Hsp90-Targeted Library

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Introduction

Protein folding and assembly in the cell often requires the assistance of a diverse set of enzymes known as molecular chaperones. The major chaperone systems in the eukaryotic cytosol, Hsp70, TRiC/CCT, and Hsp90, are each essential for viability, suggesting that they fulfill nonoverlapping functions [1]. However, despite intensive mechanistic and functional studies, the spectrum of cellular substrates and functions mediated by these different chaperones remains largely undefined.

Heat shock proteins (Hsp90s) comprise a family of highly conserved molecular chaperones that are central to protein structure homeostasis. They plays an important role in the folding and activation of a range of client proteins involved in cell cycle regulation, steroid hormone responsiveness and signal transduction as well as morphological evolution [2]. They prevent aggregation of cellular proteins and act as ‘‘biochemical buffers’’ to guard proteins against diverse types of cellular stress including heat, changes in cellular pH, and hypoxia. Even under normal conditions, Hsp90 is a highly abundant cytosolic protein and is essential for cell viability. Therefore, Hsp90 is one of the key regulatory proteins in living cells. Hsp90 is a molecular chaperone whose association is required for the stability and function of multiple mutated, chimeric and over-expressed signalling proteins that promote cancer cell growth and/or survival. These proteins, named according to the 90-kDa average molecular mass of their members, are highly conserved molecular chaperones that account for 1–2% of all cellular proteins in most cells under non-stress conditions [3]. A fully functional Hsp90 protein normally associates with other cochaperones, playing an important role in the folding of newly synthesized proteins or stabilizing and refolding denatured proteins after stress [4]. Its expression is associated with many types of tumors including breast cancer and pancreatic carcinoma, human leukemia, and systemic lupus erythematosus, as well as multidrug resistance [5]. Hsp90 inhibition provides a recently developed, important pharmacological platform for anticancer therapy [6].

The "Hsp90 client proteins" are directly associated with Hsp90 and they include a wide variety of signal-transducing molecules that regulate cell growth and differentiation, protein kinases and transcription factors are more essential [7]. Hsp90 client proteins include mutated signaling

proteins (p53, Bcr-Abl, Raf-1, Akt), HER2/Neu (ErbB2), nNos, HIF-1α, epidermal growth factor receptors (EGFRs) and growth factor receptors (IGF-1Rs), Cdk4 [8]. These mediators are very necessary for cancer cell survival and proliferation. They behave as key regulators of cellular growth, differentiation, stress-response and apoptotic pathways. Hsp90 has also been found to interact with the products of several viral oncogenes including the Src-kinases v-Src [9] and v-Fgr [10]. Previous studies have implicated a role for the cytosolic chaperone Hsp90 in the synthesis, membrane association, and maintenance of Src-kinases. The cellular forms of both Lck and Fgr have been reported to interact with Hsp90 during and after in vitro translation [11]. In addition, the catalytic activity of a constitutively active form of Lck appears to require Hsp90 when the protein is expressed in fibroblasts [12]. Thus, Hsp90 may assist in folding and stabilizing Src-kinases.

Chaperoning of these client proteins is regulated through a dynamic cycle driven by ATP binding to Hsp90 and subsequent hydrolysis [13]. Hsp90 requires a series of cochaperones to forma complex for its function. These cochaperones, including Cdc37, Hsp70, Hsp40, Hop, Hip, p23, pp5, and immunophilins, bind and release in the superchaperone complex at various times to regulate the folding, assembly, and maturation of Hsp90 client proteins. As such, Hsp90 is central to many cellular processes including growth, cell cycling, apoptosis, cancer, stress response, endocrine function, plant immunity, development and even evolution [14]. Since Hsp90 was first shown to be the target of the ansamycin anti-tumor agent geldanamycin, there has also been increasing interest in Hsp90 as a therapeutic target [15].

As a huge number of known signaling proteins such as tyrosine kinases Hsp90 has a highly conserved active ATP-binding site. The existed reports towards ATPase activity have often varied from lab. to lab. and appeared to depend on the protein source and degree of purification. Anyway, binding and hydrolysis of ATP is well known in the HSP60, HSP60 and Hsp90 classes of molecular chaperones [16]. The major functions of the HSPs in normal cells include coordination of higher-order interactions between ‘‘client’’ proteins and macromolecular machines, organelles, trafficking and metabolic vesicles, and structural proteins such as microtubules and actin filaments. The HSPs, and Hsp90 in particular, facilitate signal transduction and gene transcription by stabilizing interactions between client proteins, proteins and nucleic acids, and proteins and their ligands [17]. A unique aspect of Hsp90 biochemistry is that this protein stabilizes rather than modifies its client proteins; and this, too, seems to be how it is involved in malignant transformation [18]. Mutations and/or amplifications in oncogenic receptor tyrosine kinases, such as EGFR and HER2, or oncogenic signal transducers, such as RAF and SRC, frequently lead to constitutively active proteins.

Summarizing, Hsp90 is the central component of a complex chaperone system whose cellular functions and mechanism are still poorly understood [19]. In higher eukaryotes, Hsp90

collaborates with a large set of cochaperones to mediate the conformational regulation of tyrosine kinases and steroid hormone receptors [20]. Hsp90 has also been proposed to buffer phenotypic variation of these signaling molecules, allowing cells to maintain a wild-type phenotype in the face of genetic mutations [21]. The structures of Hsp90 and several Hsp90 cofactors have recently been elucidated [22], but it is still unclear how this chaperone recognizes its substrates or affects their conformation.

Structure and functions of Hsp90

To the current day, transcriptome and genome analyses have revealed that the human Hsp90 family includes at least 17 genes that fall into four classes. These include: Hsp90AA, Hsp90AB, Hsp90B classes and TRAP contain 7, 6, 3, and 1 genes, respectively. However, the total number and chromosomal localization of the genes encoding members of the Hsp90 family in the human genome are still unknown. Earlier efforts that contributed to identifying and mapping the human Hsp90 family of genes were fragmented or misleading due to the use of an incomplete genome assembly, and to the limitation of the experimental methods used. Mammalian cells contain three types of Hsp90s: cytosolic Hsp90, mitochondrial Trap-1, and Grp94 of the endoplasmic reticulum. Each of the Hsp90s, as well as the bacterial homolog, HtpG, hydrolyze ATP and undergo similar conformational changes. Unlike the other forms of Hsp90, cytosolic Hsp90 function is dependent on a battery of co-chaperone proteins that regulate the ATPase activity of Hsp90 or direct Hsp90 to interact with specific client proteins.

Hsp90 proteins can be found in the cytosol, nucleoplasm, endoplasmic reticulum (ER), mitochondria, and chloroplasts [23]. Most Eubacteria have a single homologue of Hsp90 known as HtpG (high-temperature protein G), whereas Archaebacteria lack a Hsp90 representative [24]. All eukaryotes possess cytosolic members, called Hsp90 (90 kDa heat-shock protein), in the sensu stricto. There are two major cytosolic isoforms of Hsp90, Hsp90AA1 (inducible form) and Hsp90AB1 (constitutive form) [25]. These two isoforms are the result of gene duplication approximately 500 million years ago [26]. A recent report added another cytosolic isoform to the Hsp90 family, Hsp90N, which is associated with cellular transformation [27]. The ER paralogue, generally called Grp94 (94-kDa glucose-regulated protein), is present in all eukaryotes except fungi, which seem to have lost it, and is suggested to have originated via gene duplication very early in evolution [28]. A chloroplast homologue that is most similar in sequence to ER Grp94 has been found in three plant species, suggesting that it originated from the common ancestor of Eubacteria and chloroplasts [29]. The mitochondrial paralogue, TRAP1 (tumor necrosis factor receptor-associated protein 1), is most closely related to Eubacterial HtpG sequences, which that suggests it

originated from a HtpG-like ancestor [30]. As a distinctive feature, TRAP1 possesses a unique LxCxE motif that is absent in all other Hsp90 family members [31].

