with the exception of a few monogenic pathologies driven by one single factor, in most cases the problem of novel drug discovery is more complex than the identification of a unique target and the consequent design of a molecule that may individually block its activity. This has often proved not sufficient for an effective treatment. It has been proposed that the limitations of the "one target, one drug" rule may indeed explain the low number of newly discovered active drugs in recent years {Hopkins, 2008 #13021;Whitesell, 2014 #13544}. In the case of cancer, for instance, it is now well appreciated that many mutated proteins or deregulated metabolic pathways are the leading cause of increasing costs and low success rate (typically 0.0001%) of 'target-centric' drug discovery. Moreover, classical strategies aimed at targeting the active sites of enzymes appear to be approaching their limits. Due to the evolutionary and structural conservation of the latter across the proteome, many instances have shown that the efficacy of orthosteric drugs is hampered by selectivity issues, off-target effects

and development of drug resistance mechanisms. {van der Greef, 2005 #12422; Steensma, 2009 #12937}

In this context, targeting Hsp90 and Hsp70 pathways presents fresh opportunities for testing broad ideas on mechanistic regulation, protein interactions and their manifestation in terms of biological functions. Hsp90 and Hsp70 pathways may in fact be viewed as modular interaction networks {Vogelstein, 2004 #2693}, in which the chaperones act as *hubs* that control the assembly of multiprotein complexes that eventually direct cells towards their final phenotypes. As noted by Gestwicki and coworkers, functional protein complexes in general, and chaperone complexes in particular, are in most cases assembled in a combinatorial manner {Lamb, 2006 #13557;Cesa, 2013 #13554}, typically involving one enzyme combined with multiple non-enzymes. The composition of the resulting systems ultimately defines the type, location, and duration of cellular activities: depending on the cell or cell-compartment types, different proteins are combined with different mechanisms, favoring adaptation to disparate functional requirements.

In addition to these aspects, it has been shown that client binding has a direct influence on the enzymatic activities of both Hsp90 and Hsp70: recruitment of the client protein has in fact been shown to stimulate ATPase for Hsp90 {Genest, 2013 #12678;Lorenz, 2014 #12777;McLaughlin, 2002 #14031}, while in Hsp70 ATP binding to the N-terminal nucleotide-binding domain (NBD) alters substrate affinity to the C-terminal substrate-binding domain (SBD) and substrate binding enhances ATP hydrolysis {Liu, 2010 #13917;Swain, 2007 #12287;Zhuravleva, 2012 #13911}. Inhibition of the enzymatic component may result in the complete, indistinct shut down of the functions of the system, while developing chemicals that mimic and/or enable the controlled modulation of fine-tuned mechanisms in the endogenous cellular environment {Rodina, 2016 #13528;Shrestha, 2016 #13020}, may generate new opportunities in therapy and chemical biology.

Allosteric modulators have represented an area of intense research in the past two decades {Nussinov, 2013 #12964;Nussinov, 2014 #12804;Ostrem, 2013 #13887;Rettenmaier, 2015 #12971;Hardy, 2004 #10215;Hilser, 2010 #12329} whereby the integration of molecular and physical biology with structure-based mechanistic studies and advances in the design and synthesis of new ligands has had a strong impact on disparate targets. In this frame of thought, three main realizations have encouraged the search for new allosteric modulators.

First, allosteric sites tend to be under lower sequence and structural conservation pressure than active sites, which can facilitate the design of target-specific ligands, with reduced risks of toxicity or side-effects {Panjkovich, 2010 #12938;Wei, 2016 #13376}.

Second, the role of protein dynamics has been increasingly recognized as fundamental for biological function: proteins carry out their tasks and participate in biochemical interaction networks by switching among a limited number of structural sub-states, which favor adaptation to different partners and allow biomolecules to fine-tune their activity in response to varying conditions. Such conformational changes are induced by several biochemical factors, including covalent modification, redox-state changes, and most importantly, ligand-binding {Panjkovich, 2012 #12692;Panjkovich, 2010 #12938;Boehr, 2009 #12276;Morra, 2012 #12592}.

Third, allosteric ligands can be exploited to reshape protein-protein interaction surfaces by binding to sites far from the actual interface, and thus influence the selection of one binding partner over another at a shared interface by favoring conformational states with specific recognition profiles.

Allosteric binding and regulation report on all three aspects: allosteric events are the prime mechanisms by which fine regulation of enzymatic activities and recognition of binding partners in the assembly of large complexes, such as those formed by Hsp90 and Hsp70, can be achieved. In a

simplified view, allostery defines how modifications at one site are propagated through the structure to a distal region {Monod, 1965 #10212;Yu, 2001 #12977}. In this framework, modulation of ligand affinity towards the primary active site can be achieved by binding of an "effector" at a different location, the allosteric site {Cui, 2008 #10234}. The end result is a shift in the structural population, whereby the activation of particular dynamic conformations may tune specific enzymatic activities and molecular recognition events {Nussinov, 2013 #12964;Hilser, 2012 #12953;Hilser, 2010 #12329}. These physical modifications affect the ways in which chaperones coordinate and organize their networks, and consequently their effects on clients and on the cellular pathways the latter belong to.

Within this framework, our perspective will focus on the development of allosteric modulators of Hsp90 and Hsp70, critically assessing the recent literature in terms of the progress made in delivering novel chemical entities with potential prospects in chemical biology and in applicative therapies. We endeavor to address the questions on how to regulate the fundamental control points at the crossroads of different biological pathways and design/discover chaperone allosteric modulators displaying interesting properties. We will also consider how structure-based computational approaches may play a role in the design of such molecules, by shedding light on the detailed molecular mechanisms of Hsp90 and Hsp70 regulation.

Hsp90.

The members of the 90 kDa Heat shock protein (Hsp90) family are homodimeric molecular chaperones that orchestrate different cellular pathways involved in cell development and maintenance, by regulating the late stage maturation, activation and stability of a plethora of client proteins (updated list in http://www.picard.ch/downloads) {McClellan, 2007 #12671}.

Different cellular compartments host different paralogs, which oversee the assembly of specific networks of interactions.

In humans, the main paralogs are Hsp90 (with isoforms alpha and beta) in the cytoplasm, Grp94 in the endoplasmic reticulum (ER), and Trap-1 in the mitochondria {Johnson, 2012 #12674}. Recently, important roles have also been invoked for extracellular Hsp90 {Cortes, 2018 #13897;Zhang, 2017 #13898}. Microbial Hsp90 has been linked to pathogenic infections in malaria and tuberculosis {Roy, 2012 #13895;Neckers, 2008 #13896}.(FIGURE 2, Hsp90 structure).

Structurally, the protein exists as a homodimer and each individual chain consists of three globular domains (N-terminal, Middle and C-terminal). Hsp90, Trap1 and Grp94 have a mutual sequence identity of about 30-40%, which reflects the high structural similarity of their individual domains {Ali, 2006 #12386; Dollins, 2007 #10008; Lavery, 2014 #12669}. However, the preferential relative orientation of the domains in the crystal structures solved so far significantly varies depending on the isoform, cellular compartment, and organism {Southworth, 2008 #10040}. X-ray crystallography, SAXS solution data and kinetic measurements have led to the proposal of a general functional mechanism based on global conformational modulations triggered by ATP binding and hydrolysis, which integrates an array of structural information{Lavery, 2014 #12669}. In the absence of nucleotide, various, mostly open, conformations co-exist. ATP binding shifts the chaperone to a partially closed and then into an asymmetric closed conformation (observed for Trap1) that is significantly strained, leading to buckling of the MD:CTD interface(FIGURE 2, HIGHLIGHT ON ASIMMETRY). Interestingly, this region has a key role in client binding{Genest, 2013 #12678}. Upon ATP hydrolysis, strain is relieved to yield a symmetric closed state reminiscent of the fully symmetric yeast Hsp90 dimer {Ali, 2006 #12386}. This model establishes a direct conformational communication between the ATP binding site and the client-remodeling site. *In vitro* experiments demonstrated that, although the fundamental conformational states are well

conserved among species (and/or paralogs), equilibria and kinetics are unique for every Hsp90 homolog {Southworth, 2008 #10040}, suggesting adaptation to the specific pool of clients in a given cellular environment.

Different Hsp90 paralogs are associated to different molecular partners. The majority of clients identified for the cytosolic Hsp90 are related to signal transduction, cell maintenance and growth, including many steroid hormone receptors and kinases {Johnson, 2012 #12674}. Grp94 clients include IgGs, some integrins, and the Toll-like receptor family. The validated list of Trap1 clients is rather small and mostly related to mitochondrial protein homeostasis and respiration, involving mitochondrial ERK kinases and SDH {Sciacovelli, 2013 #13838;Rasola, 2010 #13832}. Finally, interactions with co-chaperones also appear to be paralog specific: while cytosolic Hsp90 function is aided by numerous co-chaperones, which organize complexes for client activation (e.g. Cdc37), enhance (e.g. in the case of Aha1) or slow (e.g. Sti1) the rate of ATPase activity, no co-chaperones have been detected for the ER and mitochondrial Hsp90s (see{Johnson, 2012 #12674}).

Pharmacological inhibition of the enzymatic activity has been pursued for Hsp90 as a prominent strategy for drug development mainly focused on targeting the N-terminal ATP-binding site {Workman, 2013 #12670;Neckers, 2013 #12681;Travers, 2012 #12672;Workman, 2007 #12365;Workman, 2003 #2709}. The first candidates based on the natural product geldanamycin (i.e. 17-AAG, 17-DMAG, IPI504) showed interesting activities in clinical trials but their development was discontinued due to high toxicity. Overall, about 20 ATP-competitive chemotypes have been tested or are now in clinical trials against different types of cancers, such as HER2-positive breast cancer, myeloma, Acute Myeloid Leukemia (AML), prostate cancer, melanoma, ovarian cancer, Non-Small-Cell Lung Cancer (NSCLC), Gastrointestinal Stromal Tumors (GIST), Rectal cancer, and melanoma{Garcia-Carbonero, 2013 #13545}. They compounds include: i) the purine scaffold series (BBIIB021, BIIB028 and PU-H71); ii) the resorcylic pyrazoles and

isoxazoles (VER52296); iii) the resorcylic dihydroxybenzamides (AT13387 and KW-2478); iv) the orally active thienopyrimidine VER-82576, and v) the 8-arylthiopurine CUDC-305, which is orally bioavailable and active in an orthotopic brain tumor model{Neckers, 2013 #12681}. The main limitation of Hsp90 N-terminal inhibitors is that the drug concentration required to outcompete the abundant ATP and to induce client degradation is the same as that needed to induce the heat shock response, which results in the activation of the pro-survival factor Heat shock factor 1 (HSF1), and induction of Hsp27 and Hsp70. Hsp90 indeed sequesters HSF1 in unstressed cells, and disruption of this interaction is thought to determine release of HSF1 and initiation of the transcriptional response that leads to the heat shock response {Zou, 1998 #13874;Conde, 2009 #13981}. Such drug-induced effect creates a vicious cycle in which higher doses at an increased frequency are required, ultimately exposing the patient to toxicity risks{Powers, 2007 #12076}. Additional factors for the limited success of ATP-competitive inhibitors in the clinic have recently been discussed by Neckers *et al.* {Neckers, 2018 #13976}. One critical reason is linked to the pharmacodynamics markers of target engagement and the cell models used to identify such markers, which are commonly represented by peripheral blood mononuclear cells (PBMCs) obtainable from patients pre- and post- therapy. It has however been shown that the pharmacokinetics of Hsp90 inhibitors differs significantly between tumor and normal tissues, that some tumor cells are more sensitive to N-terminal Hsp90 inhibitors than normal ones {Woodford, 2016 #13533} and that the Hsp90 complex in cancer cells is biochemically distinct from that of normal cells. All these factors may affect the affinity for inhibitors, their distribution and pharmacodynamics properties {Chiosis, 2006 #13978;Rodina, 2016 #13528}. Together with these limiting factors, the roles of Hsp90 in mediating nuclear events beyond the stabilization of oncogenic kinases may also contribute to the limitation of success in clinical trials {Trepel, 2010 #12366}. Finally, most preclinical models rely on immune compromised mice: thus, the impact of

perspective drugs on host immunity have not been fully characterized so far {Graner, 2016 #13977}. Neckers and coworkers also provide a critical evaluation of prospects for the clinical evaluation of N-terminal directed compounds. We refer the reader to the original paper for more insight into these issues {Neckers, 2018 #13976}.

