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A Systematic Protocol for the Characterization of Hsp90 Modulators

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Abstract

Several Hsp90 modulators have been identified including the N-terminal ligand geldanamycin (GDA), the C-terminal ligand novobiocin (NB), and the co-chaperone disruptor celastrol. Other Hsp90 modulators elicit a mechanism of action that remains unknown. For example, the natural product gedunin and the synthetic anti-spermatogenic agent H2-gamendazole, recently identified Hsp90 modulators, manifest biological activity through undefined mechanisms. Herein, we report a series of biochemical techniques used to classify such modulators into identifiable categories. Such studies provided evidence that gedunin and H2-gamendazole both modulate Hsp90 *via* a mechanism similar to celastrol, and unlike NB or GDA.

Keywords

Heat shock protein 90; Novobiocin; Geldanamycin; Celastrol; Gedunin

The 90kDa heat shock proteins (Hsp90) are essential for both refolding denatured proteins as well as the conformational maturation of several nascent polypeptides into their biologically active structure. Hsp90-dependent client proteins are associated with signaling cascades in normal cells. However, regulation of cell signaling and proliferation is necessary in tumor cells due to their highly stressed environments, and Hsp90 is critical for maintaining their survival. Hsp90 inhibition has been shown to block several oncogenic signaling nodes responsible for regulating the six hallmarks of cancer simultaneously.

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Consequently, Hsp90 has emerged as a viable target for the development of cancer chemotherapeutic.[1–3]

Other disease states are also attenuated through modulation of Hsp90. Rheumatoid arthritis is an inflammation inducing autoimmune disease characterized by the aberrant penetration of immune system cells into joint synovial tissue along with the pseudo-oncogenic growth and proliferation of synovial fibroblasts and their invasion into surrounding cartilage. Inhibition of the Nf-kB pathway has been proposed as a treatment option for RA, and indeed, several proteins in this pathway are Hsp90-dependent, including RIP, IKK, and JNK, among others.[4]

Alzheimer's is a disease characterized by the accumulation of misfolded-proteins that demonstrates positive mediation by Hsp90 modulators. [5] The accumulation of β-amyloid (Aβ) oligomers in the brain has been shown to result in neurodegeneration. These Aβ aggregates initiate a cascade that leads to the hyper-phosphorylation and misfolding of tau protein, inflammatory reactions and cytotoxicity. [6] By stimulating the rematuration of misfolded proteins, Hsp90 induction has emerged as a potential strategy for the treatment of Alzheimer's disease, thereby promoting the search for non-toxic, small molecule Hsp90 modulators that induce the heat shock response, resulting in the overexpression of Hsp27, Hsp40, Hsp70, Hsp90 and associated proteins.[7]

Currently, significant efforts are underway to develop both known and novel Hsp90 inhibitors.[8–12] Such a process would likely benefit from the development of a set of experiments by which Hsp90 modulators can be mechanistically differentiated.

Modulators of Hsp90 have been found to interact primarily at two locations, the C- and N-termini of the protein. The Hsp90 N-terminus contains a nucleotide-binding domain that is responsible for the hydrolysis of ATP, which provides the requisite source of energy for the protein folding machinery. Binding and hydrolysis of ATP is mediated by at least two distinct Hsp90 conformations. Upon binding ATP, the N-terminal domains dimerize and adopt the clamped conformation. This induces protein folding, after which, ATP is hydrolyzed to ADP, forcing Hsp90 to adopt its open conformation and eventually, to facilitate client protein release.[13]

The N-terminal ATP binding pocket has been the focus of intense drug discovery efforts. The natural product inhibitors GDA (Figure 1) and radicicol, as well as their derivatives, bind to this site and prevent formation of the clamped conformation, and instead induce the open conformational state.[14,15] These compounds cause the accumulation of "intermediate" Hsp90 complexes incapable of proper folding by preventing the binding and hydrolysis of ATP (15). This serves to destabilize Hsp90-client complexes, and ultimately leads to client protein degradation *via* the ubiquitin-proteasome pathway.[8,9]

A second site of modulation is located near the N-terminus at the interface between Hsp90 and the kinase co-chaperone Cdc37. Experiments using small interfering RNA (siRNA) have shown that Cdc37 knockdown, in effect a complete disruption of Hsp90/Cdc37 interactions, leads to selective degradation of kinase client proteins, and strongly sensitizes Hsp90 to inhibition by GDA and other N-terminal inhibitors.[16–18] The natural product

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celastrol was identified as the first small molecule disruptor of this interaction.[19] Two mechanisms have been proposed to describe how celastrol manifests this activity. Through molecular modeling experiments, it was suggested by Zhang *et al*. that celastrol occupies the Cdc37 binding site on Hsp90, and blocks Cdc37 from occupancy.[20] However, Sreeramulu *et al*. observed the formation of celastrol/Cdc37 Michael adducts in solution, thereby suggesting celastrol covalently modifies Cdc37 cysteine residues, and therefore acts *via* irreversible inhibition.[21]