Hsp90 is a large, homodimeric protein with three main structural domains [32]. The *N*-terminal domain contains the ATP- and geldanamycin-binding site, and is responsible for the weak intrinsic ATPase activity of Hsp90. The middle domain, which is thought to be the major site of client protein binding, is connected to the *N*-terminal domain through a highly charged linker region. The *C*-terminal domain contains the dimerization interface and a conserved *C*-terminal MEEVD motif, which is responsible for binding TPR-containing cochaperones. Cytosolic Hsp90 binds to many cochaperones that regulate its activity and form part of the Hsp90 assembly machine (Fig. 1).

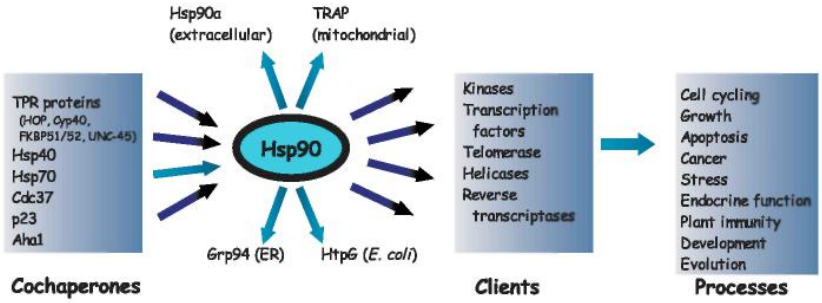


Fig. 1. Cytosolic Hsp90 and its homologs Grp94, TRAP and HTPG along with numerous cochaperones form the cellular assembly machine. They act on a range of client proteins and thereby control many cellular processes.

Structural studies on Hsp90 and its homo-logs have also provided important information on how client proteins might be recognized and bound by Hsp90. David Agard (University of California San Francisco) presented recent work on the structure of the C-terminal domain of the E. coli homolog HtpG [33]. The structure shows an unusual solvent-exposed α-helix. When the structure is docked into a 3.5-Å molecular envelope of the full-length protein, these helices project toward what is thought to be the central, client protein-binding cavity. The sequence of these helices is remarkably similar to that of helix 12 of the steroid receptor family, leading to the suggestion that this helix may displace helix 12 on the receptors, thus facilitating ligand binding.

As briefly mentioned above, the ATPase activity of Hsp90 is known to be essential for its function and mutants with hyper- and hypo-ATPase activity have compromised function *in vivo*. The middle domain of Hsp90 is also known to play a key role in the binding of client proteins, and previous structural work showed that it can interact with and affect the activity of the *N*-terminal ATPase domain. The structural basis for the stimulation of ATPase activity by the cochaperone was presented by Chris Prodromou (Institute Wof Cancer Research, London). Aha1 binding to the middle domain of Hsp90 repositions a loop in the middle domain such that it optimizes interactions with the *N*-terminal domain critical for ATP hydrolysis. Johannes Buchner presented work showing

that the cochaperone Sti1 inhibits the ATPase activity of Hsp90 but, in addition, stimulates the ATPase of Hsp70 [34]. As Sti1 binds to both Hsp70 and Hsp90, it is thus critical in mediating the transfer of client proteins. Stimulation of the ATPase activity of Hsp70 promotes dissociation of the client protein from Hsp70, whereas inhibition of the ATPase activity of Hsp90 facilitates association of the client with Hsp90.

Conformational changes within and between domains are known to be critical to Hsp90 function. Conformational changes induced in Hsp90 upon nucleotide and inhibitor binding were addressed by several speakers. Chris Prodromou presented further evidence for an *N*-terminal dimerization model for yeast Hsp90, whereas Sophie Jackson (Cambridge University) presented an alternative model for human Hsp90. In this second model, ATP binding is followed by a conformational change, but this does not involve dimerization of the *N*-terminal domains [35]. Changes to the structure of Hsp90 upon nucleotide or inhibitor binding were nicely illustrated in two talks by David Agard and Dan Gewirth (Duke University Medical Center). Dan Gewirth showed that in the structure of the Grp94 *N*-terminal domain a hydrophobic patch is exposed, leading to the suggestion that this may be another potential cochaperone or client-protein binding site. Remarkably similar conformational changes were observed by David Agard when comparing the isolated *N*-terminal domain of HtpG with ADP and the full-length apo structure. David Agard presented very new and exciting results on the high-resolution structure of full-length HtpG in both apo and ADP-bound states, revealing dramatic alterations in domain relationships and suggesting a model for how ATPase is activated. Dan Gewirth presented several structures of the *N*-terminal domain of Grp94 in complex with nucleotides and inhibitors. There are substantial differences in the mechanism of action of Grp94 compared with that of Hsp90 (no cochaperones have yet been identified and there is no detectable ATPase activity), and subtle structural differences may account for the different modes of action. In particular, nucleotides and inhibitors induce different conformations of the lid: the inhibitors stabilize a closed conformation whereas nucleotides force the lid into an open conformation that allows *N*-domain dimerization [36]. The differences can be attributed to an insert of five amino acids in Grp94 that repositions a glycine in the ATP-binding site. From all the structural and mechanistic studies, it is becoming clear that Hsp90 can adopt numerous conformations and may be very flexible in solution in the absence of client protein or cochaperones.

Hsp90 as a promising therapeutic target

The recent identification of Hsp90 as a promising target for anticancer therapies also highlights the practical implications of understanding the extent of chaperone function in the cell [37]. The fact that Hsp90 has become such a popular target is due, in part, to the intrinsic selectivity

of tumor cells toward geldanamycin (GA) and its derivatives. The molecular basis for this has not been understood. Francis Burrows (Conforma Therapeutics) presented work addressing this important issue. He showed that Hsp90 in tumor cells is present in a highly active state in which it is in complex, has a high ATPase activity and high affinity for inhibitors such as GA [38]. Although more work is needed to characterize this state in detail, this result has provided a much-needed model for the differences in Hsp90 activity in normal and tumor cells. Paul Workman (Institute for Cancer Research) reported on the success of phase I clinical trials with the geldanamycin derivative 17-AAG, describing in detail the effects of this drug on the cellular levels of important biomarker proteins such as Ck4 and Raf3. Preliminary trials using combinatorial approaches where 17-AAG is used in conjunction with other drugs such as the taxanes show much promise as an alternative therapeutic strategy. Combinatorial therapeutic approaches were also described by Len Neckers (National Cancer Institute, National Institutes of Health), in this case with inhibitors of Hsp90 and the proteasome. He also described recent experiments on the effects of Hsp90 inhibitors on cell migration and tumor invasiveness that have come up with surprising results. In these cases, even cell-impermeable inhibitors are active, leading to the discovery of an extracellular form of Hsp90, Hsp90α. Hsp90α seems to play a key role in the activity of matrix metalloproteases (MMPs), which are known to control cell migration. However, caution was stressed in a study presented by John Price (St. Vincent's Institute, Melbourne, Australia) showing an enhancement of bone loss in response to 17-AAG treatment. In a mouse model, 17-AAG increased tumor-associated bone destruction by stimulating osteoclast formation.

To date, nearly all cancer-promoting Hsp90-client interactions, including those that block programmed cell death, stem from Hsp90-stabilized, aberrant signal transduction events (Fig. 2; important exceptions include FKBP38 and p53). In most cases, Hsp90-mediated stabilization of protein–protein interactions requires an *N*-terminal ATPase activity (amino acids 1–210) and a core client binding motif (amino acids 272–629). Client proteins associate in transient low-affinity complexes with Hsp90 dimers and affiliated proteins, whereupon bound ATP is hydrolyzed at the N terminus of Hsp90, which leads to a conformational change in the Hsp90–client complex. This conformational change in the complex activates and stabilizes client proteins. Because tumor cells have significantly higher levels of activated Hsp90 than normal cells, small molecules that inhibit the *N*-terminal ATPase activity show a high degree of tumor specificity [39]. Several of these inhibitors are in early-stage cancer therapeutic clinical trials.