One more critical factor that deserves mention is that, given the similarity of the catalytic domain in all Hsp90 family members, most of these compounds lack paralog specificity and impact on the whole Hsp90 functional spectrum by indiscriminately blocking all Hsp90-dependent processes. However, differences exist among paralogs in terms of expression levels, protein clientele, interactions with co-chaperones and, consequently, molecular mechanisms at the basis of their functions. Trap1, for instance, is less expressed in some cancers than in normal tissues{Yoshida, 2013 #12675}. Indeed, recent data by the Neckers group indicate that Trap1 expression is inversely correlated with tumor grade in several cancers, suggesting that, in some settings, this chaperone may even act as a tumor suppressor{Rasola, 2017 #13810;Rasola, 2014 #12936;Liu, 2010 #12682}. In this context, the targeting of pockets different from the ATP one, shows a first example of how to interfere with Hsp90 mechanisms in an isoform selective manner exploiting the dynamics of ligand-protein cross-talk. Chiosis and coworkers {Patel, 2013 #12676;Patel, 2015 #13839} combined library screening, structural and computational analyses to unveil paralog-specific chemical tools, able to differentially modulate Grp94 versus cytosolic Hsp90. Screening identified purine based compounds (FIGURA 3, PU ETC + Gambogic acid) with a preference for Grp94 greater than 100-fold over Hsp90 and from 10- to 100-fold over Trap-1. Structural analysis indicated that specific rearrangements in and around the Grp94 NTD binding site open specific non-polar pockets that can efficaciously be engaged by aromatic modifications on the purine scaffold. This work shows that the overall protein structure and conformational flexibility play important roles in configuring the binding sites of different members of this chaperone family, and

that the consideration of ligand-protein cross-talk may provide novel opportunities for the development of selective compounds. The availability of paralog-specific chemical tools permitted to characterize different chaperoning mechanisms for the client protein HER2 used by Grp94 or cytosolic Hsp90. In particular, results suggested that Grp94 is specifically expressed on malignant HER+ breast cells surface and may translocate to other membrane locations, such as those needed by the altered function of its oncogenic client protein, HER2. In this case, inhibition of Grp94 is sufficient to destabilize membrane HER2 and inhibit its signaling properties, implicating Grp94 in regulating oncogenic signal transduction at the plasma membrane. In contrast, no major requirement for Grp94 was observed in cells with low HER2 plasma expression, where inhibition of Hsp90 was sufficient to destabilize HER2. Overall, these results indicate that chaperone conformational dynamics may be aptly exploited to develop chemical tools that can be used in different cellular contexts, allowing precision interventions that take into account protein expression levels. This series of compounds was further developed by extensive SAR investigations leading to the design of **18c**, a derivative with good potency for Grp94 (IC_{50} = 0.22 μM) and selectivity over other paralogs (>100- and 33-fold for Hsp90α/β and Trap-1, respectively). The activity of **18c** was explored in different cellular models of inflammation and cancer{Patel, 2015 #13839}.(FIGUREA 3, PU ETC).

An example of ligand that targets the N-terminal domain at a different location than the ATP-binding site is represented by Gambogic acid {Davenport, 2011 #14027}. The molecule was identified via HTS screening of natural product libraries and shown to **i**nhibit cell proliferation, determine degradation of stringent client proteins, disrupt the interaction of Hsp90, Hsp70 and Cdc37 and induce expression of Hsp70, a typical indicator of Hsp90 N-terminal inhibition. Binding to this domain with low micromolar Kd was confirmed by surface plasmon resonance, competition experiments with geldanamycin and ,olecular docking experiments.

Allosteric Inhibitors of Hsp90

Fundamental for the development of allosteric modulators was the discovery of a second druggable site in the C-terminal domain (CTD) of Hsp90{Marcu, 2000 #10032}. Early work by the Neckers group started from the investigation of the interactions between Hsp90 and coumarin antibiotics known to affect the activity of the homologous bacterial protein DNA gyrase {Marcu, 2000 #10030;Marcu, 2000 #10032}. In particular, they observed that the coumarin antibiotics Novobiocin, and chlorobiocin (FIGURE 4, STRUTTURE DI NOVOBIOCIN, CHLORBIOCIN) lead to a marked reduction in the cellular levels of Hsp90 client proteins such as $p185^{erbB2}$, $p60^{v\text{-}src}$, Raf-1, and mutated p53, using SKBR3 and MCF7 human breast carcinoma cells and v-src transformed mouse fibroblasts. Novobiocin showed an IC50 around 700 μM. Immobilized Novobiocin was used to isolate either full length endogenous Hsp90 from cell lysates or Hsp90 deletion fragments translated *in vitro*. The results showed that Hsp90 binding to immobilized Novobiocin was competed by soluble coumarins and ATP but not by N-terminally directed ligands geldanamycin or radicicol. Importantly, the authors showed that a carboxy-terminal Hsp90 fragment could bind immobilized Novobiocin but not immobilized geldanamycin. In contrast, a geldanamycin-binding amino-terminal fragment did not bind Novobiocin. These results showed for the first time the existence of an additional, alternative binding site on Hsp90, that could be targeted by chemotypes different from ATP mimics or geldanamycin- and radicicol-based molecules. Importantly, this work showed that binding to a site distal from the nucleotide orthosteric pocket resulted in the modulation of the functional properties of the Hsp90, leading to impairment of its chaperone functions and opening a whole new range of possibilities for pharmacologic interference with Hsp90 functions.

Importantly, Novobiocin and related natural products were not shown to induce the Heat Shock Response (HSR) that instead represents one of the main drawbacks in the clinical development of Hsp90 N-terminal inhibitors. This important property is shared by many of the allosteric ligands inspired by the discovery of Novobiocin activity. The reasons for this differential behavior can aptly be explained hypothesizing that Hsp90 binding to HSF1 is correlated to the chaperone conformation. Ligands targeting different Hsp90 domains can expectedly have distinct impacts on the population of the proteins' conformational states involved in the process of HSF1 recognition (REFS). Hsp90 binding to HSF1 is favored for the ATP-dependent, N-domain dimerized chaperone, a conformation only rarely sampled by mammalian Hsp90. Kijima *et al.* {Kijima, 2018 #13979} have used mutations that fix Hsp90 in this closed conformation, proving that this is indeed the one relevant for HSF1 binding. The authors also succeeded in mapping out Hsp90 binding site on HSF1, showing that the binding motif is comprised of the Heptad Repeat (HR)-A/B trimerization domain in the context of a portion of the Regulatory Domain (RD), the molecular region dedicated to sensing heat and initiating the Heat Shock Response. ATP-competitive inhibitors oppose closure of the dimer {Hessling, 2009 #12686}, thus disfavoring the conformation that is required for stable HSF1 binding. In contrast, allosteric ligands targeting regions distal from the N-domain (derived from Novobiocin or different chemotypes, *vide infra*) can expectedly induce a different conformational landscape for the chaperone, in which structural ensembles that are fit to bind HSF1 are significantly populated. Indeed, KU32, a Novobiocin mimic developed by the Blagg group (*vide infra*) was shown not to disrupt the interaction between HSF1 and closed Hsp90{Kijima, 2018 #13979}. Hsp90 dynamics thus plays a key role in modulating the heat shock response, and chemical tools that perturb such dynamics provide a chance to investigate it in signaling pathways.

While no co-crystal structure for Novobiocin or its derivatives bound to the C-terminal of the

chaperone has been solved yet, intense design, synthesis and Structure Activity Relationships (SAR) studies have been carried out to shed light on the main determinants of activity and to develop novel drug candidates with applications in cancer and neurodegeneration.

Important steps in this direction were taken by the Blagg group {Burlison, 2006 #12411;Yu, 2005 #12283}, who first developed a focused library of compounds to explore the functional relevance of the benzamide side chain, coumarin core, and noviose sugar (FIGURE 5, compound **A4** JACS 2005 di YU). The library was tested for efficacy on SKBr3 cancer cell lines at 100 μM concentration, followed by western blot analysis of cell lysates to check for the degradation of Hsp90-dependent client proteins. Compound **A4** turned out to be the most active one in this assay and was subsequently tested in a mutated androgen receptor-dependent prostate cancer cell line (LNCaP) and a wild-type androgen receptor prostate cancer cell line (LAPC-4) and showed a relevant effect on the concentrations of the mutant androgen receptor, AKT, and Hif-1R at $\sim$1 μM in the LNCaP cell line. The compound reduced levels of the androgen receptor at lower concentrations in the wild-type androgen receptor prostate cancer cell line (LAPC-4) {Burlison, 2006 #12411;Yu, 2005 #12283}.

The results highlighted that attachment of noviose to the 7-position and an amide linker at the 3-position of the coumarin ring are critical for Hsp90 inhibitory activity(FIGURE 5, compound **DHN1, DHN2**). In SKBr3 breast cancer cells **DHN1** and **DHN2** induced degradation of Hsp90-dependent ErbB2 and p53 between 5 and 10 μM and between 0.1 and 1.0 μM, respectively. It was also found that activity was better in the presence of a diol on the sugar ring. Interestingly, in contrast to N-terminal inhibitors, such derivatives of Novobiocin perturbed Hsp90 at concentrations 1000 to 10000-fold lower than that required for client protein degradation, a phenomenon that showed how allosteric compounds could be active with lower toxicity: for these reasons, these compounds

were tested as neuroprotective agents, with promising efficacy in a culture model for Alzheimer's disease (EC_{50} of 6 nM) {Zhao, 2012 #13899; Lu, 2009 #13846; Forsberg, 2018 #13841}.

These results supported the investigation of different modifications on the scaffold based on the identified pharmacophores. In this context, the sugar rings were substituted by more synthetically accessible cyclic or acyclic moieties carrying hydrogen-bonding functions, identifying the piperidine ring as the best substitution for the carbohydrate. Moreover, the chain on the amide side was extended with biphenyl moieties (FIGURE 6a) {Donnelly, 2008 #12404;Donnelly, 2008 #12648;Shelton, 2009 #12373;Zhao, 2011 #12587}. A relevant example of these compounds, KU135, inhibited Jurkat T-lymphoblastoid leukemia cells proliferation with an IC_{50} of 416 nM after 48 hours of treatment {Shelton, 2009 #12373}. In this test, KU135 turned out to be ~3, ~10, and ~600 times more potent than etoposide, 17-AAG, and novobiocin, respectively. Furthermore, KU135 proved to inhibit cell division and induce apoptosis in the leukemic cells, together with the degradation of Hsp90 client proteins pAkt and Hif1-a without inducing the overexpression of Hsp70, indicative of an adverse HSR. Surface plasmon resonance (SPR) experiments showed that KU135 could bind Hsp90β K_d of 1 to 2 μM.

A further interesting evolution of this class of allosteric modulators/inhibitors of Hsp90 was represented by the introduction of a biphenyl scaffold in lieu of the coumarin ring system found in Novobiocin. Initial structure-activity studies showed that the distance between the nitrogen atoms on the piperidine ring and the amide was important for Hsp90 C-terminal inhibition {Zhao, 2015 #12789}. Computational docking studies identified the biphenyl scaffold in FIGURE 6 Bifenili containing the *para-meta* substitution pattern as the optimal core surrogate to mimic the stereochemical relationships among the pharmacophores presented by the coumarin rings. Initial

tests in SKBr3 and MCL7 breast cancer cells yielded low micromolar IC_{50} values (between ~ 0.5 and ~ 5 μM) and degradation of oncogenic Hsp90 clients Her2, Raf and Akt. {Zhao, 2015 #12789}.

Extensive SAR analyses were then performed on these compounds, exploring modifications of the core-biphenyl scaffold, the amide chains, and different types of amine substituents on the other side of the molecule (FIGURE 6b). Many of these derivatives manifested interesting antiproliferative activities, reaching nanomolar IC_{50}'s against SKBr3 and MCF-7 breast cancer cell lines {Garg, 2017 #13840}, the best compounds being as potent as ~ 150nm. The compounds led to degradation of several client proteins (Her2, EGFR, ERα, and Akt) and left Hsp90 levels constant without causing Hsp70 overexpression, the latter being a hallmark of cytotoxic Hsp90 C-terminal inhibitors.

(FIGURE DIEFENILI)

A different set of molecules from this series (both coumarin- and biphenyl-based derivatives) was shown to modulate Hsp90 functional responses in a different manner. Some of these compounds (e.g. KU-32, KU-596 (FIGURA 5)) raised interest for their cytoprotective effects {Lu, 2009 #13846;Ansar, 2007 #13847}, since they could induce the pro-survival HSR yet at significantly lower concentrations than those needed for client protein degradation {Lu, 2009 #13846;Ansar, 2007 #13847;Kusuma, 2012 #13842;Forsberg, 2018 #13841;Zhang, 2012 #13845}. Such properties made them interesting as agents able to protect neural cells from degeneration. Indeed, KU-32 and KU-596 (FIGURA 5) turned out to be efficacious in cellular models of Alzheimer's disease: their modulation of chaperone functions resulted in an induction of Hsp70, which could refold denatured proteins, decrease the levels of abnormal proteins and prevent protein aggregation, ultimately alleviating the phenotypes associated with Alzheimer's and other neurodegenerative

diseases. Further studies showed the possibility to reverse clinical symptoms of Diabetic Peripheral Neuropathy *in vivo* and protect against neuronal glucotoxicity {Urban, 2010 #13848;Ma, 2015 #13849}.

KU-32, specifically, protected against glucose-induced death of embryonic DRG (dorsal root ganglia) neurons cultured for 3 days in vitro. Similarly, KU-32 significantly decreased neuregulin 1-induced degeneration of myelinated Schwann cell DRG neuron co-cultures prepared from WT (wild-type) mice {Urban, 2010 #13848;Ma, 2015 #13849}.

KU-596 has recently been examined for its potential in decreasing c-jun expression and improving motor function in an inducible transgenic model of a Schwann cell-specific demyelinating neuropathy (MPZ-Raf mice) {Zhang, 2018 #13989}. KU-596 therapy reduced c-jun expression, significantly improved motor performance, and ameliorated the extent of peripheral nerve demyelination in both prevention and intervention studies. These effects were reconnected to KU-596 controlled induction of Hsp70 expression: indeed the drug's neuroprotective efficacy was absent in Hsp70 knockout mice. These data are very interesting as they show that modulation of chemical pathways, namely Hsp70 expression levels, via designed chemicals provides the basis for novel therapies to improve conditions in demyelinating neuropathies in humans.

The accessibility and usability of this class of Hsp90 C-terminal allosteric modulators were further expanded by substituting the synthetically complex noviose sugar with simple cyclohexyl moieties while maintaining biological efficacy {Forsberg, 2018 #13841} (FIGURA 6c). Some of the derivatives once more proved to induce Hsp70 levels. Activities were further probed by the c-jun assay and by measuring mitochondrial bioenergetics. Reduction of c-jun and increased mitochondrial respiratory capacity (MRC) were observed for selected noviomimetics, lending support to the notion that designed modulators of Hsp90 can also impact mechanistic features that are associated with neuroprotective efficacy.