The C-terminus of Hsp90 is responsible for formation of the stable Hsp90 homodimer complex and contains a second nucleotide-binding sit.[1,22,23] The C-terminus, in concert with the middle domain of Hsp90, is also capable of mediating Hsp90's interactions with client proteins, promoting or preventing the binding of co-chaperones and immunophillins, and modulating ATPase activity. [24–26] It has been demonstrated that NB (Figure 1), a previously identified DNA gyrase inhibitor, binds the C-terminal nucleotide pocket and displaces both ATP and other ligands from the N-terminus. However, NB is a poor inhibitor, and modulates Hsp90 function at ~700 μM. In addition, Neckers and coworkers have shown that the N-terminal ATP-binding site must be occupied before the C-terminal site is accessible.[27,28]

An advantage of C-terminal versus N-terminal inhibition is lack of the heat shock response. [29] N-terminal inhibition results in displacement of heat shock factor 1 (HSF1), the transcription factor responsible for activation of the heat shock response.[30] Under normal conditions, Hsp90 binds HSF1, but in the presence of cellular stress or N-terminal inhibitors, this complex is disassembled and HSF1 translocates to the nucleus as a phosphorylated trimer that binds the heat shock binding elements.[31] Several modulators of Hsp90 function have been identified based on the NB scaffold and have been shown to induce client protein degradation without induction of the heat shock response.[29,32–34]

Some members of the NB-based class of compounds exhibit differing biological profiles. For example, KU32 induces a pronounced heat shock response upon incubation with cells at concentrations far below that needed to induce client protein degradation (unpublished results). The natural product gedunin has demonstrated Hsp90 inhibitory activity but does not compete with GDA for binding to the N-terminus.[19,35] Similarly, H2-gamendazole manifests Hsp90 inhibitory activity *via* an unknown mechanism of action.[36] In an effort to delineate the varying modes of Hsp90 modulation, as well as to develop techniques to affirm an Hsp90 inhibitor's mechanism of action, several Hsp90 inhibitors, including GDA, NB A4, A4-dimer, KU32, KU174, KU135, celastrol, gedunin, and H2-gamendazole (Figure 2) have been systematically evaluated for their effects on Hsp90 and their phenotypic profiles in cancer and yeast cells through proteolytic fingerprinting, affinity chromatography, co-immunoprecipitation, and induction of the heat shock response.

NB and GDA are two well-known Hsp90 inhibitors that have undergone extensive biochemical characterization.[10,15,27,28,37–40] They serve as both chemical tools and standards for which the biological activities to which other Hsp90 modulators are compared. Proteolytic fingerprints of Hsp90 in the presence of GDA or NB are quite different. Hsp90 in the presence of GDA or without ligand provides the same proteolytic fingerprint upon

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incubation with trypsin, i.e. proteolytic cleavage occurs at Arg 291, Lys 399, and Arg 612 (corresponding to mammalian Hsp90β numbering). Trypsin treatment of Hsp90 incubated with NB provides a fingerprint that results in cleavage at Arg 291 and Lys 399, but not Arg 612 (mHsp90β). In a complementary manner, affinity chromatography provides additional data for GDA and NB.[39,41] For example, when rabbit reticulocyte lysate (RRL) is applied to a GDA-sepharose column, Hsp90 is retained on the column, and can be eluted with increasing concentrations of GDA or NB. Conversely, when RRL is applied to a NB-sepharose column, Hsp90 is retained on the column and can be eluted with increasing concentrations of NB, but not GDA.[27,28] This differential activity for N-terminal and C-terminal inhibitors can be applied to further classify new modulators.

For Hsp90 modulators that do not result in either GDA or NB proteolytic fingerprints or similar chromatographic results, other mechanisms, such as disruption of Hsp90-cochaperone interactions, may be responsible for the observed inhibitory activities. In such cases, co-immunoprecipitation experiments can be used to further clarify the mechanism of action.

A library of NB Analogues

A library of NB analogues was designed to probe the essential nature of several residues found on the natural product and to expeditiously reveal modifications that could enhance Hsp90 modulation. All compounds were prepared in 1 M stock solutions in DMSO.

Reagents

The anti-Hsp90 antibodies used to detect proteolytic fragments of Hsp90 were obtained as follows. The AC88 monoclonal antibody recognizes the C-terminus of Hsp90 and was obtained from Stressgen. The affinity-purified polyclonal rabbit antibodies raised against a peptide representing the N-terminus of mouse Hsp86 were obtained from Affinity BioReagents, Inc (PA3-013). GDA was a gift from Infinity Pharmaceuticals. Molybdate was obtained from Sigma Chemical Co. All drugs were prepared as a 1M stock and stored as single use aliquots at −20°C.