More than a decade of work has established that Hsp90 affects all of the ''hallmarks of cancer'' (Fig. 2) [40]. Hsp90 interacts with several components of the apoptosis machinery to promote survival, and the best-described interactions feed through the AKT signaling pathway. Dysregulation of the phosphoinositol 3-kinase/AKT pathway is a common event in human

malignancies and usually occurs through overactivation of AKT by mutation/amplification of genes in the pathway, or inactivation of PTEN by mutation or promoter hypermethylation [41]. Constitutively active AKT is stabilized by its interaction with active Hsp90-cdc37, which facilitates phosphorylation of AKT by pyruvate dehydrogenase kinase [42]. Hsp90 also seems to protect phospho-AKT from dephosphorylation and deactivation by PP2A. Activated AKT perpetuates survival signaling in several ways: AKT phosphorylates (and inhibits) several members of the proapoptotic Bcl2 family, inhibits caspase-9 activity, and promotes survival indirectly by stimulating NF-kappaB-dependent transcription by phosphorylating IKK [43].

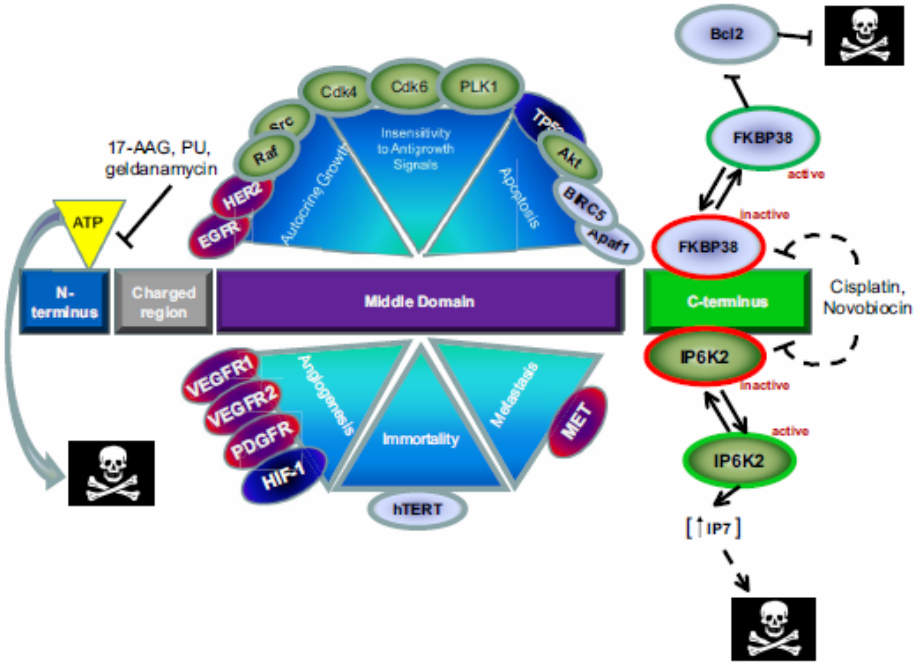


Fig. 2. Schematic representation of Hsp90 binding to client proteins, how these proteins affect six hallmarks of cancer, and drug target sites within Hsp90.

As shown in fig. 2, Hsp90 interacts with proteins that contribute to at least six hallmarks of cancer. Hsp90 binds to many of the expressed kinase domains within the human genome. Hsp90 stabilizes the active conformations of both WT and mutant tyrosine kinase receptors (red-purple), cytosolic serine-threonine and tyrosine kinases (green), transcription factors (blue), structural proteins and other enzymes (gray). It should be noted, that no specific sequence within the middle domain as a binding site is implied by the cartoon. Client binding occurs through the middle domain of Hsp90 (purple rectangle),whichleads to Hsp90 dimerization, cochaperone binding (HSP70, HIP,HOP,cdc37), and ATP binding and hydrolysis. Many of these interactions are inhibited by small molecules that compete for the *N*-terminal ATP binding pocket such as the benzoquinone, ansamycins (geldanamycin, 17-AAG), radicicols, their derivatives, and purine analogues (PU). Thus, many signal transduction pathways require Hsp90 to perpetuate growth promoting signals, and attenuation of these signals by inhibition of Hsp90 ATPase activity leads indirectly to cell

death. On the other hand, proteins that bind to the C terminus (green rectangle) of Hsp90, such as FKBP38 and IP6K2, are maintained in a constitutively inactive state by the interaction (red border). Drugs such as cisplatin and novobiocin (which interact with the C terminus of Hsp90 at high concentrations) appear to disrupt these interactions, leading to the release and activation of the now cytosolic C-terminal binding partners (green border) and subsequent apoptosis, either through inhibition of Bcl2 (FKBP38) or increased cytosolic concentrations of IP7 (IP6K2). Dashed arrows indicate interactions that have yet to be demonstrated biochemically.

The diverse activities of Hsp90 seem to be coordinated in part by numerous cochaperone proteins that can bind Hsp90 and contribute specialized functions to the chaperone machine. The actual functions of most of these cochaperones are still unclear. They may modulate specific steps in the chaperone pathway, assist in recruiting or chaperoning client proteins, or direct trafficking of chaperone complexes. However, it now seems that some Hsp90 cochaperones have a variety of activities, including some that aren't necessarily linked to Hsp90. This is the case for the small, deceptively simple cochaperone p23, which binds to Hsp90 complexes late in the chaperoning process.

Prokaryotic HtpG and the organelle-specific Hsp90s, GRP94 and TRAP1, seem to act without cochaperones. A cellular function for these 'lonely' chaperones is established only for GRP94, whereas we know little about the bacterial homolog HtpG, which lacks a marked mutant phenotype and is absent in many prokaryotes. Thus far the only prokaryotic organism with an established function for HtpG is the cyanobacterium Synechococcus sp. PCC7942. HtpG is required for basal and acquired thermotolerance, acclimation to low-temperature stress, and oxidative stress [[44]]. Hitoshi Nakamoto (Saitama University) reported that htpG mutants show reduction of photosynthetic pigments under normal conditions. This reduction involves instability of linkers that connect hexamers of the pigment phycocyanin to assemble phycobilisomes, the primary light-harvesting complexes. A yeast two-hybrid approach identified a protein functioning in the biosynthesis, the uroporphyrinogen decarboxylase HemE, as an HtpG-interacting protein, possibly representing a client. Queitsch used phylogenetic profiling to determine genes whose presence or absence is correlated with HtpG presence across 63 sequenced organisms. Among the genes associated with Hsp90 presence are some that are involved in stress responses and motility as well as others implicated in DNA repair after UV treatment.

Based on his data on the regulation of GRP94-client interaction by ATP and ADP, Chris Nicchitta (Duke University Medical Center) proposed that levels of adenosine nucleotides may serve as a 'stress sensor' for the ER-specific Hsp90. Decreases in adenosine nucleotide levels would allow for derepression of GRP94, facilitating interaction with clients.

As Yair Argon (University of Pennsylvania) reported, loss of GRP94 function in mice causes embryonic lethality, owing to a failure to develop the mesoderm germ layer. Mutant embryonic stem (ES) cells give rise to a number of cell types with the exception of muscle cells and show inhibition of Ca2+ homeostasis and hypersensitivity to serum deprivation. Both mutant animals and ES cells represent excellent models for studying mammalian GRP94 function in vivo.

Although Hsp90 interacts with a relatively small set of client proteins as compared with other general chaperones, these clients have quite different structures and functions. Understanding how Hsp90 specifically recognizes its clients, therefore, represents a considerable challenge. Several talks addressed this important question by identifying the binding regions on client proteins as well as in Hsp90 itself. Ami Citri from the Weizmann Institute presented recent work on the ErbB receptor family establishing a key sequence in erbB2 that mediates recognition by Hsp90. This sequence is located on a surface loop in the kinase domain of the receptor and is important in regulating dimerization and therefore the kinase activity.