One important aspect of the results described above is that target engagement was demonstrated in all cases by affinity purification with isolated Hsp90 domains, proteolytic fingerprinting, SPR, mutation or competition assays. The importance of target engagement issues in Hsp90 drug development is thoroughly discussed by Neckers and coworkers {Neckers, 2018 #13976}.

An additional example of a small molecule modulator targeting the Hsp90 C-terminal domain is represented by a natural rotenoid called Deguelin and related compounds {Lee, 2015 #13850}. In this context, Lee *et al.*{Lee, 2015 #13850} introduced L80 (FIGURA 7), a derivative which, according to biochemical and biophysical analyses, showed to target the C-terminal region of Hsp90. L80 decreased the expression of various Hsp90 client proteins and disrupted the interaction between hypoxia-inducible factor (HIF)-1a and Hsp90, led to downregulation of HIF-1a and its target genes, including vascular endothelial growth factor (VEGF) and insulin-like growth factor 2 (IGF2). This translated into inhibition of viability, colony formation, angiogenesis-stimulating activity, migration, and invasion in a panel of non–small cell lung cancer cell lines and their paclitaxel-resistant sublines.

Regan and coworkers reported on the discovery of modulators of the chaperone's PPIs by targeting the interaction of Hsp90 with the co-chaperone named Hsp Organizing Protein (HOP) {Yi, 2008 #13856;Yi, 2009 #13857;Kajander, 2009 #13858;Pimienta, 2011 #13859;Speltz, 2015 #13860}. HOP acts as a linker protein that holds together Hsp90 and Hsp70 by forming complexes with its C-terminal domain and modulates the activities of these chaperones. The authors used the AlphaScreen high throughput technology to identify compounds that inhibit this interaction. The discovered compounds showed activities *in vivo* against cancer cell lines BT474 and SKBR3, inducing decreased levels of client protein HER2, with associated cell death {Yi, 2008 #13856}. One

compound was thoroughly characterized, namely 1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione (**C9,** (FIGURA 7) {Pimienta, 2011 #13859}. *In vitro* experiments using purified protein components were used to prove that C9 antagonizes Hsp90/HOP interaction. *In vivo*, C9 was cytotoxic also against the highly metastatic MDA-MB-231 breast cancer cell line and did not induce the transcriptional upregulation of Hsp70, a hallmark of Hsp90 C-terminal inhibitors' activity. Interestingly, the authors treated cells with a combination of C9 and either 17-AAG or NVP-AUY922, two active N-terminal directed ATP competitive inhibitors: their results indicated that the overexpression of Hsp70, typically induced by the NTD ligands, could be significantly counteracted by C9. Moreover, in this setting, the lethal dose of C9 was also decreased compared to the use of C9 alone.

This data supports the concept that combinations of ATP-competitive inhibitors and allosteric modulators may synergize to induce cancer cell death *via* a combination of different molecular mechanisms, while potentially minimizing undesired effects.

The McAlpine group characterized allosteric modulators derived from the natural compound SansalvamideA, which were shown to bind at the interface between the N-terminal and Middle domain of Hsp90 and to interfere with the recruitment of HOP, IP6K2 and FKBP52 at the C-terminal {Vasko, 2010 #12464;Kunicki, 2011 #13862;Alexander, 2011 #13863}. This group also introduced peptide-based macrocycles that, targeting the C-terminal domain, blocked the recruitment of co-chaperones and clients without inducing the HSR {Wang, 2015 #13861;Koay, 2016 #13541;Koay, 2014 #13864} (((FIGURA 7)).

Finally, it is worth mentioning that other natural products have been used as a source of inspiration for the development of mimics able to target Hsp90 C-terminal domain and

allosterically inhibit it. Among these, the most relevant ones are Epigallocatechin-3-gallate (EGCG), gambogic acid and coumermycin A1.

EGCG {Yin, 2009 #14025} was demonstrated to bind the C-terminal domain of Hsp90 by affinity chromatography. The molecule was successively developed into a series of derivatives with reaching nanomolar IC_{50}'s against SKBr3 and MCF-7 breast cancer cell lines as low as 4 μM {Khandelwal, 2013 #14026}. Coumermycin A1 was identified in the same context of Novobiocin {Marcu, 2000 #10030;Marcu, 2000 #10032} and subsequently used for the development of novel derivatives and SAR studies {Burlison, 2006 #14028}. The best derivative showed an IC50 of about 2 μM in SKBr3 and MCF-7 breast cancer cell lines.

In the case of large peptides and macrocycles as well as in that of natural products and their mimics, considering their potential points of metabolic liability, large molecular weight and aggregation tendencies, caution should be given to the issue of ruling out off-target effects, problems with adsorption and distribution, and PAINS (Pan Assay INterference compoundS) {Aldrich, 2017 #13990}.

Overall, the synthetic development of C-terminal allosteric modulators, started from the initial discovery of a secondary binding site in Hsp90 able to host the natural compound Novobiocin {Marcu, 2000 #10030;Marcu, 2000 #10032}, shows that different cellular functions can be modulated by modifying selected scaffolds. It is thus possible to develop chemical tools to direct cells towards different fates, regulating the types and levels of Heat Shock Response and acting on the folding/unfolding of client proteins associated with different biochemical pathways.

Allosteric Activators of Hsp90

Allosteric modulators offer a chance to garner new mechanistic information on biochemical

processes by enhancing protein functions at specific levels along their pathways, as opposed to completely abrogating their activity {Zorn, 2010 #12298}. This interesting opportunity applies to Hsp90 as allosteric stimulators of its catalytic activity have recently appeared. Molecules with these properties represent new chemical tools useful to gain insights into the role of Hsp90 conformational dynamics in determining its function as well as for the design of novel therapeutic strategies {Yokoyama, 2015 #13865;Zierer, 2014 #12772; Sattin, 2015 #13061;D'Annessa, 2017 #13561}.

In this context, Yokoyama and coworkers reported on the stimulatory effects of the natural lactone goniothalamin on the ATPase activity of the bacterial homolog of Hsp90, HtpG. Goniothalamin induced a two-fold increase in both kcat and kM. Competition assays with *in vitro* reconstituted truncated versions of the chaperone identified the N-terminal domain of HtpG as the domain hosting the goniothalamin binding site. Importantly, the stimulator had a negative impact on the client refolding activity of HtpG in cooperation with Hsp70 {Yokoyama, 2015 #13865} (FIGURE 8 a (giappi); b (buchner); c (nostri)..

The Buchner group used a FRET based screening, reporting on the conformational rearrangements of Hsp90, to identify allosteric activators of the chaperone closure kinetics {Zierer, 2014 #12772}. The authors reasoned that, as the conformational changes are the rate-limiting steps of Hsp90 ATPase, {Weikl, 2000 #12719;Hessling, 2009 #12686} molecules that influence such kinetics should aptly influence the ATPase activity. Upon screening a library of 10000 compounds, the authors identified 5 compounds accelerating the ATPase activity two- to five-fold (Figure 1 B Angewante buchner). The authors further mapped the binding site of a selected activator using NMR spectroscopy, identifying the NTD region close to helix α2 as the pocket engaged by the ligand. Targeting this region induces structural changes in the active site that reverberate on nucleotide hydrolysis rates.

Next, the authors used a yeast model to test the effect of the stimulators on the folding of the stringent client Glucocorticoid Receptor (GR) *in vivo*. Administration of accelerators reduced GR activity, while increasing Hsp90 affinity for the client. Collectively, these results indicate that the presence of the stimulators negatively affects client processing *in vivo*.

Our group reported for the first time on the *rational* design of activators by combining molecular simulations and theoretical characterization of allosteric communication mechanisms in Hsp90{Sattin, 2015 #13061;D'Annessa, 2017 #13561}. First, the allosteric site was identified through the analysis of the patterns of long-range coordination among residue-pairs: the sets of aminoacids endowed with potential allosteric control properties were designated as the ones belonging to the M- and C-terminal domains and characterized by minimal values of the coordination propensity (CP) parameter. This parameter evaluates the coordination between any two residues (separated by a defined cut-off distance) as a function of the mean square fluctuation of their distance. Small CP values define (groups of) mechanically connected aminoacids that move in a cooperative manner giving the highest contribution in modulating functional motions. The CP profile may change in the presence/absence of a ligand allowing to the connection between the internal dynamic and the observed ligand effects {Morra, 2010 #12346;Morra, 2012 #12592;Morra, 2009 #10228;Rehn, 2016 #13041}. With this approach, we identified residues at the CTD interface with the M-domain that respond to the specific bound nucleotide and define a pocket with stereochemical properties apt to bind drug-like molecules. Interestingly, later independent experimental results reported consistency between the predicted allosteric site and the client binding regions {Street, 2011 #12460; Genest, 2013 #12678} (FIGURE 8 a (giappi); b (buchner); c (nostri). Possible complementary interactions with the putative site were thus mapped with probe molecules, identifying conserved regions in ensembles of Hsp90

conformations where the same interactions were consistently made with the majority of structures. Translating this information into pharmacophore models to screen drug databases allowed the identification of several Hsp90 allosteric binders with interesting anticancer properties. It is worth noting that the use of pharmacophores derived from molecular dynamics and the avoidance of constraints on chemical scaffolds permitted to expand the chemical diversity of the predicted ligands {Morra, 2010 #12690}. Compound **1** (FIGURE 8 a (giappi); b (buchner); c (nostri) {Morra, 2010 #12690} turned out to be the molecule with the most promising anticancer properties, also showing the possibility to be evolved in a number of different derivatives.

Using structural and dynamic information obtained from various sets of MD simulations of Hsp90 in different conditions, we were successively able to evolve **1** into new chemical entities that enable controlled activation of Hsp90 ATPase function. Designed derivatives showed: *i)* a structure-dependent ability to stimulate Hsp90 ATPase; *ii)* the capability to alter conformational dynamics favoring synergistic effects with the activating co-chaperone Aha1; *iii)* modulation of Hsp90 direct interactions with the co-chaperone Sba1 (p23); *iiii)* competition with the model client protein Δ□□□Δ {Genest, 2013 #12678}. The putative binding site was also validated through mutational analysis (FIGURA NUOVA). The analyses of protein responses to first-generation activators in computational models and in different sets of experiments were exploited to guide the design of a second generation of derivatives with improved ability to stimulate ATP hydrolysis. We could thus deliver a series of ligands that permitted to enhance ATPase turnover by up to 7-fold {Sattin, 2015 #13061;Vettoretti, 2016 #13054;D'Annessa, 2017 #13561;Bassanini, 2018 #13964}. Furthermore, decorating the best activator with a mitochondrial penetrating group determined cytotoxicity versus cancer cells in the order of 500 nM {Sattin, 2015 #13061;Vettoretti, 2016 #13054;D'Annessa, 2017 #13561}. The combination of experimental and MD-based investigations of binding and activation trends for different derivatives also facilitated the

development of a quantitative SAR model that correlates Hsp90 activation to the presence of a certain allosteric activator. Such model integrates the information on the dynamic cross-talk that allows protein conformations to adapt to the presence of the ligand, selecting sub-states that favor the activation process.

We characterized the impact of compound-induced activation on Hsp90 interactions *in vitro* and on the stability of a number of clients in cellular models: consistent with observations from other groups, administration of accelerators induced a marked decrease in levels and stability of stringent client proteins, supporting the concept that acceleration of conformational dynamics may in fact represent a new way of perturbing the chaperoning mechanisms that underlie cell viability: indeed, some of our compounds inhibit the proliferative potential of tumor cells including those resistant to Hsp90 ATP-competitive inhibitors {Sattin, 2015 #13061; D'Annessa, 2017 #13561}.

Finally, it is to be noted that the detrimental effect of conformational closure and ATPase stimulation on cell viability was also observed through careful mutational and biophysical analysis {Zierer, 2016 #13520;Rehn, 2016 #13041}.

Very recently, Prodromou and coworkers {Roe, 2018 #14024} showed that also dihydropyridine derivatives could target the M-C-terminal region of Hsp90 and induce activation of its ATPase activity. Interestingly, dihydropyridines were shown to upregulate the heat shock response (HSR) inducing a neuroprotective effect in an APPxPS1 Alzheimer's Disease (AD) mouse model. Importantly, such HSR response could be observed only in diseased cells. The binding site on Hsp90 was validated through mutagenesis. Based on their data and starting from the observation that dihydropyridines could function as co-inducers upregulating chaperones and co-chaperones only when a pathological state is present, the authors suggest that allosteric ligand binding compromises Hsp90's ability to chaperone, by modulating its ATPase activity, which consequently

induces the HSR in diseased cells. Interestingly, this may represent the mechanism by which reduced amyloid plaque and increased dendritic spine density in the APPxPS1 Alzheimer's mouse model could be initiated, indicating the compounds as therapeutic leads for neurodegenerative disease, such as AD.

At this point, it is tempting to speculate on the molecular reasons why both inhibition and acceleration of the Hsp90 cycle would lead to client degradation and impact on cell viability. Hsp90 functions depend on a delicate balance between ATP hydrolysis, large conformational changes and assembly of multiprotein complexes that include clients, stimulator and/or inhibitor co-chaperones at different points in time. In such a highly regulated system, it is crucial for Hsp90 to reach, populate and spend the correct amount of time in the conformations required to correctly assemble the functional complexes and as a consequence process client proteins. Artificial accelerators would force the system to spend less time than that required in such conformations. Inhibitors, on the other hand, could potentially stall the cycle at specific conformations. Overall, the perturbation of Hsp90 conformational landscape and timing of conformational transitions by both accelerators and inhibitors could potentially reverberate in the non optimal processing of client proteins, with effects on relative downstream pathways and ultimately on cell viability.