Proteolytic Fingerprinting of Hsp90

TnT rabbits reticulocyte lysate was incubated under conditions for protein synthesis at 37 °C in the presence of drug or vehicle control for 5 min. The protein folding reaction mixtures contained 50% (v/v) rabbit reticulocyte lysate, an ATP regenerating system (10mM creatine phosphate and 20 units/mL creatine phosphokinase), and 75 mM KCl. The reaction mixtures contained drug/control as indicated. After incubation, the reaction mixtures were chilled on ice, followed by digestion with the indicated amount of TPCK-treated trypsin (Worthington) at the indicted concentrations. After digestion on ice for 6 min, reactions were stopped by immediate boiling in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and subjected to Western blot analysis using antibodies specific to the N-terminus of Hsp90 (PA3-013 Affinity BioReagents) or the C-terminus of Hsp90 (AC88, Stressgen).

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NB affinity column chromatography

Three grams of epoxy-activated sepharose 6B (Sigma Chemical Co.) was washed and swollen in 100 mL of distilled water for 1 hr at room temperature. The resin was washed further with coupling buffer (0.3 M sodium carbonate pH 9.5). The gel was mixed with 400 mg of NB (sodium salt, Sigma Chemical Co.) in 10 mL of coupling buffer and incubated at 37 °C with gentle rotation for 20 hours. The excess ligand was washed away with coupling buffer, and the remaining epoxy-active groups were blocked with 1 M ethanolamine in coupling buffer for 12 hours at 30 °C with gentle shaking. The gel was thoroughly washed sequentially with coupling buffer, 0.5 M NaCl in coupling buffer, distilled water, 0.5 M NaCl in 0.1 M sodium acetate (pH 4.0), and again in distilled water. It was then equilibrated in 25 mM HEPES (pH 8.0) containing 1mM EDTA, 10% ethylene glycol, and 200 mM KCl and kept at 4 °C protected for light.

Preparation of GDA affinity beads

Briefly, GDA was derivatized and immobilized as follows: 1,6-hexanediamine was added to GDA (10 mM in chloroform) at a 10 fold molar excess and allowed to react for 2 hours. After aqueous extraction, 17-hexamethylenediamine-17-demethoxyGDA was dried, redissolved in DMSO, and reacted with AffiGel 10 resin (Bio-Rad Laboratories). Before use, the GDA AffiGel beads were washed in buffer (50 mM Tris-HCl (pH 7.4), 1% P-40, 2 mM EDTA, 100 mM NaCl, and 1 mM sodium orthovanadate) and blocked with 1% bovine serum albumin.

RRL system and ATP regenerating reaction mixtures were incubated for 5 min under 37 °C in order to induce Hsp90 expression. After the RRL system was applied to the column and equilibrated with the coupling buffer (0.3 M sodium carbonate pH 9.5), it was washed with 20 volumes of the buffer containing 0.15 M NaCl. After washing the column, binding proteins were eluted with a linear gradient of drugs (0~5 mM) in the same buffer. All these experiments were carried out in the dark at 4 °C. The eluents were analyzed on SDS/PAGE (12.5 % gel) with anti-Hsp90 antibody.

Hsp90 coimmunoprecipitation

SKBr-3 cells were treated with vehicle, celastrol, gedunin and H2-gamendazole. GDA and NB were used as control. Cells were lysed in 20 mM Tris HCl (pH 7.4), 25 mM NaCl, 2 mM DDT, 20 mM Na_2MoO_4, 0.1 % NP-40, and protein inhibitors. Lysates were incubated for 2 hr at 4°C while rotating, and then centrifuged at 14,000×g for 10 min. Protein (500 μg) was incubated with anti-Hsp90 antibody for 2 hrs at 4°C. Protein G agarose (40 μl) was added to each sample, and samples were then incubated overnight at 4°C. The beads were washed with the same lysis buffer. Bound proteins were isolated by boiling in sample buffer, and subjected to SDS-PAGE, coimmunoprecipitating proteins were analyzed by western blot analysis.