Recent work provided evidence that the chaperone Hsp90 can serve as such a buffer in Drosophila melanogaster. Remarkably, it can do so in a multitude of different morphological pathways. In all eukaryotes tested, Hsp90 is essential, abundant at normal temperatures, and induced by stress. Under physiological conditions, Hsp90 dynamically interacts with a diverse but highly select set of inherently unstable ‘client’ proteins (as mentioned above - kinases and transcription factors). Typically, it keeps these metastable proteins poised for activation until they are stabilized by conformational changes, such as those associated with signal transduction. The requirement of many principal regulatory proteins for Hsp90 renders entire pathways sensitive to decreases in its function. In Drosophila, challenging Hsp90 function by mutation, pharmacological inhibition or environmental stress can produce a profusion of morphological changes affecting virtually every structure of the fly. Notably, the particular change observed in an individual fly depends on previously silent genetic variation. In the two cases tested, multiple polymorphisms affecting specific developmental pathways could be enriched by selection so that the traits were expressed even after Hsp90 function was restored. Thus, it appears that Hsp90 allows the storage and release of genetic variation in Drosophila6 as a consequence of its essential function in chaperoning regulators of growth and development. If so, this effect might be conserved in other organisms, potentially influencing the pace and nature of evolution.

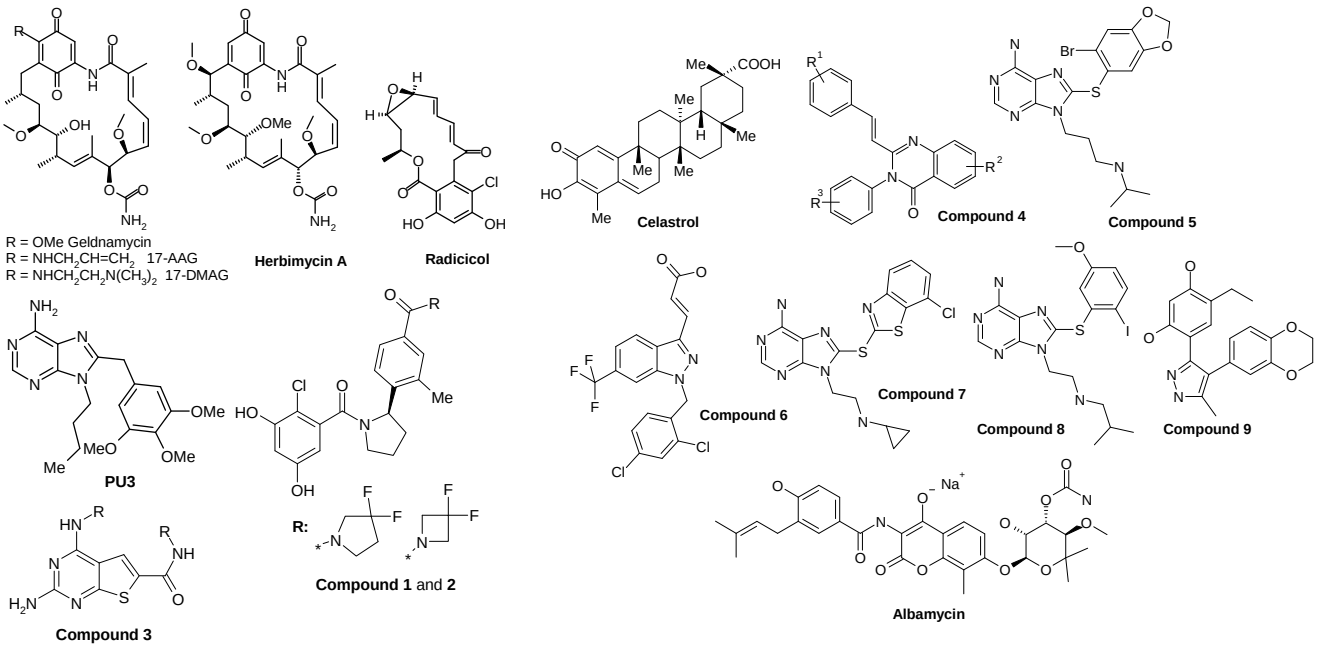
Inhibitors of Hsp90 activity

With the rapid rise of tumor resistance, combinatorial anticancer therapies have gained favor over single-molecule inhibition to maximize the suppression of oncogenic pathways. In this regard, Hsp90 inhibitors have rapidly emerged as a class of promising drugs that can target multiple

oncogenic pathways simultaneously [45]. In the last decade, a large number of oncogenic client proteins have been identified to associate with Hsp90 and contribute to malignant transformation. Development of Hsp90 inhibitors, derived from the natural compound geldanamycin that mimics the ATP binding site of Hsp90, was designed to target Hsp90 and allow degradation of these client proteins. Preclinical and clinical data with Hsp90 inhibitors in various cancer models are promising, and evidences also hint at the potential for tumor-selective cytotoxicity as well as enhanced sensitization to chemo- and radiotherapy.

It has been proposed that Hsp90 inhibitors, by interacting specifically with a single molecular target, cause the destabilization and eventual degradation of Hsp90 client proteins. As such they have shown promising levels of anti-tumour activity in preclinical model systems, and one Hsp90 inhibitor, 17-AAG (Fig. 3), is currently in Phase II clinical trials [46]. In addition, several synthetic Hsp90 inhibitors are currently in Phase I evaluation, including a purine-scaffold agent CNF-2024 [47] and SNX-5422 [48] developed by Serenex and Novartis, have also recently moved into clinical evaluation. Hsp90 inhibitors are unique in that, although they are directed towards a specific molecular target, they simultaneously inhibit multiple signalling pathways on which cancer cells depend for growth and survival. In addition, anti-cancer selectivity may derive from the simultaneous combinatorial effects of Hsp90 inhibitors on multiple cancer targets and pathways. Therefore, Hsp90 is an ideal protein target for anti-cancer research and this activity has been reviewed recently [49].

Well known Hsp90 inhibitors include: geldanamycin (GM; 50), 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG; 51), 17-allylamino-17-demethoxygeldanamycin (17-AAG; NSC 330507; 52), herbimycin A (NSC 305978; 53), radicicol [54] and PU3 [55]. These represent a wide range of structures.



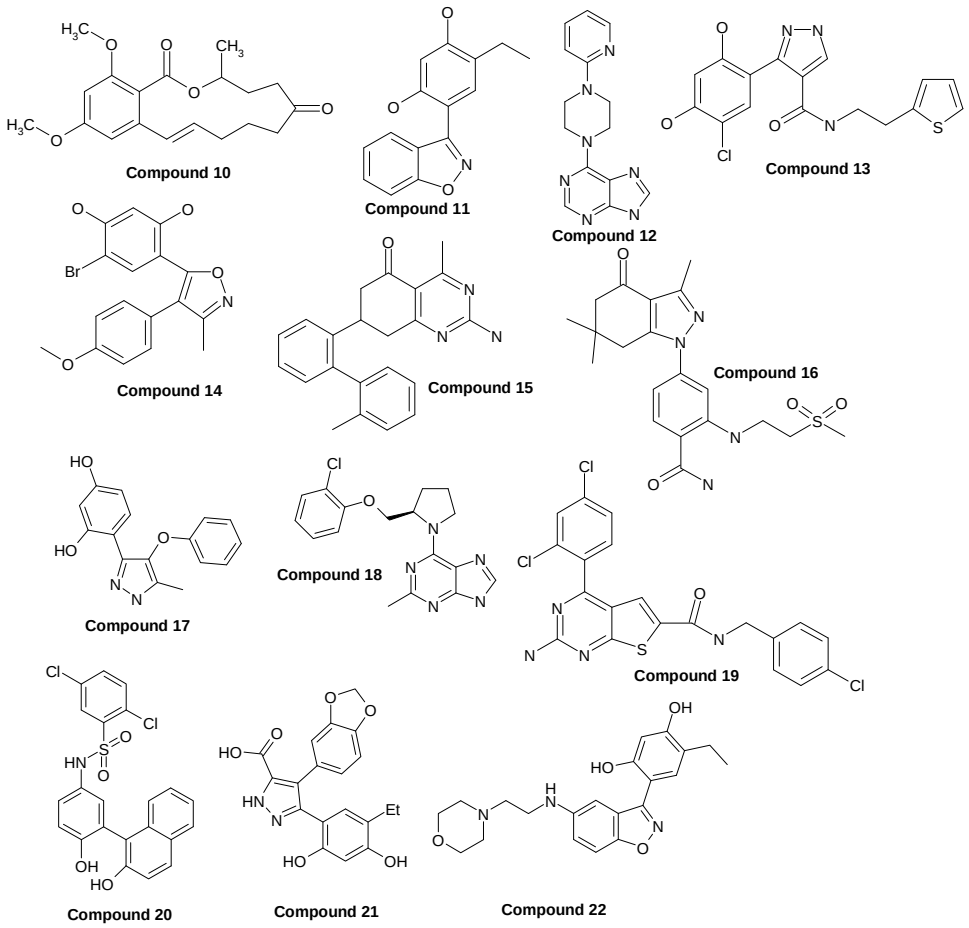


Fig. 3. Representative Hsp90 inhibitors (The shown chirality for the Geldanamycin and the 17-DMAG here is the same as that in the crystal structures).