In general, it appears that perturbing the system by artificial acceleration can provide novel modes to investigate the relationships between ATP processing and *in vivo* activities of the chaperone in the cell, in the absence of genetically induced perturbations to Hsp90 levels. The available data underscore the fact that ATPase does not fully correlate with chaperoning activity. Rather, an ordered and coordinated sequence of conformational events is required for correct Hsp90

function and client processing{Zierer, 2016 #13520; Sattin, 2015 #13061}.

The idea to perturb Hsp90 functions through accelerators may lead to the identification of new chemical tools that complement biochemical and molecular biology approaches in the investigation of the complex and multifaceted mechanisms of Hsp90 at different stages of the chaperone cycle {Pullen, 2011 #12767; Beebe, 2013 #12785}. Furthermore, observed cytotoxic activities and, in some cases, the observed lack of heat shock response induction indicate new potential therapeutic opportunities.

Hsp70

The Hsp70 family of molecular chaperones consists of ubiquitous and highly conserved proteins. The most abundant members are cytosolic constitutive (Hsc70) and stress-inducible (Hsp70) (encoded by *HSPA1A* and *HSPA1B*) {Daugaard, #13901} forms; ER and mitochondrial paralogs (*HSPA5* also known as BiP or GRP78 in the ER; *HSPA9*, also known as mortalin, GRP75, or mtHsp70, in mitochondria) have also been characterized {Daugaard, #13901}. Hsp70 proteins share a highly conserved bipartite structure composed by two domains, connected by a flexible linker: a ~44 kDa N-terminal nucleotide binding domain (NBD) with ATPase activity, and a ~25 kDa substrate binding domain (SBD) {Kityk, 2012 #12955; Swain, 2007 #12287;Mayer, 2005 #13910;Zhuravleva, 2012 #13911}. The SBD and the NBD communicate *via* an allosteric cross-talk that, in turn, controls the conformational cycle: the ATPase driven inter-conversion between open (low substrate affinity) and closed (high substrate affinity) states regulates the folding machinery, which leads to binding or release of SBD substrates, typically linear hydrophobic polypeptides, or partially folded proteins. The binding (or release) of these substrates can, in turn, enhance (or decrease) NBD ATPase activity.

One additional level of regulation is provided by a number of co-chaperones that modify Hsp70 functions not only by modulating ATPase rates and substrate specificity (Hsp40 and DNAJ) {Kampinga, 2010 #13913}, but also exerting control on nucleotide exchange (BAG and HSp110, also known as Nucleotide Exchange Factors (NEFs)) {Bracher, 2015 #13912}, and client substrates for ubiquitination. Indeed, co-chaperones such as the J proteins and NEFs coordinate ATP cycling and client loading onto Hsp70 {Li, 2015 #13881}. (Figure 9)

Hsp70 participates in mediating correct folding as well as buffering the toxicity of denatured/misfolded proteins, and its dysregulation has been linked to several diseases, ranging from cancer to neurodegenerative disorders.

Hsp70 forms complexes with the Hsp90 chaperone machinery through the action of the co-chaperone HOP to buffer the destabilizing effect of oncogenic mutations on the structural stability of client proteins, mainly kinases {Soti, 2002 #13907}. In this context, Hsp70 inhibition alone has proven to effectively disrupt the Hsp90-centered folding machinery{Powers, 2010 #13908}. This evidence supports Hsp70 as an effective cancer drug-target, offering not only an alternative approach to antagonize Hsp90, but also providing the possibility to pursue combination strategies with Hsp90 inhibitors, eventually maximizing therapeutic efficacy.

In neurodegeneration, Hsp70 has been linked to several diseases such as Alzheimer's disease, Pick's disease, progressive supranuclear palsy, corticobasal degeneration, and others. All these pathologies share the common trait of an aberrant accumulation of hyperphosphorylated tau, called tau-tangles{Avila, 2004 #13909}. The main accepted strategy against neurodegeneration focuses on eliminating toxic oligomers or preventing the misfolding/aggregation of the relevant proteins. In some cases, inhibition of Hsp70 has been shown to correlate with a rapid increase of tau ubiquitination and proteasome-dependent tau degradation. However, Hsp70 also participates in the clearance of tau misfolded aggregates {Jinwal, 2009 #13873} *via* mechanisms that have not fully been understood yet. In this framework, it may be hypothesized that these disorders may also be approached with drugs that promote Hsp70-dependent degradation. Drugs that modulate Hsp70-dependent degradation may be combined with Hsp90 targeted modulators to maximize effects on the elimination of aggregated client proteins.

Interestingly, while Hsp70 is known to interact with HSF1 {Shi, 1998 #13985;Karagoz, 2016 #13986; Mendillo, 2012 #13987}, its inhibition does not result in the induction of the heat shock response {Kijima, 2018 #13979;Powers, 2007 #2925;Powers, 2010 #13908;Le Breton, 2016 #13984;Assimon, 2013 #13914}, while leading to the degradation of many clients that are also common to Hsp90. This may be ascribed to different possible reasons. Hsp70 binds to different

regions of HSF1 than Hsp90 {Kijima, 2018 #13979}, and small molecules may be unable to outcompete such interactions. Alternatively, small molecules may target a specific subset of Hsp70 isoforms or populations that are sufficient to block chaperoning of client proteins but not repressor activity on HSF1: In the titration model of heat shock response {Zheng, 2016 #13982;Krakowiak, 2018 #13983;Le Breton, 2016 #13984}, a subset of Hsp70 in the cell binds Hsf1, repressing its transcriptional activity. Upon other stress, client proteins can start to unfold and misfolded conformation can accumulate. Such misfolded structures may compete Hsp70 away from Hsf1, eventually activating transcriptional activity. In this context, it is possible to speculate that small molecules target the subpopulation/isoform of Hsp70 relevant to disrupt the formation of the chaperone complex with the client, but not the Hsp70 subpopulation/isoform that binds and represses HSF1. Finally, it should also be considered that Hsp70 perturbation induces apoptosis in transformed cells {Howe, 2014 #13988;Powers, 2007 #2925;Powers, 2010 #13908}: directing transformed cells towards this state may precede the observation of the adverse heat shock response displayed by Hsp90 N-terminal targeted, ATP-competitive inhibitors.

These facts vividly illustrate the complexity of mechanisms presiding Hsp70 biological roles, a complexity that can be effectively disentangled *via* chemical tools that modulate chaperone activities at different levels.

Allosteric modulators of Hsp70.

The allosteric mechanism of Hsp70 inter-domain communication and the interdependence between substrate affinity and nucleotide processing {Mayer, 2010 #12456;Zhuravleva, 2012 #13911} provide interesting opportunities for chemical modulation. In the context of Hsp70 targeting, the classical drug-discovery approach focused on ATP-competing ligands has met several disappointments. Hsp70 functional promiscuity and the nature of the nucleotide binding

site, highly conserved among Hsp70 members and similar to that of other ATP-regulated proteins, pose selectivity issues{Assimon, 2013 #13914}. Some compounds were in fact developed to target the ATP binding site of the protein but showed low potencies and pleiotropic effects on cells {Powers, 2010 #13908;Rerole, 2011 #13915}, which translated into toxicity issues.

To proceed towards the identification of novel modulators of Hsp70, Gestwicki and collaborators utilized a “gray box” approach: their method consists in screening plant-derived extracts against an *in vitro* reconstituted mixture of bacterial Hsp70 (namely DnaK) and its partner Hsp40 (namely DnaJ). In contrast to cell-based phenotypic screenings that may reveal effects due to both on-target and off-target effects, perturbing the components of a multiprotein complex *in vitro* in a purified and reconstituted form {Chang, 2011 #13866;Miyata, 2010 #13867} permits to identify the actual target in a framework that approximates native, physiological conditions. In general, one component may have measurable enzymatic activity (e.g. ATPase), while the other proteins in the complex may play ancillary roles that impact reaction kinetics, turnover rates or substrate affinities. In their “gray box” experiment, Chang and collaborators screened ligands in the presence of high concentrations of ATP ([ATP] >> KM) and against the combined DnaK-DnaJ complex{Chang, 2011 #13866;Miyata, 2010 #13867}. The first choice was made to favor the discovery of allosteric rather than competitive ligands, while the second, linking measured effects to the presence of a stimulatory co-chaperone, was selected to directly include the impact of DnaJ on observed activities. As a consequence, compounds selected as active in this screen should expectedly be the ones influencing the activity of the full complex.

This approach permitted the identification of myricetin, one of the first allosteric inhibitors of Hsp70, able to inhibit its activity by about 75%. (FIGURA 10a- MIRICETINA; BINDING SITE su 70). The binding site of myricetin was determined by NMR; the ligand was found to engage a previously unknown pocket between the IB and IIB domains of Hsp70. As an allosteric modulator,

myricetin prevented DnaJ-mediated stimulation of ATPase activity, with minimal impact on either Hsp70 intrinsic turnover rate or its stimulation by another co-chaperone, GrpE, which binds DnaK at a different location.

Chemical screens against multiprotein complexes were further used to uncover probes that may be able to control Hsp70 within the intact chaperone machinery {Chang, 2008 #13870; Wisen, 2010 #13868;Wisen, 2008 #13871;Wright, 2008 #13872}. These studies indicated that some compounds may impact ATPase activity of Hsp70 only in the presence of specific co-chaperones. One such compound, dihydropyrimidine 115-7c ((FIGURA 10b- 115-7c; derivato con steric hindrance; BINDING SITE su 70).),was shown to bind to a site close to the region engaged by J-domains for stimulation of Hsp70 activities. In this context, 115-7c activities mirrored those of the co-chaperone Hsp40. NMR-TROSY, docking studies and site-directed mutagenesis further corroborated these observations, and led to the indication that the small molecule modulator and DnaJ may act in concert {Wisen, 2010 #13868}. The structural model was used to introduce steric hindrance on the initial lead to outcompete DnaJ (Hsp40) interaction. Interestingly, the authors developed an analog labeled 116-9e, which, in the ATPase assay, had little effect on the DnaK-GrpE combination, but was found to inhibit the DnaK–DnaJ complex formation by 80%. These data indicated selective interference with J-domain for the stimulation of Hsp70 ATPase activity.

The possibility to activate or inhibit Hsp70 activities in a controlled fashion using small molecules as regulators opens up fascinating therapeutic perspectives. This is mainly the case of Alzheimer's and tau-related diseases, where neurodegeneration and toxicity can be traced back to common protein misfolding events. In this context, deciphering the unclear pathogenic role of the chaperone machinery can generate precious information eventually leading to novel pharmacological treatments. Jinwal *et al.* used selective Hsp70 inhibitors (e.g. myricetin) and

activators (e.g. 115-7c) to gain insight into these mechanisms {Jinwal, 2009 #13873}. In a cell-based model of Alzheimer disease, inhibitors led to rapid tau degradation by the ubiquitin proteasome system (UPS). In contrast, activators preserved tau levels. These results were apparently paradoxical, as activation of Hsp70 was expected to increase tau clearance. This led the authors to the interesting hypothesis that activation may mimic the effects of stress-induced increase in Hsp70 levels, a mechanism needed to ensure that more Hsp70 is available to bind larger amounts of unfolded substrates. Because of the very slow kinetics of processes related to tau accumulation, an acute increase in Hsp70 levels would shift the equilibrium toward larger amount of Hsp70/tau complexes, suggesting that apparent reduction in free tau levels are only due to stoichiometric considerations and not to end-point pharmacological response of the activator. In a model where Hsp70 is genetically overexpressed, the authors also indicated that pharmacological activation of ATPase activity favors formation of a pro-folding complex preluding to tau accumulation and hyper-phosphorylation. In contrast, in the same conditions, myricetin and other Hsp70 ATPase inhibitors determine a selective increase in the amount of low-affinity Hsp70/tau complexes that have to be cleared by the UPS. Hence, whereas the overexpression of Hsp70 or overactivation of its ATPase activity does not consequently alter the balance of folding/degradation, its inhibition is directly connected to increased tau binding for exclusive degradation.

These considerations, suggested by the use of selective allosteric modulators of Hsp70 and of its functional complexes, may also have therapeutic implications: higher levels of Hsp70 can in fact be induced by the use of Hsp90 inhibitors (the two chaperone systems are connected through HSF1) {Zou, 1998 #13874;Dickey, 2005 #13875} such as celastrol or geldanamycin. This would provide more Hsp70/tau complexes to target for efficacious inhibitors of Hsp70 ATPase, augmenting tau clearance by ubiquitination.

This strategy would also represent a possible alternative use of Hsp90 inhibitors that showed toxicity when administered for long times: one would use Hsp90 inhibitors for a limited amount of time to upregulate Hsp70, which would be consequently inhibited to degrade toxic aggregates.

Another class of Hsp70 allosteric ligands is represented by the derivatives of MKT077, a molecule belonging to the class of rhodacyanines. MKT077 was shown to have interesting anticancer effects against a number of human cancer cell lines, with IC50 between 0.35 and 1.2 uM {Wadhwa, 2000 #13876;Propper, 1999 #13877}. NMR, biochemical and computational studies were used to show that the compound has affinity for the ADP-state but not for the ATP-state of the protein and that it engages a pocket, which is close, but not superimposable to the ATP-binding site in the NBD {Rousaki, 2011 #13878} (FIGURA 11,con BINDING SITE E MKT077 E YM…). Due to the presence of a charged pyridinium, MKT077 has poor pharmacological properties, which may explain the failure in Phase I cancer clinical trials and the very limited performances in passing the Blood Brain Barrier (BBB) in tests against neurodegeneration models. MKT077 was thus evolved into neutral analogues (YM series) {Miyata, 2013 #12771}. The resulting compounds retained affinity for Hsp70 *in vitro* and reduced pathogenic tau in brain slices. *In vivo*, one of the YM compounds, YM-08, crossed the BBB and maintained a brain/plasma (B/P) ratio greater than 0.25 for 18h, with no renal toxicity. Interestingly, MKT-077 and YM-1 substitute for the co-chaperone Hip in promoting Hsp70-dependent nNOS maturation {Morishima, 2011 #13879}. Thus, MKT derivatives showed interesting effects against tau aggregates in several models.