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Regulation of oncoproteins involved in tumor proliferation

MCF-7 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium, Ham's F-12 (Gibco) supplemented with nonessential amino acids, L-glutamine (2 mM), streptomycin (500μg/mL), penicillin (100 units/mL), and 10% fetal bovine serum. Cells were grown to confluence in a humidified atmosphere (37°C, 5% CO2), seeded in culture dishes (1×10^6/dish), and allowed to attach overnight. Compounds were added at varying concentrations and returned to the incubator for 48 h. Cells were harvested and analyzed for Hsp90 client protein degradation as described previously.[42]

β-galactosidase assay

PP30 yeast strain expressing Hsp90a as their sole Hsp90[43] was transformed with the centromeric *URA3* vector, pHSE,[44] constitutively expressing β-galactosidase (encoded by *lacz*) as a reporter gene under control of a promoter bearing 3× Heat Shock Element (HSE) response elements.[44] Transformants were selected by DO medium (dropout 2% glucose medium) supplemented with appropriate amino acids without uracil.[45] Yeast cells were grown overnight to exponential phase with a cell density of $2–3\times10^6$ cells per ml in 50ml of the same medium at 30°C. Then, appropriate compounds were added to a final concentration of 30μM, followed by incubation at 30°C for 2h. Cells were additionally heat shocked at 39°C for 1h, collected by centrifugation (2000×g; 5 minutes), washed once with ddH_2O, and frozen at −80°C. The proteins were extracted as previously described,[46] except for exclusion of EDTA in the extraction buffer. β-Galactosidase activities of HSE were measured as previously described.[47] Cell lysate (10μl) was mixed with equal volume of 2×buffer Z (0.12M Na2HPO4.7H2O, 0.08M NaH2PO4.H2O, 0.02M KCl, 0.002M MgSO4) pH 7.0. The mixture was added to 700μl of 2mg/ml ONPG solution in 1×buffer Z prewarmed at 30°C and incubated at 30°C for 5–30 minutes. The reaction was stopped by adding 500μL of 1M Sodium Carbonate. The optical density at 420nm (OD) of each reaction mixture was determined. The protein concentration of the lysate was 420 determined by the BioRad assay (BioRad). The β-galactosidase activity was calculated using the following formula:

$$\text{Enzyme Activity} = 1000\times \text{OD}_{420}/\text{minute}/[10\mu\text{l}\times\text{protein concentration }(\mu\text{g}/\mu\text{l})].$$

Small molecule effects on the proteolytic fingerprint of Hsp90

We first studied the effect of GDA and NB on the conformation of Hsp90 from RRL in order to reproduce previously reported fingerprints. In agreement with previous work (39, 41), Hsp90 exhibited a different proteolytic fingerprint upon treatment with NB or GDA. The proteolytic fingerprint of Hsp90 in the presence of GDA was identical to the fingerprint produced upon treatment with vehicle control (Hsp90 is completely degraded by high concentrations of trypsin, suggesting that GDA has no effect on the protease sensitivity of Hsp90) (Figure 3). This fingerprint corresponds to trypsinolysis at several amino acid residues, including Arg 291, Lys 399, and Arg 612 (mHsp90β). Also in agreement with literature precedent, the proteolytic fingerprint of Hsp90 upon incubation with increasing concentrations of NB (Figure 3), in the presence of relatively high concentrations of trypsin (~125 μg/mL), resulted in a prominent band at 40 kDa, and no 78 kDa band was observed with antibody to the N-terminal domain. In contrast, the most prominent band occurs at 50

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kDa upon immunoblotting with a C-terminus antibody. When using relatively low concentrations of trypsin, both NB and GDA treated assays failed to show a strong band at 40 kDa (data not shown). With the proteolytic fingerprints of both NB and GDA re-established, trypsinolysis of Hsp90 in the presence of other Hsp90 modulators were compared.

We applied the fingerprint evaluation protocol to RRL incubated with Hsp90 modulators KU32, KU174, KU135, A4, A4-dimer, coumermycin A1, H2-gamendazole, celastrol and gedunin, and compared these data to those obtained with NB and GDA (Figure 3). The Hsp90 fragments resulting from trypsinolysis after incubation of RRL with NB-derived compounds KU32, KU174, KU135, A4, and A4-dimer, as well as coumermycin A1 were similar to those observed for NB. The most prominent band occurred following immunoblot with the C-terminal antibody. All of the NB analogues demonstrated the property of efficiently blocking trypsinolysis at the same amino acid residue as NB, Arg 612 (mHsp90β). This amino acid residue is proximal to the amino acid sequence between 657 and 677, which was discovered by Marcu *et al*. to be critical for C-terminal ligand binding.[27,28] These data support the conclusion that NB and its derivatives induce similar conformational effects on Hsp90, thereby suggesting a similar mode of binding for these compounds.