It was also reported that celastrol (Fig. 3) disrupted Hsp90-Cdc37 interaction in the superchaperone complex to exhibit antitumor activity *in vitro* and *in vivo*, particularly against pancreatic cancer [[56]]. The related molecular docking and molecular dynamic simulations have showed that celastrol blocked the critical interaction of Glu33 (Hsp90) and Arg167 (Cdc37). In contrast to classic Hsp90 inhibitor (geldanamycin), celastrol (0.1-100 Mmol/L) did not interfere with ATP binding to Hsp90. However, celastrol (1-5 Mmol/L) induced Hsp90 client protein degradation (Cdk4 and Akt) by 70% to 80% and increased Hsp70 expression by 12-fold. Celastrol induced apoptosis *in vitro* and significantly inhibited tumor growth in Panc-1 xenografts. Moreover, celastrol (3 mg/kg) effectively suppressed tumor metastasis by more than 80% in RIP1-Tag2 transgenic mouse model with pancreatic islet cell carcinogenesis. This data suggest that celastrol is a novel Hsp90 inhibitor to disrupt Hsp90-Cdc37 interaction against pancreatic cancer cells.

Many natural-occurring compounds, such as Geldanamicyn or Radicicol, act as Hsp90 inhibitors [[57]]; however, to date, only the 17-allylamino-geldanamicyn has shown to exert a potent antitumor activity in a preclinical model and it is currently in clinical trials. A well-known mechanism of Hsp90 inhibition involves the compounds geldanamycin and its derivative 17-

allyamino-geldanamycin blocking ATP binding to Hsp90. Thus, treatment of cells with geldanamycin results in inactivation, destabilization, and degradation of Hsp90 client proteins. These compounds cause the catalytic cycle of Hsp90 to arrest in the ADP-bound conformation, subsequently leading to premature release and degradation of client proteins. This method has proven to be feasible therapeutically, such that 17-allyamino-geldanamycin has entered clinical trials [58]. In fact, a modified geldanamycin with lower toxicity, 17-AAG, has been examined in phase I clinic trials with encouraging results. However, a number of current Hsp90 inhibitors employ the same mechanism of ATP blockage for inactivating this chaperone. None of these inhibitors has received the Food and Drug Administration approval. It would be premature to conclude that the strategy of blocking the ATP binding to Hsp90 is a viable approach for the development of Hsp90 inhibitors. In addition, many compounds that might have inhibited the function of Hsp90 were probably excluded during drug screening simply because they could not bind to the ATP pocket. Because the Hsp90 chaperoning process involves the transient formation of multiprotein complexes with cochaperones, halting the chaperoning cycle at various stages is also likely to achieve Hsp90 inhibition.

While 17-(allylamino)-17-demethoxygeldanamycin has shown promise in clinical trials, this compound class has significant template-related drawbacks. Because of this limitation, novel chemotypes are strongly needed. Thus, a new, potent non-ansamycin small-molecule inhibitor of Hsp90, BX-2819, containing resorcinol and triazolothione rings was recently described [59]. Structural studies have demonstrated binding of BX-2819 to the ADP/ATP-binding pocket of Hsp90. The compound blocked expression of Hsp90 client proteins in cancer cell lines and inhibited cell growth with a potency similar to 17-(allylamino)-17-demethoxygeldanamycin. In a panel of four cancer cell lines, BX-2819 blocked growth with an average IC_{50} value of 32 nM (range of 7-72 nM). Efficacy studies have demonstrated that treatment with BX-2819 significantly inhibited the growth of NCI-N87 and HT-29 tumors in nude mice, consistent with pharmacodynamic studies showing inhibition of Hsp90 client protein expression in tumors for greater than 16 h after dosing. The obtained data support further studies to assess the potential of BX-2819 and related analogs for the treatment of cancer.

The structure-based design, synthesis, structure-activity relationships (QSAR) and pharmacokinetics of potent small-molecule inhibitors of Hsp90 based on the 4,5-diarylisoxazole scaffold were recently presented in [60]. Analogues from this series have high affinity for Hsp90, as measured in a fluorescence polarization (FP) competitive binding assay, and are active in cancer cell lines where they inhibit proliferation and exhibit a characteristic profile of depletion of oncogenic proteins and concomitant elevation of Hsp72. One compound from this series was suggested to be highly potent in the Hsp90 FP binding assay (IC_{50} = 21 nM) and inhibit

proliferation of various human cancer cell lines *in vitro*, with GI_{50} averaging 9 nM. It is retained in tumors *in vivo* when administered i.p., as evaluated by cassette dosing in tumor-bearing mice. In a human colon cancer xenograft model, this compound inhibits tumor growth by ~50%.

Information from X-ray crystal structures were used to optimize the potency of a HTS hit in the Hsp90 competitive binding assay. A class of novel and potent small molecule Hsp90 inhibitors were thereby identified. Thus, two enantio-pure compounds (Compound 1 and 2, fig. 3) were potent in PGA-based competitive binding assay and inhibited proliferation of various human cancer cell lines *in vitro*, with IC_{50} values averaging 20 nM.

As part of an oncology chemistry program directed toward discovery of orally bioavailable inhibitors of Hsp90, several solution-phase libraries were designed and prepared [61]. A library of racemic resorcinol amides was prepared affording more than hundred purified compounds. After evaluation in a binding assay, followed by an AKT-Luminex cellular assay, three potent analogs had functional activity between 0.1 and 0.3 μM. Resolution by preparative chiral SFC chromatography led to 3 compounds having functional IC_{50} = 27, 43, and 190 nM, respectively. One of these exhibited high clearance in human hepatocytes driven primarily by glucuronidation as confirmed by metabolite identification. A second exploratory library was designed to investigate heterocyclic replacements of the resorcinol ring. The second library highlights the use of the (-)-sparteine-mediated enantioselective Pd-catalyzed α-arylation of *N*-Boc-pyrrolidine to prepare chiral 2-arylpyrrolidines in parallel.

Brough and coworkers [62] have recently described novel 2-aminothieno[2,3-*d*]pyrimidine ATP competitive Hsp90 inhibitors, which were designed by combining structural elements of distinct low affinity hits generated from fragment-based and *in silico* screening exercises in concert with structural information from X-ray protein crystallography. Examples from this series have high affinity (IC_{50} = 50-100 nM) for Hsp90 as measured in a fluorescence polarization (FP) competitive binding assay and are active in human cancer cell lines where they inhibit cell proliferation and exhibit a characteristic profile of depletion of oncogenic proteins and concomitant elevation of Hsp72. Several examples (3 compounds) caused tumor growth regression at well tolerated doses when administered orally in a human BT474 human breast cancer xenograft model.