Another series of derivatives was developed for cancer treatment, designed to improve the metabolic stability of MKT077. The most potent molecules, such as 30 (JG-98), turned out to bind at the same site and to be 3-fold more active than MKT-077 against breast cancer cell lines with EC_{50} values in the mid-nanomolar range. In spite of a modest destabilization of chaperone

clients, Akt1 and Raf1, the ligand was able to induce apoptosis in cancer cells {Li, 2013 #13880}. From the mechanistic point of view, JG-98 effects were due to allosteric perturbation of Hsp70 binding with Bag3, a co-chaperone that is necessary to promote cell survival pathways (FIGURA 11E SCHEMA DA GESTWI MOL CANCE THERAPEUTC) {Li, 2015 #13881}.

Chiosis and coworkers combined structural homology modeling, binding site prediction and druggability score evaluations to design allosteric ligands of Hsp70. Their model represented the more open ADP-bound state of the chaperone, revealing pockets, which were not visible in the crystal structures of ATP-bound homologs. One of the pockets, located in the NBD, had a high druggability score, and was designated as the chosen pocket to target allosteric Hsp70 mechanisms. Interestingly, this site exposed a Cys residue, which could be targeted by a Michael acceptor establishing a covalent link between a hypothetical ligand and the protein. On this basis, they designed and synthesized 2,5'-thiodipyrimidines (FIGURE 12 YK5, CHIOSIS CELL CHEM AND BIOL), carrying an acrylamide moiety to covalently bind the allosteric Cystein residue (Cys267) {Rodina, 2013 #12769}. Cell experiments, pulldowns with biotynilated ligands, and trypsin digestion followed by mass-spec fragment identification verified that YK5 bound to the allosteric pocket and could covalently inhibit Hsp70. This was further tested in cancer cells, where the cytotoxic activity was linked to the disruption of the formation of active oncogenic Hsp70/Hsp90/client protein complexes {Rodina, 2013 #12769}. YK5 was subsequently optimized with extensive SAR studies {Kang, 2014 #13882}, reaching potencies ((FIGURE 12 17a 20a di JMED CHEM1) between high nanomolar to low micromolar range and leading to a reduction of oncoproteins typically protected by Hsp70. The inhibitors containing acrylamide, that ensures covalent binding to Hsp70, were further modified to identify derivative 27c ((FIGURE 12 27c di JMED CHEM2), which had biological effects comparable to those observed for the irreversible

inhibitors at comparable concentrations {Taldone, 2014 #13884}.

Interestingly, the affinity and selectivity of these compounds for Hsp70 was further exploited for the development of chemical tools that enable the analysis of the Hsp70-regulated proteome in tumor *vs.* normal cells as well as comparative analysis of complexes in different tumor types. In this context, YK5 was further modified with suitable spacers and terminal biotin (FIG FORM ACS CHEM BIOL). The new derivatives permitted the isolation of large molecular assemblies with Hsp70 in complex with onco-client proteins, effectively facilitating their characterization *via* biochemical techniques {Rodina, 2014 #13883}.

Using a chemo-genomics screening on yeast cells, Hassan *et al.* {Hassan, 2015 #13885} identified the novolactone XX (FIGURE 13 FUGURA FROM CELL CHEM BIOL MAYER) as a potential modulator of Hsp70. Indeed, biochemical, structural and functional analysis revealed that this allosteric inhibitor could covalently bind at the interface between the SBD and the NBD, disrupting inter-domains communication and locking Hsp70 in a conformation not compatible with ATP-induced substrate release. This, in turn, resulted in the inhibition of Hsp70 refolding activities.

Finally, it is worth mentioning that a small molecule inhibitor targeting the Substrate Binding Domain has shown interesting activities against human Hsp70 in cultured tumor cells and the bacterial Hsp70{Leu, 2009 #13886;Leu, 2014 #13891}. 2-phenylethynesulfonamide (PES) was selective for Hsp70 vs. Hsp90 and induced disruption of the association between Hsp70 and its co-chaperones as well as clients FIGURE 13 FUGURA. PES determined tumor cell death through protein aggregation, impaired autophagy and effects on lysosomes. The molecule was also tested in an *in vivo* tumor model, proving able to enhance survival in mice with Myc-induced lymphomagenesis. Overall, while the SBD has been a rather unexplored site so far, it may offer novel possibilities for allosteric interventions in the Hsp70 system{Leu, 2014 #13891}.

Overall, allosteric modulation may hold lots of promise for targeting Hsp70 pathways, defying chemical intervention based on ATP competitive molecules. Furthermore, it is interesting to observe how Hsp70 modulation *via* stimulators or inhibitors generated novel hypotheses, in particular on the mechanisms of tau-aggregate degradation and processing. In perspective, a combination (possibly with coordinated administration times) of Hsp90 and Hsp70 modulators/inhibitors could prove useful in the treatment of difficult molecular diseases.

Structural and Computational approaches for the discovery and design of Chaperone allosteric modulators.

The results discussed above clearly show that allosteric modulators of Hsp90 and Hsp70 have reached a stage where they can be aptly exploited for the investigation of molecular mechanisms of client binding and processing, as well as for drug development projects. The discovery and optimization of new allosteric effectors would greatly benefit from the availability of detailed structural information on their complexes with the target chaperone. In this respect, recent progress in structural biology has allowed to overcome limitations imposed by the intrinsic flexibility of Hsp90 and Hsp70 protein families in their complex endogenous environment. Thanks to the extraordinary power of cryoEM, it has been possible to glimpse into how Hsp90:Cdc37:Cdk4 {Verba, 2016 #13559} and Hsp90:Hsp70:Hop:GR {Kirschke, 2014 #12762} are organized and how the Hsp70 and Hsp90 ATPase cycles might be coupled.

However, while an increasing number of crystal structures have appeared that show the full-length chaperones bound to mimics of ATP in the nucleotide active site, very little or no experimental information is available to provide insight at atomistic resolution on the complexes with allosteric modulators (inhibitors or accelerators). This is particularly true for Hsp90, where

available structural characterization on the modes of binding of allosteric modulators is due mainly to NMR {Kumar Mv, 2018 #14023;Sattin, 2016 #13050} and cross-linking experiments {Matts, 2011 #12655}. In the case of Hsp70, X-ray information has been obtained for covalent {Hassan, 2015 #13885} and non-covalent {Leu, 2009 #13886;Leu, 2014 #13891} ligands, while NMR has been used to generate binding modes for Rhodacianines as 115-7c, and related compounds{Jinwal, 2009 #13873;Rousaki, 2011 #13878;Wisen, 2010 #13868}.

The difficulties in obtaining experimental molecular details of allosteric ligand-chaperone binding stem from the expected highly dynamic nature of the resulting complexes, making the latter refractory to crystallization. Indeed, kinetic and binding experiments {Wisen, 2010 #13868;Cesa, 2013 #13554;Zierer, 2014 #12772;Rehn, 2016 #13041;Zierer, 2016 #13520;Sattin, 2015 #13061} indicate that allosteric perturbation on the one hand modifies the timing of the conformational cycle, while on the other hand reshapes protein-protein interaction surfaces guiding the protein in preferential partner selection.

To gain atomistic insights into the determinants of chaperone dynamics, and how they are related to and influenced by allosteric ligand binding, we can turn to computational/theoretical approaches {van Gunsteren, 2006 #2798;van Gunsteren, 2008 #12707; Pontiggia, 2007 #10033;Monticelli, 2005 #13969;Meli, 2008 #13854}. While plastic and finely regulated systems like Hsp90 and Hsp70 are a challenging task, in particular for all-atom models, they also represent prime test cases to evaluate the reach and significance of computational approaches. Herein, we will focus on results reported for the full-length proteins and their complexes.

The dynamics of Hsp90 and their relationships to allosteric regulation have been studied with a multiplicity of approaches, ranging from plain all-atom MD simulations to biased methods, from

network analysis to coevolution studies.

Using statistical mechanics based methods, we carried out comparative analyses of Molecular Dynamics (MD) trajectories of multiple structural representatives of Hsp90 (yeast Hsp90, mammalian Grp94, bacterial HtpG), in different nucleotide states (ATP-bound, ADP-bound, apo state) {Morra, 2009 #10228;Morra, 2010 #12930;Morra, 2012 #12592}. Through this approach, we were able to identify potential points of allosteric control distal from the nucleotide binding-site. Specifically, we made use of *i)* the coordination propensity (CP) analysis previously described {Morra, 2012 #12592} to identify (groups of) mechanically connected aminoacids; *ii)* the analysis of the *mechanical strain* experienced by the protein in the presence of different nucleotides (ATP, ADP, Apo-state), to calculate how much the instantaneous distance of a residue from neighboring amino acids differs from the time-average. By evaluating the time evolution of the parameters in MD runs with different ligands, it was possible to identify distal regions that undergo consistent nucleotide-dependent local deformations in different Hsp90 homologs. Statistical and comparative analysis of the trajectories also unveiled the residues that move in a cooperative manner and may give the highest contribution in modulating functional motions. Interestingly, analysis of mechanical coordination and structural deformation revealed that the ligand-dependent structural modulations mostly consist of relative rigid-like movements around two common hinges of a limited number of quasi-rigid domains, shared by all three proteins. The first hinge, whose functional role has been demonstrated by several experimental approaches, is located at the boundary between the N-terminal and Middle-domains. The second hinge is located at the end of the H4–H6 three-helix bundle, located at the boundary between the M-large and M-small structural subdomains where three Phe residues (Phe484, Phe488 and Phe432) form a well-packed hydrophobic core which unfolds/unpacks going from the ATP- to the ADP-state. Interestingly, the latter hinge was located in the vicinity of Cys597 (human Hsp90 alpha

numeration), whose S-nitrosylation was experimentally observed to alter both the C-terminal and N-terminal association properties of Hsp90, affecting its conformational equilibrium, the ATPase cycle and recruitment of clients {Retzlaff, 2009 #12653}. The results of computational analyses were used to guide the design of point mutations of residues distal from the ATP site whose dynamics turned out to be most affected by the nucleotide exchange. In particular, it was interesting to observe that Phe to Ala mutations at the end of the aforementioned three-helix bundle could stimulate the ATPase and closure kinetics of the chaperone. From the conformational dynamics point of view, such mutations increased the overall flexibility in the chaperone, speeding up the search for the closed, catalytically competent state. Other mutations, located in particular in the C-terminal domain, were in contrast found to rigidify the protein leading to inhibition (FIGURE WITH MUTATIONS AND RIGIDIFICATION). Furthermore, mutations in allosteric points also showed an effect on co-chaperone recognition and binding {Rehn, 2016 #13041}.

Seifert and Graeter {Seifert, 2012 #14016} made use of MD simulations of HtpG followed by force-distribution analysis to identify internal allosteric communication pathways that connect the nucleotide-binding site to a region in the M-domain, 2-3 nm distant from the catalytic site. In this model, the long helix in the M-domain (helix H18 in the original paper) serves as dynamic hinge region, consistent with the previously defined model {Morra, 2012 #12592}, and its terminal part nicely overlaps with the client protein-binding site mapped out by Genest and coworkers {Genest, 2013 #12678}. Importantly, the work of Seifert and Graeter {Seifert, 2012 #14016} stresses the importance of the detailed molecular characterization of the mechanism: force transmission is in fact triggered only by ATP, which induces the bending of the proximal H3 helix in the N-terminal domain and represents the major force channel into the middle domain. In the ADP and *apo* states, this allosteric mechanism is different. This strategy, which has been extended to

other allosterically regulated systems, indicates how an external perturbation, represented e.g. by ligand binding, can be efficiently modeled as an external force acting on the macromolecule, making techniques able to take this perturbation into account a very general approaches to study the mechanisms involved in protein allostery {Seifert, 2013 #14017}.

All atom MD simulations were also used to study the role of the unusual asymmetry in the dimeric structure of Trap1 (the mitochondrial Hsp90 homolog) on the mechanisms of ATP processing and conformational cycle regulation {Elnatan, 2017 #13725;Moroni, 2018 #13836}. Using a combination of X-ray crystallography, FRET analyses, DEER, mutational and biochemical experiments, Agard and coworkers proved that ATP hydrolysis on the two protomers is sequential and deterministic {Elnatan, 2017 #13725}. Microsecond all-atom MD simulations showed that the ATP g-phosphate in the buckled protomer has fewer water molecules in proximity than the one in the straight protomer. At higher temperature, the same trend is maintained. While the two nucleotide-binding pockets are essentially identical and the average RMSD between the N-terminal domain of the two protomers is 1.74 A, water dynamics in the active site (not only occupancy) was different between the two protomers, in particular near the two ATP b- and g-phosphates. Most of the waters turned out to spend only a few nanoseconds in the buckled protomer. In contrast, in the straight protomer, there are longer-lived waters positioned between E130 and the g-phosphate and also more waters positioned above E130. The asymmetry in water occupancy and dynamics between two protomers was found to correlate well with the observed preferential hydrolysis of the buckled protomer ATP. Expanding on the issue of how structural asymmetry may determine function in Trap1, the internal dynamics of the mitochondrial chaperone was investigated applying the mechanical, coordination and fluctuation measures introduced for Hsp90 {Morra, 2009 #10228;Morra, 2010 #12930;Morra, 2012 #12592}. The substructures at the interface between the Middle domain (M-domain) and the C-terminal domain

(CTD) of Trap1 were shown to be specifically modulated by the type of nucleotide bound and by the specific protomer to which it was bound. Specific coordination was defined between the nucleotide site and distal client binding sites: In particular, the most intense coordination was observed between the ATP-binding sites and the buckled region in the small M-domain of the distorted protomer {Moroni, 2018 #13836}. This asymmetric communication between the nucleotide and client sites supports the observation that structural asymmetry plays a role in functional regulation and tuning of the chaperone properties.