The Hsp90 proteolytic fingerprints observed from RRL incubated with H2-gamendazole, celastrol, and gedunin were unique when compared to the fingerprints obtained from RRL incubated with GDA or NB (Figure 3). When immunoblotting with the N-terminal antibody, a 78 kDa band was observed as the major band, suggesting that celastrol, gedunin, and H2-gamendazole all bind Hsp90 and efficiently block trypsin cleavage at amino acid residues Arg 291 and Lys399, unlike GDA, but do not block cleavage at Arg 612 (mHsp90β), unlike NB. When the C-terminal antibody was used, several weak bands appeared, however, no major band at 50 kDa was observed, unlike both GDA and NB. This provides further evidence to support the conclusion that these compounds are blocking cleavage at Hsp90 residues Arg 291 and Lys 399 (mHsp90β), and are inducing conformational effects distinct from GDA, NB, and NB derivatives. Also, this provides strong evidence that celastrol, gedunin, and H2-gamendazole exhibit their effects by direct interactions with Hsp90.

Computational rationalization of Hsp90 conformations and trypsinolysis results

To provide a plausible structural basis to explain these experimental data, computational analyses were conducted that included sequence-based analyses of cleavage sites and subsequent structural analyses of the cleavage sites environment in different Hsp90 crystal and solution structures.

Probable cleavage of the HtpG (bacterial Hsp90) sequence by trypsin was first explored *in silico* by using PeptideCutter[48] and OPAL (http://www.oppf.ox.ac.uk/opal/OPAL.php) approaches and respective web-based servers with the objective of gaining information about potential cleavage sites and their location. PeptideCutter[1] is a specialized tool that allows searching for possible cleavage sites in a protein sequence and predicting the

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probability of cleavage. The OPAL approach and server provides information about the probable cleavage sites ranked according to cleavage rate. A comparative analysis of the cleavage sites in the E. coli Hsp90 sequence has revealed a number of possible cleavage sites in the Hsp90 sequence. The experimentally observed Arg-291, Lys-399, and Arg-612 residues, which correspond to HtpG residues Arg-291, Lys-398, and Arg-616 respectively, were predicted by PeptideCutter to be amongst the sites that exhibit the highest cleavage probability of 100%, 94.9 %, and 82.6% respectively. A similar result was obtained from the OPAL server (Table 1), indicating these sites are predictable by different *in silico* tools.

Emerging structural and biochemical data has suggested that the Hsp90 molecular chaperone may exist in a conformational equilibrium between the open apo-form, the ADP-bound compact, and the ATP-closed bound protein state, which have been reported in previously solved crystal and solution structures. Comparison of Arg-291, Lys-398, and Arg-616 (HtpG) residues in the three conformations (open solution, semi-closed and closed) provided insight into the potential structural form of Hsp90 that binds NB (Figure 4). In the semi-closed and closed conformation of Hsp90, Arg-616 (HtpG) resides on the surface in an α-helix, and is fully exposed to the solvent. In the open, solution form, Arg-616 (HtpG) lies at the interface between the two monomers and is slightly obscured due to the presence of other residues. Hence, the probability of trypsinolysis at the Arg-616 (HtpG) position may be greater for the semi-closed and closed conformation as compared to open conformation in which it is less solvent exposed.

We have followed with the analysis of solvent accessible surface area (SASA) for the cleavage sites in different crystal structures of Hsp90 to evaluate whether Arg616 can be accessible by trypsin in these conformational states. Our calculations rationalized the experimental data and asserted structural preferences for NB binding, since it is reasonable to assume that NB may bias the Hsp90 equilibrium toward a structural form that is not readily amenable to trypsinolysis.

When the solvent accessibilities were calculated for the three conformations, it was found that in the open conformation, the Arg-616 (HtpG) residue resides in a low solvent accessibility (~24 $Å^2$) as compared to the semi-closed (~93 $Å^2$) conformation (Table 2), since it exists in a cavity at the interface of the two monomeric species, while in other conformations it remains on the surface. Thus, cleavage in the open structure is likely to be more difficult, since there is insufficient space for a protein the size of trypsin to bind Arg616 (HtpG).

Effects of NB analogues, celastrol, gedunin, and H2-gamendazole on affinity chromatography using NB- and GDA-sepharose beads

Determination that NB interacted with the C-terminus of Hsp90 was originally reported based on affinity purification experiments.[27,28] NB immobilized on sepharose beads demonstrated the retention of a C-terminal fragment of Hsp90, but not an N-terminal fragment. Also, increasing concentrations of GDA were found insufficient for the elution of Hsp90 from a NB-sepharose column. In contrast, however, an increasing concentration of NB eluted Hsp90 from GDA-immobilized sepharose beads. Other compounds that manifest

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similar activity to NB indicate a similar mode of binding and would provide a confirmational screen for newly discovered Hsp90 modulators that bind the C-terminus.

To reproduce the previously reported data, RRL was applied to either GDA or NB immobilized on sepharose beads and washed with 0.15M NaCl to remove non-specific binding proteins. The beads were then subjected to elution conditions with either increasing concentrations of GDA or NB (Figure 5, A and B). Results from these experiments confirmed those previously reported. With the proper conditions in hand, we evaluated the NB derived compounds KU32, KU135, KU174, A4, and the A4 dimer, as well as celastrol, gedunin, and H2-gamendazole in identical experiments (Figure 5, A, B, and C).