A novel class of 3-phenyl-2-styryl-3*H*-quinazolin-4-one Hsp90 inhibitors (for example Compound 4, see fig. 3) with *in vitro* anti-tumor activity has been recently identified by structure-based virtual screening of a chemical database with docking simulations in the *N*-terminal ATP-binding site (Fig. 4), *in vitro* ATPase assay using yeast Hsp90, and cell-based Her2 degradation assay in a consecutive fashion [63]. These results exemplify the usefulness of the structure-based virtual screening with molecular docking in drug discovery. The structural features responsible for a tight binding of the inhibitors in the active site of Hsp90 were discussed in detail.

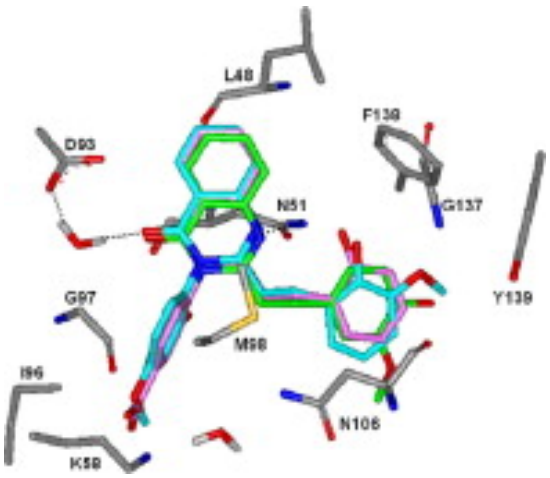


Fig. 4. 3-Phenyl-2-styryl-3*H*-quinazolin-4-ones in the active site of Hsp90.

In addition, docking-based virtual screening identified 1-(2-phenol)-2-naphthol compounds as a new class of Hsp90 inhibitors of low to sub-micromolar potency [[64]]. The paper has reported the binding affinities and cellular activities of several members of this class. A high resolution crystal structure of the most potent compound reveals its binding mode in the ATP binding site of Hsp90, providing a rationale for the observed activity of the series and suggesting strategies for developing compounds with improved properties.

Concept and Applications

Hsp90-targeted library design at CDL involves:

• *A combined profiling methodology that provides a consensus score and decision based on various advanced computational tools*:

1. Unique bioisosteric morphing and funneling procedures in designing novel potential TK ligands with high IP value. We apply CDL's proprietary Chemosoft^{TM} software and commercially available solutions from Accelrys, MOE, Daylight and other platforms.

2. 3D-molecular docking and 3D-pharmacophore modeling/searching.

3. Computational-based `*in silico*` ADME/Tox assessment for novel compounds includes prediction of human CYP P450-mediated metabolism and toxicity as well as many pharmacokinetic parameters, such as Brain-Blood Barrier (BBB) permeability, Human Intestinal Absorption (HIA), Plasma Protein binding (PPB), Plasma half-life time ($T_{1/2}$), Volume of distribution in human plasma (V_d), etc.

The fundamentals for these applications are described in a series of our recent articles on the design of exploratory small molecule chemistry for bioscreening [for related data visit ChemDiv. Inc. online source: www.chemdiv.com].

3D-Molecular docking

As mentioned above, the Hsp90 family is composed of four members: Hsp90a, Hsp90b (cytoplasm), Grp94 (endoplasmic reticulum) and Trap-1 (mitochondria). The *N*-terminal domain (hereafter Nt-Hsp90) has been studied by crystallography and contains an unusually shaped ATP binding cleft, known as the Bergerat fold, responsible for the ATPase activity important for function [[65]]. The Nt-Hsp90 domain is shown in figure 5. In the current study, we have focused on human Hsp90α in complex with the PU3 (1UY6; [66]). to design our Hsp90-targeted library.

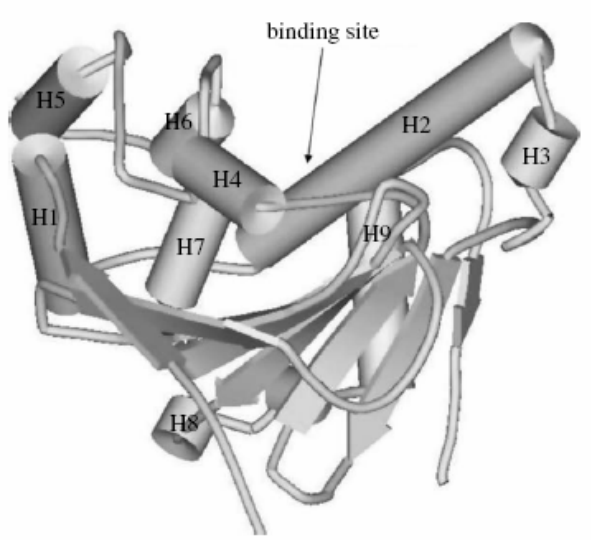


Fig. 5. Crystal structure of human Hsp90α.

A number of crystal structures of the Hsp90α-ligand complexes have demonstrated that at least two principal conformations exist: the 'open' conformation and the 'closed' conformation [for example, see: [67],[68]]. It was observed that these structures are mainly essentially identical. The main variation between the open and closed structures is in the conformation of residues 104–111. These comparisons demonstrate that there is considerable plasticity in this region of the structure, which is at the entrance to the ATP binding site. Some of the residues seen as important for binding to ligands are in this loop, e.g. Leu 107.

Crystal structures are also available for a series of complexes between Nt-Hsp90α and the ligands PU3 [[69]], ADP [[70]], geldanamycin [[71]] and radicicol [[72]]. The key interaction with Asp93 is preserved in all complexes as part of a network of hydrogen bonds around the carboxylate of Asp93 involving Asn51, Ser52, Thr184, Gly97 and four water molecules consistently found in the same positions. Three of these waters are hydrogen bonded to ADP, PU3 and geldanamycin. The important role played by the crystallographic waters in the recognition of the ligands by Hsp90 is further supported by the almost identical location of these water molecules across all the structures so far available. Of the 46 waters that show small fluctuations, all but eight appear to be close to the boundary of the simulation. These remaining eight water molecules are of considerable interest as

four correspond to the four conserved water molecules positions seen in all Hsp90 crystal structures. The remaining four immobile waters are found at the position near to the methoxy groups of the PU3, which make some hydrogen bond interactions with themethoxy group of thePU3 and form several hydrogen bonds to Gln23, Leu103 and Tyr139 (see figure 6 below). These four conserved water molecules are more stable at their positions than the others. Such information should be taken into account in future inhibitor design. As such the conserved water molecules determine the shape of the protein active binding site, and become a major factor in rational drug design.

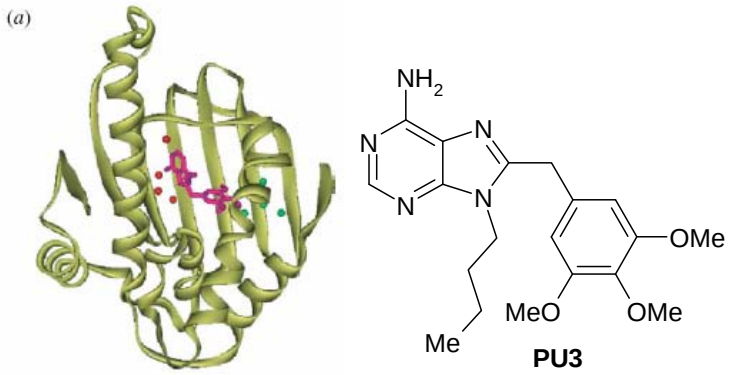


Fig. 6. The structure of the protein Hsp90 (in yellow), PU3 (in purple) and the position of the eight fixed waters during the molecular dynamics simulation (red: water 56, 133, 137 and 262; green: water 10, 148, 149 and 166).

The simulations also highlight the fact that Hsp90 is a difficult protein target because of the flexibility of the helix (residues 101–136 for 1UY6) and because there are several conserved waters participating in the interaction between it and its inhibitors. The simulations reveal that only four conserved water molecules (water molecules labelled as 56, 133, 137 and 262) are important for new inhibitor design, because their positions are stabilized when the conformation of the protein is changed.