Verkhivker and coworkers used a combination of MD simulations and computer science techniques, ranging from network modeling to community analysis, to identify preferential control points and communication mechanisms in Hsp90 {Blacklock, 2013 #14003;Blacklock, 2014 #13927;Czemeres, 2017 #13996;Stetz, 2018 #13926;Verkhivker, 2018 #14018}. In particular, structural stability analysis using force constant profiling of the inter-residue fluctuation distances permitted to identify networks of conserved structurally rigid residues that were defined as mediators of allosteric communication. Moreover, by connecting network analysis to conformational landscape concepts, the authors established a relationship between structural stability and the centrality in networks of allosterically relevant residues involved in chaperone regulation: in this picture, allosteric interactions appear to be mediated by modules of structurally stable residues. Importantly, stable and connected residues corresponded to regions in the M-domain, relevant for client binding {Blacklock, 2014 #13927}. Interestingly, this kind of analysis was also used for studying large Hsp90 complexes {Stetz, 2018 #13926}. In this work, MD simulations and network analysis revealed the central role of Cdc37 in mediating client recognition and defining allosteric regulation of the chaperone-kinase complex. The analysis was also reconnected with the role of specific sites of Hsp90 post-translational modifications in regulating Hsp90 function {Stetz, 2018 #14019}.

Overall, computational analyses carried out by different groups using different approaches converge in indicating preferential coordination pathways between the N-terminal ATP-binding site and the client binding site located between the M-small and C-terminal domain of Hsp90. The dynamics of such coordination pathways are particularly dependent on the type of bound nucleotide, establishing a direct connection between the site of nucleotide hydrolysis and the location where client recognition and processing take place. The specific cross-talk between the two different functional sites in Hsp90 couples the conformational dynamics of the client-recognition region to that of ATP hydrolysis region. It is thus conceivable to try and exploit this knowledge for the discovery of small molecule modulators targeting Hsp90 and its complexes.

In terms of allosteric lead discovery, information on the points of regulation defined through the application of the coordination propensity (CP) and strain analysis described above, was used to define potential complementary interactions with the allosteric site{Colombo, 2008 #10014;Morra, 2009 #10228;Morra, 2010 #12930;Morra, 2012 #12592}, and then translated into pharmacophore models used for virtual screening of large libraries of drug-like molecules {Morra, 2010 #12930}. This approach revealed series of compounds based on the benzofuran moiety (FIGURE XXX) (see above, allosteric activators paragraph) that proved to be genuine allosteric activators of the chaperone with important biological effects when tested in cancer cell models {Sattin, 2015 #13061;Vettoretti, 2016 #12933;D'Annessa, 2017 #13561}.

Furthermore, the structural information on the potential allosteric site could be combined with docking approaches to generate SARs for known allosteric compounds and to guide the design of novel derivatives {Zhao, 2015 #12789;Zhao, 2014 #13074;Zhao, 2013 #13083}. In particular, analysis of the fitness of known active ligands (inhibitors or activators) for the allosteric pocket

was combined to the characterization of their effects on Hsp90 structural dynamics. The investigation of the mechanisms of formation/disappearance of pockets around the allosteric site, driven by conformational Hsp90 response to first-generation ligands was then exploited to define the positions where the addition/variation of specific chemical functionalities on existing scaffolds allowed for optimization of binding interactions. This approach was used to improve on the stimulating properties of activators {Sattin, 2015 #13061;D'Annessa, 2017 #13561;Bassanini, 2018 #13964}, as well as to ameliorate potencies of coumarin-based allosteric inhibitors {Moroni, 2014 #12684}.

Simulative approaches {Bopp, 2016 #14015;Ciglia, 2014 #14020} based on the use of MM-GB/SA calculations combined with a decomposition of the effective energy (i.e., the sum of gas phase and solvation free energy) of dimerization on a per residue level were used to detect the aminoacids that are important to stabilize the C-terminal dimerization interface. This information was next translated into peptidic constructs that were successfully used to block C-terminal dimerization{Bopp, 2016 #14015;Ciglia, 2014 #14020}.

A similar approach was used by Wang and coworkers {Wang, 2017 #14021} to target the Hsp90-Cdc37 protein-protein interaction (PPI). Using interface mimicry and analysis of peptide binding patterns with MD simulations, the authors identified the short sequence HFGMLRR (Pep-5) as the one able to bind Hsp90 with a Kd of 6.90 micromolar and to interfere with the recruitment of Cdc37.

Molecular simulations are playing a more and more important role also in shedding light on the mechanisms of allosteric regulation of Hsp70: analyses of allosteric (DnaK) and non-allosteric homologs (Hsp110-Sse1) of the chaperone in different nucleotide states could identify relevant populations on the free energy landscape, underlining the high flexibility and plasticity of this

chaperone {Chiappori, 2016 #13055;Maisuradze, 2010 #12645;Nicolaï, 2010 #13922;Gołaś, 2012 #13918;Nicolai, 2013 #14012;Nicolai, 2016 #14008}. Communication patterns, internal fluctuations analysis and characterization of local deformations determined by ATP-ADP exchange were reconnected to the mechanisms of open to closed conformational transition {Chiappori, 2012 #13084; Stetz, 2017 #13923}. The results of these simulations were nicely consistent with independent results that identified important hinge residues presiding nucleotide processing and exchange {Ung, 2013 #13919; Chang, 2010 #13925}, relevant dynamic domains {General, 2014 #13920}, and inhibition mechanisms {Stetz, 2016 #13924}. In general, the results converge on highlighting the importance of the linker between the NBD and SDB as a dynamic allosteric switch {English, 2017 #13916; Zhuravleva, 2012 #13911} and of the nucleotide-dependent domain motions of the IA and IIA lobes of the NBD as the initiators of the functionally-oriented conformational reorganizations.

Recently, the interaction between the Hsp70 and Hsp40, fundamental for ATPase regulation, was studied by Malinverni et al. {Malinverni, 2017 #14007} using an elegant combination of molecular simulations based on coarse-grained and atomistic models with coevolutionary sequence analysis. Focusing on a model of DnaK(Hsp70)-DnaJ(Hsp40) interaction, the authors were able to identify an interaction surface formed by helix II of the DnaJ J-domain (Hsp40) and a structurally contiguous region of DnaK conserved throughout evolution. The latter surface involved involving lobe IIA of the nucleotide binding domain, the inter-domain linker, and the p-basket of the substrate binding domain. This integrated approach proved able to rationalize a number of experimental observations, at the same time providing a new high-resolution dynamic model to target for the design of molecules able to interfere with the Hsp70-Hsp40 interaction.

Another example of integrated approach to the study of Hsp70 functional dynamics comes from the Carlson lab {Ung, 2013 #13919}. They combined MD simulations, mutagenesis, and enzymatic

assays to identify the key residues responsible for regulating ATP hydrolysis. Focusing on the DnaK's nucleotide-binding domain (NBD) in the apo, ATP-bound, and ADP-bound states, the authors showed that each nucleotide state populates a distinct conformation. Interestingly, this was supported by biochemical mutation and binding data. MD results also identified a shearing motion between subdomains IA and IIA, which governed the structural interconversion. Coupling MD data with evolutionally analysis identified G228 and G229 as a hinge for the nucleotide dependent closing/opening motions: alanine mutations of these hinge residues caused DnaK to have reduced chaperone activities in vitro and in vivo. (FIGURA)

It is tempting at this point to suggest that this wealth of information on the correlations among structure, communication mechanisms, dynamics, function and ligand binding constitutes a solid basis to predict the allosteric response of Hsp70: upon targeting regions endowed with functionally-oriented dynamic properties, it may be possible to rationally optimize existing allosteric effectors and to discover novel chemical entities. The new molecules would have interesting applications in understanding how the chaperone functional cycle is linked to clients/co-chaperones recruitment and, hence, to cell viability. Success in this kind of design would open up new possibilities for the development of useful chemical tools and, in the long term, for the discovery of new therapeutic drugs.

Conclusions and perspectives

Hsp90 and Hsp70 are essential molecular machines that control the folding process to activate a broad and disparate array of substrate ”client” proteins. Despite their fundamental roles, many questions are still open regarding the regulation and fine-tuning of their functions in both normal and disease conditions. The development of chemical tools and drug-like molecules that *not only*

abolish the activities but rather modulate *in vitro*/*in vivo* conformational sub-states of the chaperone is playing an increasingly prominent role in elucidating the coupling between ATP processing, the conformational cycle and client remodeling. At the cell level, chemical modulators (effectors) may even help direct cells towards different destinies. In this context, the main chemical challenges are represented by: *i)* the need to establish computational methods that include the explicit characterization of functional dynamics and its influence on possible interaction sites of the target protein; *ii)* the development of novel classes of compounds that directly modulate the distribution of functional sub-states through rationally selected functional groups; *iii)* the characterization of (potentially unexpected) effects of targeting unexplored allosteric sites.

Achieving these goals will provide new chemical tools that can be used to take action at different stages, from ATP processing phase to multiprotein complex assembly. By exerting modular tuning of the chaperone machinery, it may conceivably be possible to elucidate the intricate mechanisms behind clients folding and co-chaperones recognition. Such approaches will provide important complements to more classical molecular biology methods. The availability of these data represent a new exciting possibility to design molecules that perturb enzymatic ATPase activities or modulate protein-protein interfaces, generating a direct link between structural information and functional outcomes.

In conclusion, we expect that the integration of structural dynamics studies with the development of new compounds will contribute to disentangle the intricate relationships between the selection of states through allosteric binding and the resulting functional implications. The synergy we propose will enable a dynamic chemical and structural picture for deciphering Hsp70 and Hsp90 functions as well as a rationalization of chaperone modulation operated by small molecules within a physiologically dynamic framework.

Captions for Figures

Figure 1. Schematic simplified representation of the client processing by the Hsp70 and Hsp90 chaperone systems. Only the main co-chaperones cited in the text are represented.

Figure 2. Upper panel: three-dimensional structures of the mitochondrial asymmetric (Trap1, pdb code 4ipe.pdb) and cytosolic symmetric (Hsp90, pdb code 2cg9.odb) representatives of the Hsp90 family of chaperones. Lower panel: a schematic representation of the complex with co-chaperone and client (left), together with the EM reconstruction of the complex from citation {Verba, 2016 #13559} (right).

Figure 3. Grp94-selective, purine based compounds and the identification of their targeted domain.

Figure 4. Chemical structures of the C-terminal inhibitors Novobiocin and Chlorbiocin.

Figure 5. Chemical structures of the C-terminal inhibitors developed by the Blagg group from Novobiocin. The circles indicate pharmacophoric points that resulted important for activity.

Figure 6. a) Coumarin Structure Activity Relationships (SARs). **b)** Biphenyl derivatives and modifications of the biphenyl scaffold in the study of SARs discussed in {Garg, 2017 #13840}. **c)** Byphenil compounds with noviose substitutions.

Figure 7. Chemical structures of the Deguelin analog **L80,** of the C-terminal targeted compound **C9,** and of the cyclopeptide SanA. Their binding on Hsp90 domains is schematically presented.

Figure 8. a) Chemical structure of the activator Goniothalamin. **b)** Chemical structures of the artificial accelerators discovered via FRET screening by the Buchner group. **c)** Rationally designed stimulators of the ATPase activity of Hsp90, together with the structure of the complexes on which the design was successfully based. The black square indicates the allosteric binding site for the ligand, partially overlapping with the client binding region.

Figure 9. Three dimensional and schematic structures of Hsp70 in the presence of ATP or ADP.

Figure 10. Chemical structures of Hsp70 activators Myricetin and 115-7c, and of the inhibitor 116-9e developed by the Gestwicki group, together with their binding sites on Hsp70.

Figure 11. Chemical structures of MKT-077 and YM-08 developed by the Gestwicki group together with the binding site on Hsp70.

Figure 12. Chemical structures of allosteric Hsp70 ligands developed by the Chiosis group.

Figure 13. Chemical structures of small molecules targeting the SBD of Hsp70, together with crystal structures of the respective complexes (4wv7.pdb for Novolactone and 4r5g.pdb for PES-16).

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Biographical Sketch

Mariarosaria Ferraro got her Master Degree in Chemistry and Pharmaceutical Technologies at the University of Bologna in 2012, under the supervision of Prof. Andrea Cavalli. During her Ph.D. she focused on modeling lipid rafts and membrane proteins involved in drug addiction. In 2016, after a period as a visiting student in the lab of Prof. Ross Walker at the San Diego Supercomputer Center, she obtained her Ph.D. in Drug Discovery at the University of Genoa in collaboration with Istituto Italiano di Tecnologia (IIT) under the supervision of Dr. Giovanni Bottegoni. She is currently a post-doc researcher at ICRM CNR in the group of Prof. Giorgio Colombo, where she works on anticancer compounds targeting molecular chaperones.

Ilda D'Annessa obtained her M.Sc in Bioinformatics in 2007 and her Ph.D. in Biochemistry and Molecular Biology in 2010 at the University of Rome Tor Vergata. She worked in the group of Prof. A. Desideri at the Dept. of Biology. Here, she used computational biophysics approaches to study the functional properties of protein-DNA interactions, focusing mainly on human topoisomerase IB as a target for anticancer therapy, through the characterization of its interaction with inhibitor compounds and functional consequences of mutants with altered sensitivity. In 2016 she joined ICRM CNR as a post doc in the group of Prof. Giorgio Colombo, focusing on the study of Hsp90/co-chaperone-client interactions and in the development of allosteric modulators.