When using increasing concentrations of the NB-derived compounds to elute the bound Hsp90 from either the NB- or GDA-sepharose beads, Hsp90 was eluted from the column. Washing the NB- or GDA-sepharose beads with NB derived compounds produced the same effect as that observed with the parent compound. These data suggest that the NB-derived compounds bind Hsp90 similarly to NB.

When using celastrol, gedunin, or H2-gamendazole in increasing concentrations with GDA-sepharose, Hsp90 was eluted, but only at very high concentrations. However, Hsp90 was not eluted when the NB sepharose beads were washed with these compounds at high concentrations. These data suggest that celastrol, gedunin and H2-gamendazole affect Hsp90 in a distinct manner from that of N- and C-terminal inhibitors.

Celastrol, gedunin and H2-gamendazole disrupt interactions between Hsp90 and the cochaperone Cdc37 in SKBr-3 cells

Recently, it was reported that celastrol manifests anti-proliferative activity by disrupting formation of the Hsp90/Cdc37 complex, however, the mechanism by which celastrol manifests this activity has been disputed. It has been reported that celastrol binds Hsp90 at the Cdc37/Hsp90 protein interface. In contrast, it has also been reported that celastrol manifests its activity by covalently modifying cysteine residues on Cdc37, and subsequently preventing Cdc37 from binding Hsp90. Since the observed effects of gedunin and H2-gamendazole on Hsp90 in both the proteolytic fingerprint and affinity chromatography assays were identical to the effects of celastrol, gedunin and H2-gamendazole may elicit their anti-proliferative effects *via* a similar mechanism.

To determine whether gedunin and H2-gamendazole disassemble the Hsp90/Cdc37 complex, co-immunoprecipitation experiments were conducted. SKBr-3 breast cancer cells were incubated for 24 hours with increasing concentrations of celastrol, gedunin, H2-gamendazole, NB, and GDA and then lysed with NP-40 lysis buffer (Figure 6).

Lysates were immunoprecipitated with an anti-Hsp90 antibody and Protein G agarose. Protein was submitted to SDS-PAGE and analyzed by immunoblot, using the Hsp90 and Cdc37 antibodies. The control compounds, GDA and NB, exhibited no effect on the concentrations of Cdc37 following Hsp90 immunoprecipitation, in agreement with previously reported data,[39] as these compounds are unable to disrupt the Cdc37/Hsp90

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complex. However, celastrol, gedunin and H2-gamendazole, decreased the levels of Cdc37 in a concentration-dependent manner (Figure 7), supporting the hypothesis that all three compounds manifest a similar mechanism of action that results from disruption of the Hsp90/Cdc37 complex.

Effects of Hsp90 modulators on client proteins

A hallmark of Hsp90 inhibition is the degradation of Hsp90-dependent client proteins. Hsp90 inhibition prevents the maturation of client protein substrates by the generation of destabilized complexes between client proteins and the chaperone, resulting in ubiquitinylation and subsequent degradation of the client by the proteasome.[49] This leads to decreased levels of Hsp90 clients in cells and detection of this phenomenon by Western blot is commonly employed to confirm Hsp90 inhibition. In addition, a common hallmark of N-terminal inhibition is the up-regulation of Hsp's, such as Hsp70, which stems from induction of the heat shock response.

MCF-7 cells were incubated with GDA, NB, NB analogues, celastrol, gedunin, or H2-gamendazole for 24 hours at increasing concentrations (Figure 8). Cells were lysed, submitted to SDS-PAGE, and immunoblotted to detect protein levels of the client proteins AKT and HER2, and also the cochaperone Hsp70. All compounds demonstrated the characteristic degradation of both Her2 and AKT in a dose-dependent manner (Figure 8), confirming Hsp90 client protein degradation. This provides strong evidence that Hsp90 is the cellular target for these compounds.

In an effort to investigate induction of the heat shock response, Hsp70 levels were also evaluated. As an indicator that the NB derivatives and celastrol, gedunin, and H2-gamendazole do not bind the N-terminal ATP binding pocket, administration of these modulators at various concentrations did not result in increased Hsp70 levels.