The structural, dynamical and functional importance of water molecules for biomacromolecular structure and recognition is well appreciated. Water is known to contribute significantly to the stability of biomacromolecules and to play a crucial role in molecular association [[73]]. Complex structures have shown that waters can be very important in mediating the interaction between ligand and protein. That particular structural waters are crucial for the binding affinity or specificity of protein–ligand complexes is an important issue in the design of new ligands [[74]]. For example, Yan and coworkers [[75]] have recently shown that ligand binding (PU3) strongly requires water bridging via these conserved waters. Therefore, authors have shown which water molecules are persistent components of the binding site. Conserved water molecules play an important role in the interactions between the Hsp90 and ligands. Having defined the binding site based on the protein structure with several conserved water molecules, virtual docking of a series of

ligands of PU3 family molecules was performed, in order to show how conserved water molecules affect the interaction between the protein and the different ligands, which have the same scaffold and different substituents.

A comprehensive docking-based virtual screening has identified 1-(2-phenol)-2-naphthol compounds 20 and 21 (see fig. 3) as a new class of Hsp90 inhibitors of low to sub-micromolar potency [76]. The binding affinities and cellular activities of several members of this class were recently reported. A high resolution crystal structure (Fig. 7) of the most potent compound reveals its binding mode in the ATP binding site of Hsp90, providing a rationale for the observed activity of the series and suggesting strategies for developing compounds with improved properties.

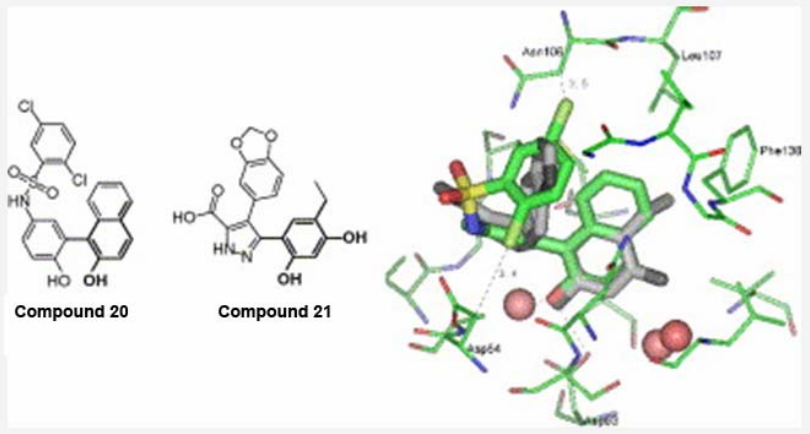


Fig. 7. Compounds 20 and 21 (see fig. 3) in the active site of Hsp90.

Gopalsamy and colleagues have recently identified small-molecule benzisoxazole derivatives (for instance, Compound 22, fig. 3) as Hsp90 inhibitors. Crystallographic studies show that these compounds bind in the ATP binding pocket interacting with the Asp93 (Fig. 8) [77]. Structure based optimization led to the identification of potent analogues, such as 13, with good biochemical profiles.

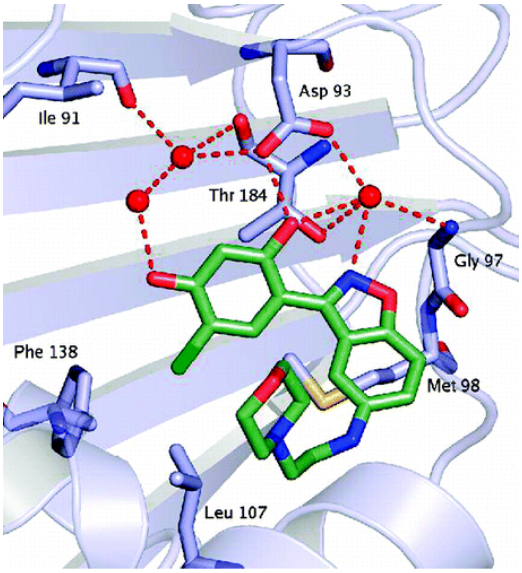
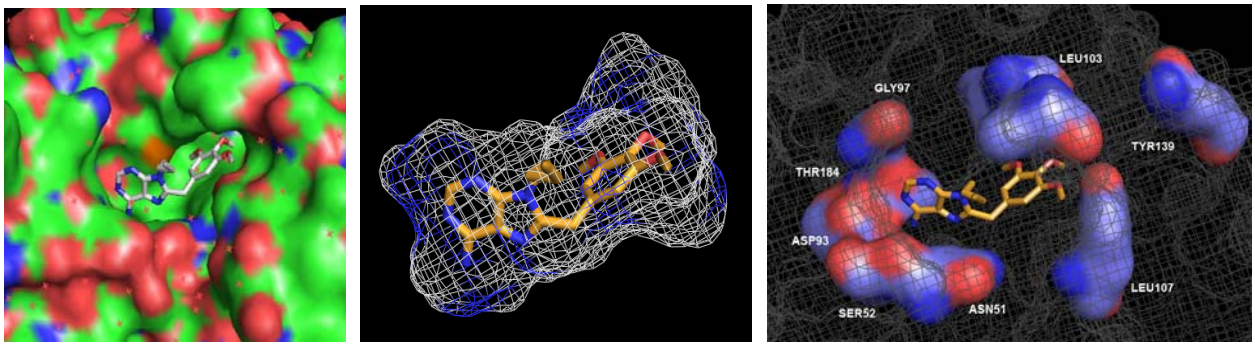


Fig. 8. Compound 22 (see fig. 3) in the active site of Hsp90.

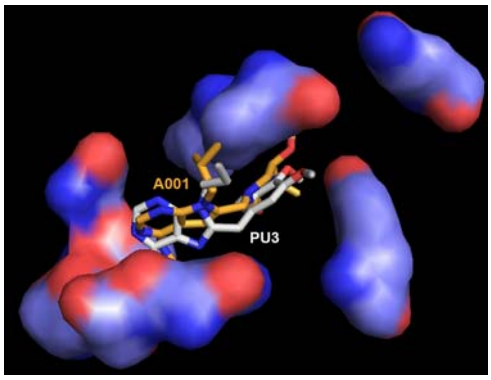
The mentioned data is absolutely invaluable for discovery of ligands with both diverse chemotypes and binding modes. Following the core concept, we have used a guided pharmacophore-constrained structure-based screening strategy (the related illustrations are not present here) for our focused-library design followed by 3D-molecular docking (see below). Thus, we have produced the respective 3D-pharmacophore space available to conduct virtual screening and to prioritize our compounds. Using sets of overlapping spheres derived from the protein-ligand complex crystallographic data, the active site of a receptor can be modeled. Sphere centers were used to define atom positions of a potential ligand as well as excluded volumes. We have generated 3D-molecular conformations using standard stereochemical rules and molecular mechanics refinements using a specific module integrated in ChemoSoft^{TM}. The obtained conformers were used as starting points for iterative modification of molecular geometry to obtain better fit for a previously generated 3D-pharmacophore. Results of this analysis were prioritized and 60K compounds with the best fit were selected for further evaluation.

Following the applied methodology, an initial round of our virtual screening against Hsp90 was accomplished for a set of 60K compounds selected from our collection in ChemDiv. Based on the structure of known Hsp90 inhibitors (see fig. 3) and using the constructed 3D-pharmacophore model, we yielded 20K high-score compounds. They were further scored against Hsp90 using 3D-molecular docking approach.

To design the title targeted library as well as to understand the interactions between Hsp90 active site and various small molecules, to find the key principles of the interactions and to provide the enhanced hit rate in the current drug design, the related docking studies were successfully carried out. Thus, molecular docking of the previously selected compounds was performed using Surflex Docking computational program *Version 1.24* (BioPharmics LLC). Structures successfully passed the 3D-pharmacophore searching were then accurately docked into the active binding site of Hsp90. Ten conformations for each structure were generated and docked into the binding site. There are two scores for each docked conformation: an affinity (-log(K_d)) (named as "polar") and a pen score (arbitrary units named as "penetration"). The pen score is the degree of the tested molecule penetration into the binding site of enzyme as well as the degree of internal self-clashing that the ligand is experiencing. Penetration scores that are close to 0.0 are favorable, however visual analysis of each conformer is more preferable. For instance, the penetration score of the reference compound (Ac-P(DON)LPF-NH_2) is close to 0.1 (*dimensionless*). Binding site and 'active' sphere-surface are shown in fig. 9a-c.