Elisabetta Moroni got her Ph.D. (2006) in Complex systems in post-genomic biology at the University of Torino, focusing on developing effective energy functions to evaluate DNA-protein binding, under the supervision of Prof. M. Caselle. Next, she joined the group of Profs. L. Belvisi and A. Bernardi at the University of Milano as a post doc working on the development of anticancer drugs. She then moved to the pharma company NMS (Nerviano, Italy) where she

worked on the design of kinase inhibitors. She continued her research activity at ICRM CNR in the group of Prof. Colombo, focusing on theoretical methods to study functional mechanisms of proteins and the design of cancer therapeutics, before taking up her current position as researcher at IRCCS MultiMedica in Milano.

Giulia Morra obtained her M.Sc. (1998) in Physics in Milan and her PhD in Chemistry (2005) at FU Berlin under the supervision of E.W. Knapp, focusing on electrostatics and MD simulations of proteins. In 2006 she joined the Biocomputing lab at ICRM CNR as a post doc under supervision of Giorgio Colombo, where she worked on developing methods for the analysis of allosteric phenomena in proteins, focusing on Hsp chaperone proteins. Since 2011 she is appointed as permanent research staff at the same institute.

Silvia Rinaldi obtained her MSc from the University of Perugia (2009) in the Center of Excellence for Innovative Nanostructured Materials. After receiving her PhD in 2013 in the lab of Prof. Olivucci at the University of Siena working on computational photochemistry and photobiology, she moved to ICRM-CNR as a Post doc researcher in the group of Prof. Giorgio Colombo. Her research interests are focused on the computational modeling of protein dynamics, analysis of molecular recognition processes and enzyme catalysis.

Federica Compostella received her Laurea Degree in Chemistry at the University of Milano. She worked in the laboratory of Profs. Scolastico and A. Bernardi at the University of Milano on the synthesis of compounds of pharmaceutical interest. After a period as a research fellow for the pharmaceutical companies Recordati SPA and Dompè Farmaceutici SPA, she became Assistant Professor at the University of Milano. She set up and is currently in charge of “Chemistry and

Introductory Biochemistry" courses at the recently founded International Medical School of the University of Milano. Her research focuses on the synthesis of compounds with biological and pharmaceutical applications with particular attention to glycoconjugates and saccharide antigens.

Giorgio Colombo is Professor of Organic Chemistry at the University of Pavia and Senior Researcher at ICRM CNR in Milano, where he set up and heads the Biocomputing lab. He received his M.Sc. and Ph.D. in Chemistry from the University of Milano, under the supervision of Profs. C. Scolastico and A. Bernardi. Before establishing his group, he worked with Prof. Ken Merz at Penn State University and Prof. A.E. Mark at the University of Groningen on computational approaches to enzyme reactivity and protein folding.

His research focuses on the study of the dynamic and energetic determinants of protein structural organization, molecular recognition and on the development of methods to design molecules with useful biological properties. He applied these concepts to chaperones and structural vaccinology.

Fig 1

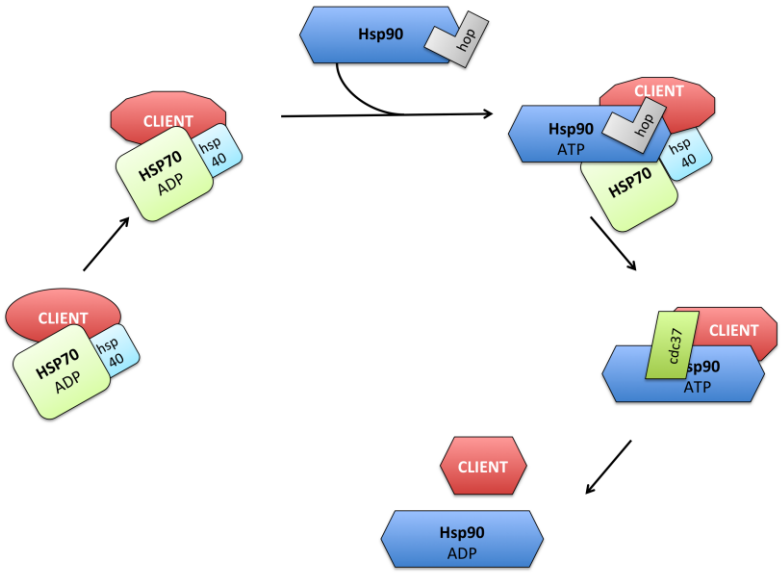


Fig 2

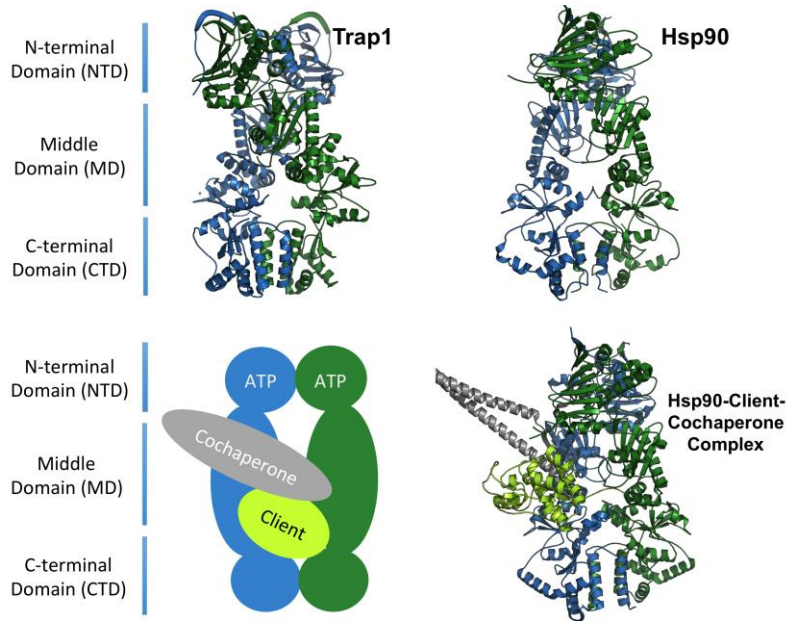

N-terminal Domain (NTD)
Middle Domain (MD)
C-terminal Domain (CTD)
Trap1
Hsp90
N-terminal Domain (NTD)
Middle Domain (MD)
C-terminal Domain (CTD)
ATP
ATP
Cochaperone
Client
Hsp90-Client-Cochaperone Complex

Fig 3

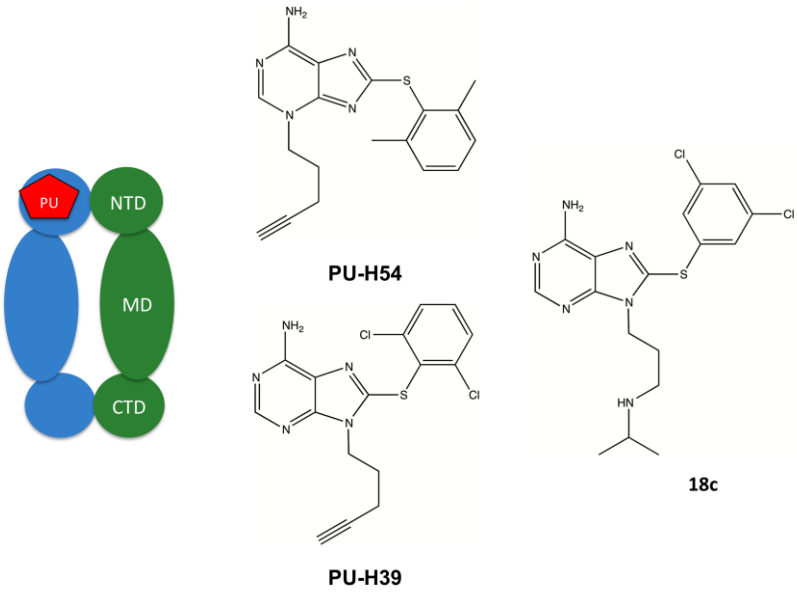
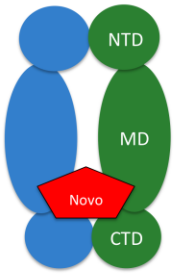
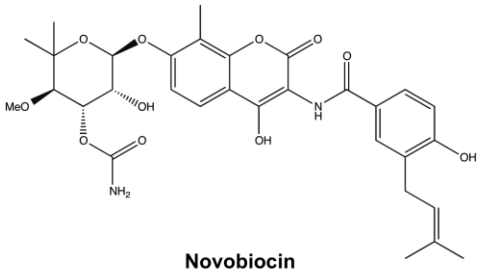
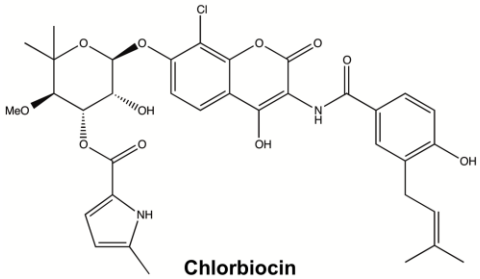

PU
NTD
MD
CTD
PU-H54
PU-H39
18c

Fig 4



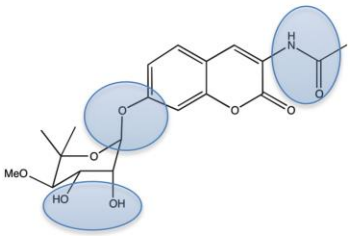


Novobiocin

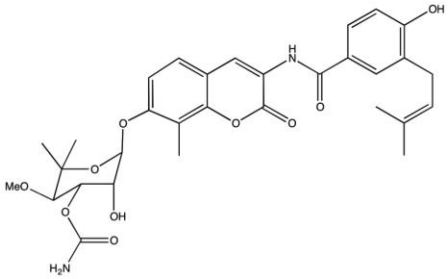


Chlorbiocin

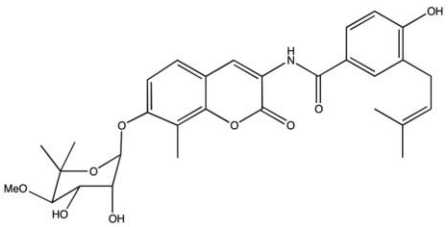
Fig 5



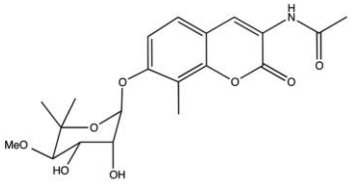
A4



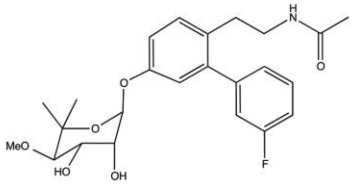
DHN1



DHN2



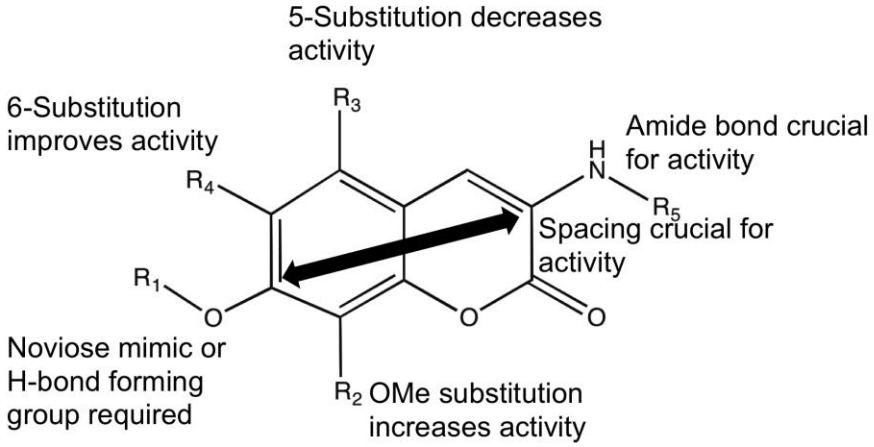
KU-32



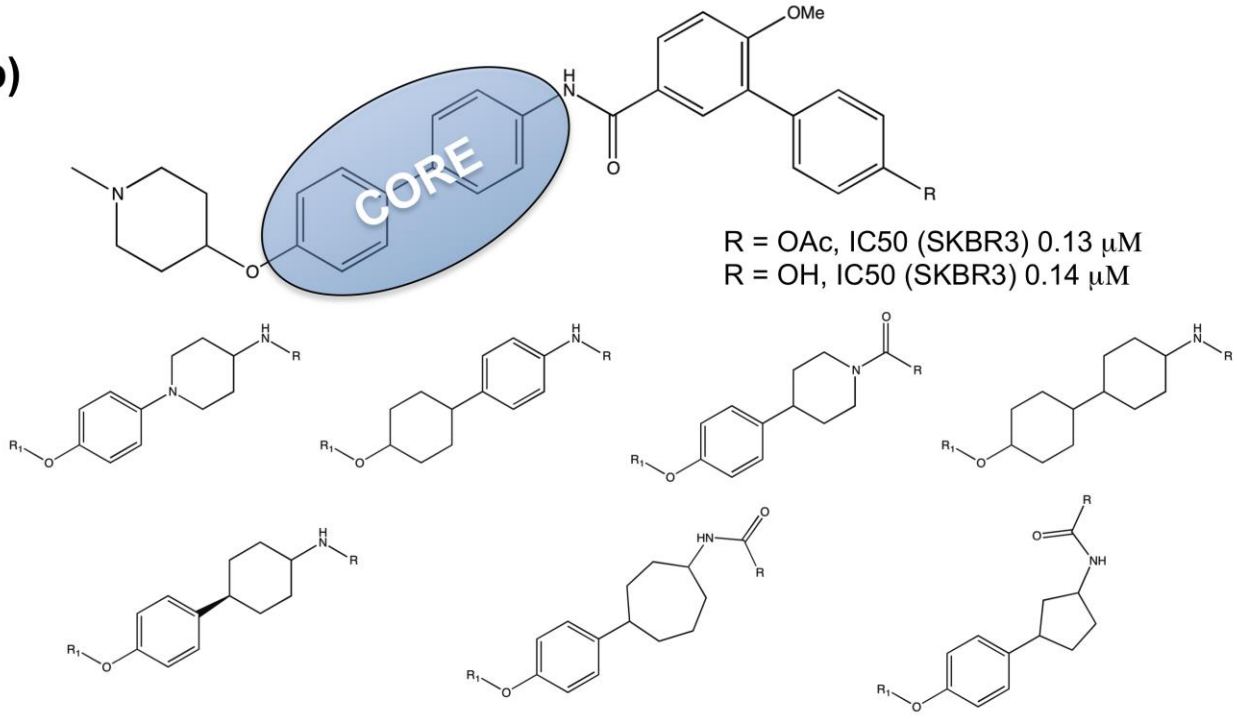
KU-596

Fig 6

a)