Evaluation of the heat shock response induced by Hsp90 modulators

Heat shock transcription factor 1 (HSF1) is an Hsp90-dependent client protein that, when displaced from Hsp90, results in the heat shock response upon transcriptional activation of the heat shock genes by binding to the heat shock binding element. Previous work has shown that human Hsp90 is functional in yeast lacking endogenous Hsp90. Therefore, we examined the effects of NB and its analogues; KU32, KU135, and KU174, as well as coumermycin A1, celastrol, gedunin, and H2-gamendazole on HSF activity. PP30 cells expressing hHsp90α (as the only copy of Hsp90) and containing the HSF-*lac*Z reporter (Heat Shock Binding Element, HSE-*lac*Z) were treated with 100μM of the above compounds for 3 hours. In addition, these cells were subsequently stressed upon heat shock (39°C) for 1 hour.

Similar to GDA, KU32 induced a heat shock response even in the absence of heat stress (Chart 1), whereas NB and KU135 blocked induction of the heat shock response, even upon exposure to subsequent heat stress. Finally, KU174, coumermycin A1, celastrol, gedunin, and H2-gamendazole exhibited no altered effect on the heat shock response in yeast, suggesting these inhibitors do not interfere with Hsp90/HSF1 interactions (Chart 1).

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Hsp90 has emerged as an important molecular target for a variety of therapeutic uses. Consequently, a large number of Hsp90 modulators exist, however, it remains unclear as to the mechanism by which some of these modulators function. To delineate structure-function relationships, we provide a systematic approach toward unraveling the mechanistic features manifested by Hsp90 modulators. The combination of proteolytic fingerprinting, affinity chromatography, co-immunoprecipitation, and evaluation of the heat shock response have been used to characterize and differentiate the modes exhibited by three classes of Hsp90 modulators. While each technique provides valuable insight into a specific modulatory attribute of a given compound, the combined results provide insights into both old and new mechanisms of modulation as well as subtle differences between compounds within the same inhibitory class.

Among the compounds evaluated by this protocol, several are characterized by inclusion of the coumarin heterocyclic scaffold as found in the natural product NB. These compounds include the natural product coumermycin A1 and the NB-derived compounds KU32, KU135, KU174, A4, and the A4-dimer. A comparison of the proteolytic fingerprint of Hsp90 induced upon incubation with these compounds supports a similar mode of binding as NB. Unlike the proteolytic fingerprint of Hsp90 when incubated with GDA, these compounds blocked trypsin mediated cleavage of Hsp90 at residue Arg 612 (mHsp90β). *In silico* experiments provide additional evidence that these compounds bind Hsp90 in the open state, thereby shifting the conformational equilibrium into the open state, wherein residue Arg 612 (mHsp90β) is sequestered from the protein surface and unavailable for trypsin binding/cleavage.

Affinity chromatography and co-immunoprecipitation provide further, corroborating evidence that KU32, KU135, KU174, A4, and the A4-dimer bind similarly to NB. These NB-derived compounds displace N-terminal ligands, as demonstrated by their ability to elute Hsp90 from a GDA-sepharose column in the same manner as that previously reported for NB. In addition, NB and the NB-derived compounds are unable to disrupt interactions between Hsp90 and Cdc37, further delineating them from other N-terminal binders.

While effects on Hsp90 conformation, location of binding, and effects on Hsp90 cochaperone interactions are similar for NB and the NB-derived compounds, their ability to induce a heat shock response, are divergent. The NB-derived compound KU32, which was similar to NB in all prior evaluations, exhibits a strong induction of heat shock response upon incubation with yeast cells at 30°C and 39°C. In contrast, compound KU135, which manifests anti-proliferative activity ~1000 fold greater than NB (IC_{50} of 420 nM in LNCaP and 1.05 μM in LN3 prostate cancer cell lines), elicits no heat shock response in yeast, similar to NB, and furthermore, prevents induction of the heat shock response at elevated temperatures. The remaining NB-derived compound, KU174, which manifests an IC_{50} of 2.3 μM in LNCaP and 110 nM in LN3 prostate cancer cells, elicits no heat shock response upon incubation with yeast cells, but unlike NB, displays no protective effects against the heat shock response upon incubation at higher temperatures.

Strong evidence has been provided that the NB-derived compounds modulate Hsp90 *via* a similar mechanism as NB and proximal to the Hsp90 C-terminal dimer interface. However,

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the β-galactosidase assay has provided evidence for the existence of potentially three unique modes of C-terminal inhibition, which differ by their effects on induction of the heat shock response. Exactly how the C-terminal inhibitors are manifesting this differential heat shock response profile is unknown, and further investigation is required.

The proteolytic fingerprint of Hsp90 upon incubation with celastrol, gedunin, and H2-gamendazole revealed these modulators to induce similar Hsp90 conformational changes that are distinct from both N- and C-terminal inhibitors. This provides strong evidence that these compounds interact directly with Hsp90, and modulate its activity in a manner distinct from GDA and NB. Affinity chromatography revealed these compounds do not compete directly with either N- or C-terminal inhibitors of Hsp90.