(**a**) (**b**) (**c**)



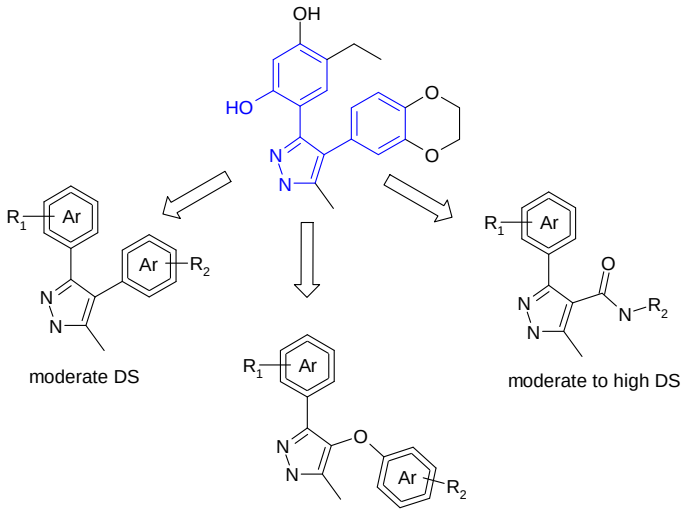
(**d**)

Fig. 9. (**a**,**c**) PU3 (orange) in the active binding site of Hsp90; (**b**) docking shape 3D-model; (**d**) PU3 (white) and the docked compound (A001, orange, see fig. 10) in the active binding.

As show in fig. 9, the selected molecule (A001) successfully penetrates into the active site of Hsp90. The "core head" which contains PU moiety is located deep in the binding pocket described above making the Asp93, Thr84, Gly97, Ser52 and Asn51 quite convenient to form corresponding hydrogen bonds. In turns, methoxy piperazine fragment is firmly embedded within the hydrophobic pocket via conserved water molecules fixing the molecule in a more suitable orientation thereby leading to tight binding. More than 10K ChemDiv compounds have been successfully passed through the docking trials; these compounds are the relevant content of the focused library developed. Representative examples of compounds from the resulting Hsp90-focused library are shown in fig. 10 (see below).

Close structure analogues and bioisosteric modifications

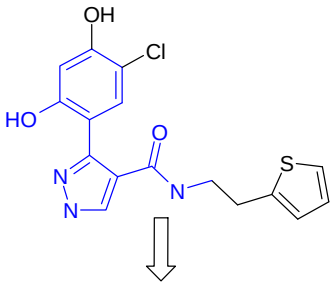
We have effectively applied the fundamental bioisosteric approach and structure diversity to design our Hsp90-targeted library. Corresponding bioisosteric modifications were successfully generated using a specific module integrated in the core ChemoSoft software. Several representative examples with the relative docking score (DS) are presented within fig. 10.



moderate DS

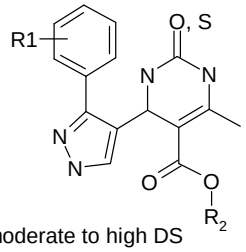
moderate to high DS

moderate DS

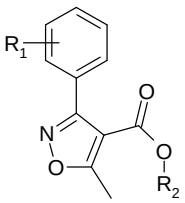


Bioisosteric replacement
More diversity
More interesting heterocyclic system

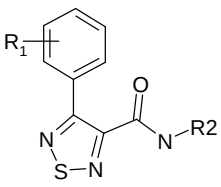
Diverse substituents looking at donating/withdrawing properties, H-bond acceptor/ donors as well lipophylicity and solubility



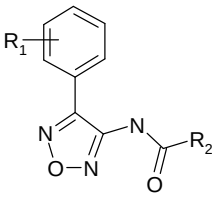
moderate to high DS



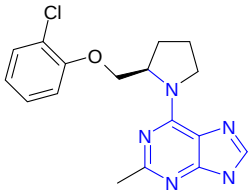
moderate DS

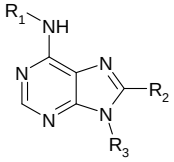


moderate DS

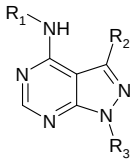


moderate to high DS

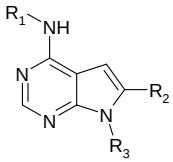




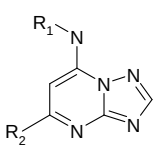
70 cmpds



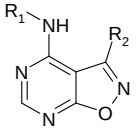
2000 cmpds



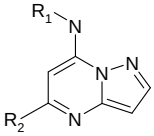
650 cmpds



650 cmpds



550 cmpds



380 cmpds

All the presented compounds with high DS

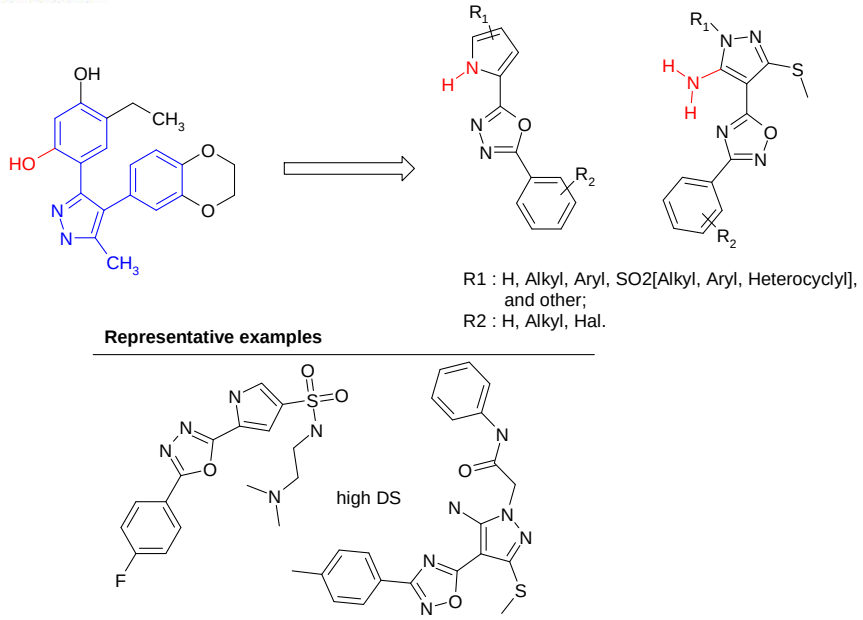


Fig. 10. Representative templates from our Hsp90-targeted library.

Conclusion

It should be especially noted that relatively modest progress in deep understanding pharmacology and clinical role of Hsp90s has been made since their discovery. From this point of view, specific natural and synthetic inhibitors of this chaperone are useful tools for dissecting Hsp90 role in both normal and aberrant biological processes. Further optimization of these molecules into clinical candidates may yield promising drugs with enhanced therapeutic potential, especially against cancer. As outlined in this study, successful discovery of novel Hsp90 leads relies on a combination of techniques from a wide range of disciplines, including 3D-molecular docking and 3D-pharmacophore-based design as well as on the traditional medicinal chemistry approach, such as bioisosteric transformations. The integration of high-throughput screening strategies with advanced virtual screening technologies holds great promise for more efficient discovery of Hsp90-targeted leads. Thus, here we provide efficient tools for *in silico* design of novel small molecule Hsp90 inhibitors. Based on the accumulated knowledgebase as well as unique structure- and target-based models we have been designed more than 10,000 small molecule compounds targeted specifically against Hsp90. As a result, the library is renewed each year, proprietary compounds comprising 50-75% of the entire set. Clients are invited to participate in the template selection process prior to launch of our synthetic effort.

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