5-Substitution decreases activity
6-Substitution improves activity
Amide bond crucial for activity
Spacing crucial for activity
Noviose mimic or H-bond forming group required
R2 OMe substitution increases activity

b)


CORE
OMe
R = OAc, IC50 (SKBR3) 0.13 μM
R = OH, IC50 (SKBR3) 0.14 μM

c)

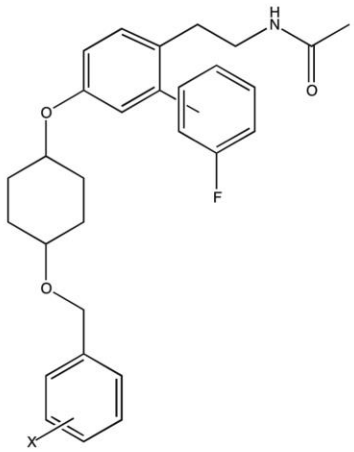

X = H, o-OCH3, o-CH3, p-Br, p-Cl

Fig 7

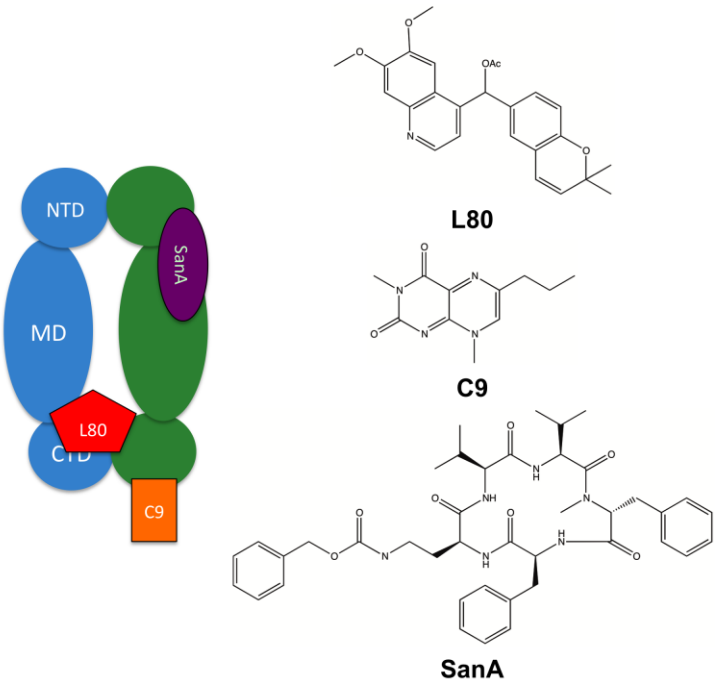

NTD
MD
SanA
L80
CTD
C9
L80
C9
SanA

Fig 8

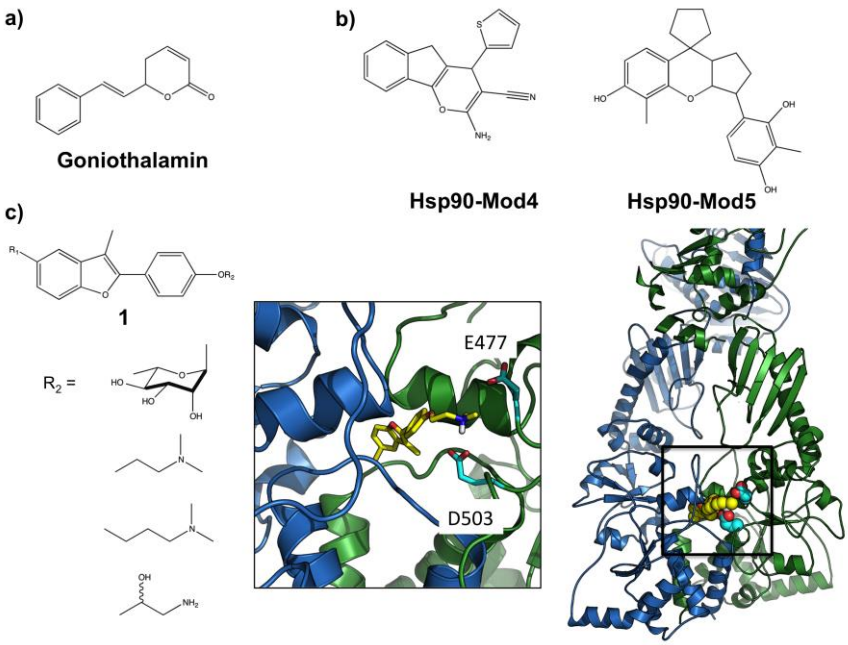

a)
Goniothalamin
b)
Hsp90-Mod4
Hsp90-Mod5
c)
1
R2 =
E477
D503

Fig 9

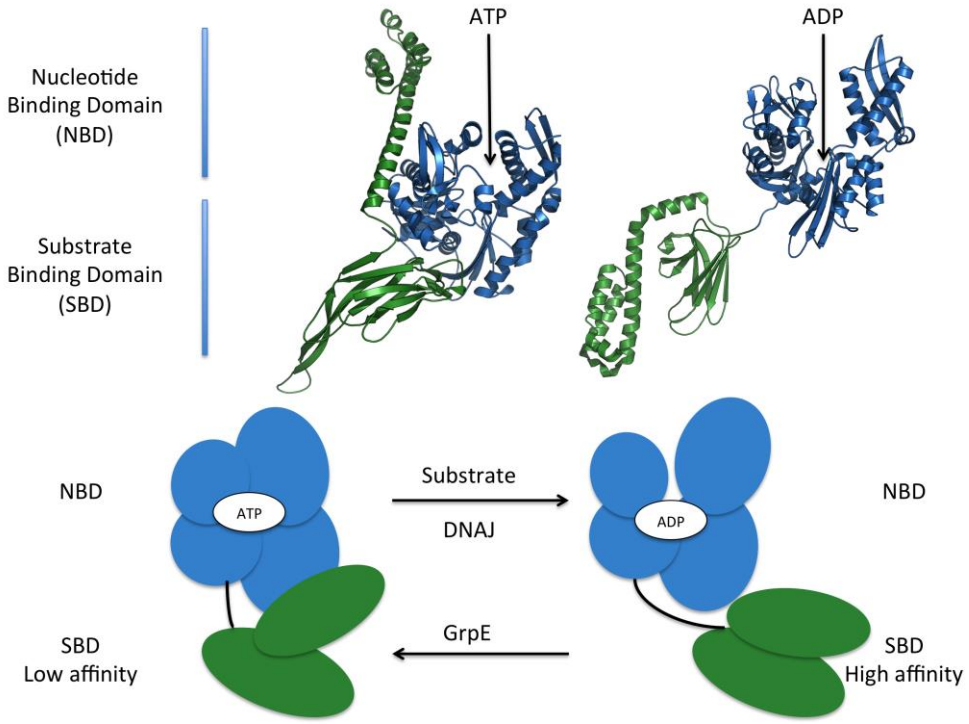

ATP
ADP
Nucleotide Binding Domain (NBD)
Substrate Binding Domain (SBD)
NBD
ATP
Substrate
DNAJ
ADP
NBD
SBD Low affinity
GrpE
SBD High affinity

Fig 10

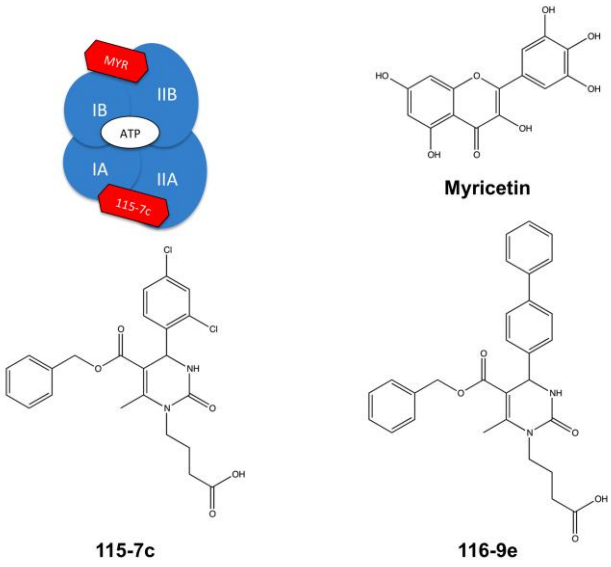
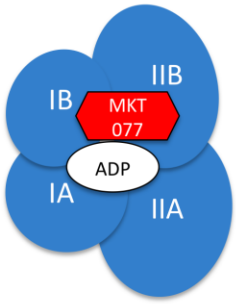
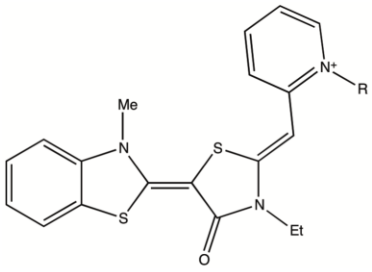

MYR
IIB
IB
ATP
IA
IIA
115-7c
Myricetin
115-7c
116-9e

Fig 11

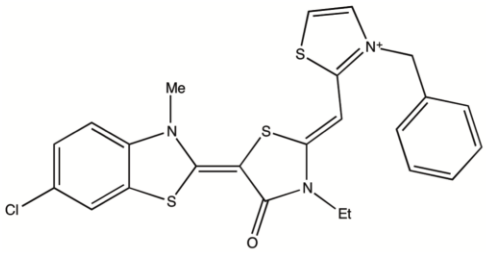




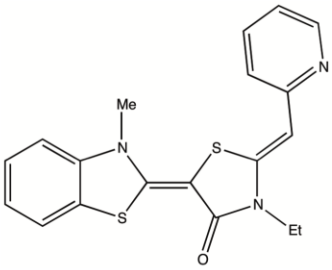
MKT-077

R = -Et MKT-077

R = -Me YM-01

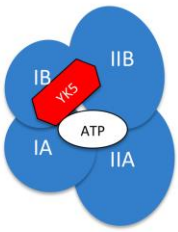


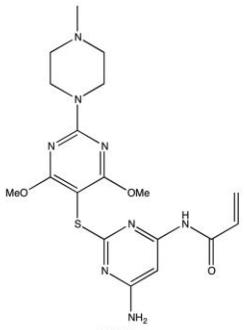
JG-98



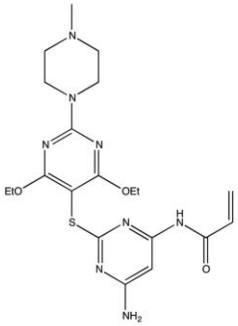
YM-08

Fig 12

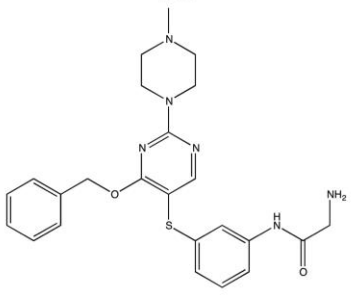




YK5

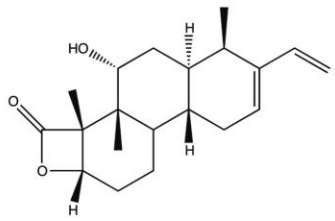


20a

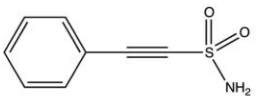


27c

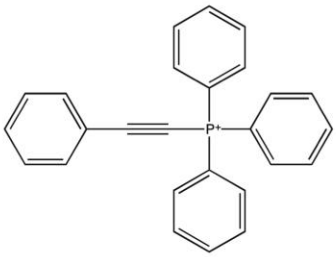
Fig 13



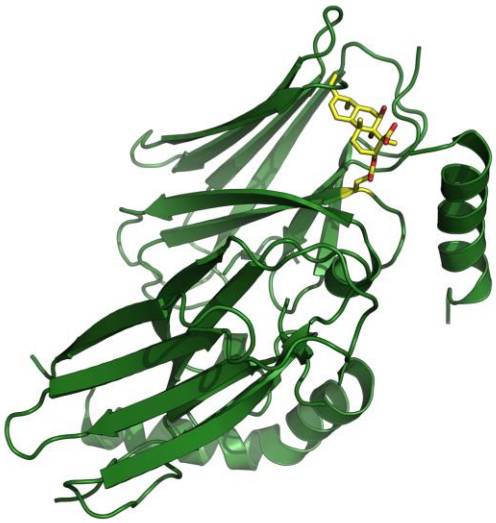
Novolactone



PES



PES-16



SBD

SBD