Celastrol and gedunin were originally identified through the “Connectivity Map” screen, which can be used to compare the effects of small molecules on the expression levels of mRNA in order to gather information about potential molecular targets. Both of these natural products yielded similar results as 17-AAG, and were subsequently validated as Hsp90 inhibitors. H2-gamendazole was identified through whole cell studies,[36,50] and is structurally dissimilar to celastrol and gedunin, but manifests potent anti-spermatogenic activity as a result of Hsp90 modulation. The experiments described in this manuscript suggest a common mechanism of action for these three compounds, and indeed, co-immunoprecipitation experiments confirmed that all three compounds effectively disrupt Hsp90/Cdc37 interactions.

The natural sources of both celastrol and gedunin, *Tripterygium wilfordii* Hook F. and *Azadirachta indica* respectively, have also been used in traditional medicine for their anti-spermatogenic properties. This work has confirmed that all three compounds manifest Hsp90 modulation *via* similar mechanisms, suggesting that gedunin and celastrol may be responsible for the observed anti-spermatogenic activity of the whole plant extracts through Hsp90 modulation.

This manuscript has detailed the application of proteolytic fingerprinting, affinity chromatography, co-immunoprecipitation, and evaluation of the heat shock response as tools for exploring and characterizing various mechanisms of Hsp90 modulation (Chart 1).

This systematic protocol can be used as an investigational tool for newly discovered Hsp90 modulators. While subsequent compound analysis including X-ray crystallography, NMR studies, and degradation studies would provide more specific details, the approach outlined herin rapidly segregates compounds into various mechanistic classes. As a proof of principle, this process has provided evidence that the NB-derived compounds manifest their activity through interactions with the C-terminus of Hsp90, similarly to NB. These C-terminal modulators are promising lead compounds for the development of clinical agents with potential applications toward Ahlzeimer’s disease and/or cancer. In addition to supporting this hypothesis, this protocol has provided insight into factors governing Hsp induction by elucidating three distinct heat shock response profiles that occur through C-terminal modulation of Hsp90. Finally, three structurally dissimilar Hsp90 modulators, celastrol, gedunin, and H2-gamendazole, were verified as disruptors of Cdc37/Hsp90

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interactions through modulation of Hsp90. These agents are now under investigation for the development of male contraceptive and/or anticancer agents.

Acknowledgments

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References

1. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-KDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther. 1998; 79:129–168. [PubMed: 9749880]
2. Pearl LH, Prodromou C, Workman P. The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J. 2008; 410:439–453. [PubMed: 18290764]
3. Bishop SC, Burlison JA, Blagg BSJ. Hsp90: a novel target for the disruption of multiple signaling cascades. Curr Cancer Drug Targets. 2007; 7:369–388. [PubMed: 17979631]
4. Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, Barta TE, Dubois LG, Huang KH, Mabbett SR, Silinski MA, Steed PM, Hall SE. Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. Arthritis Rheum. 2008; 58:3765–3775. [PubMed: 19035474]
5. Ansar S, Burlison JA, Hadden MK, Yu XM, Desino KE, Bean J, Neckers L, Audus KL, Michaelis ML, Blagg BSJ. A non-toxic Hsp90 inhibitor protects neurons from Abeta-inducted toxicity. Bioorg Med Chem Lett. 2007; 17:1984–1990. [PubMed: 17276679]
6. Peterson LB, Blagg BSJ. To fold or not to fold: modulation and consequences of Hsp90 inhibition. Future Medicinal Chemistry. 2009; 1:267–283. [PubMed: 20161407]
7. Breinig M, Caldas-Lopes E, Goeppert B, Malz M, Rieker R, Bergmann F, Schirmacher P, Mayer M, Chiosis G, Kern MA. Targeting heat shock protein 90 with non-quinone inhibitors: a novel chemotherapeutic approach in human hepatocellular carcinoma. Hepatology. 2009; 50:102–112. [PubMed: 19441108]
8. Amolins MW, Blagg BSJ. Natural product inhibitors of Hsp90: potential leads for drug discovery. Mini Rev Med Chem. 2009; 9:140–152. [PubMed: 19200020]
9. Blagg BSJ, Kerr TD. Hsp90 inhibitors: small molecules that transform the Hsp90 protein folding machinery into a catalyst for protein degradation. Med Res Rev. 2006; 26:310–338. [PubMed: 16385472]
10. Chiosis G, Lopes EC, Solit D. Heat shock protein-90 inhibitors: a chronicle from geldanamycin to today's agents. Curr Opin Investig Drugs. 2006; 7:534–541.
11. Chiosis G, Rodina A, Moulick K. Emerging Hsp90 inhibitors: from discovery to clinic. Anticancer Agents Med Chem. 2006; 6:1–8. [PubMed: 16475922]
12. Huang KH, Veal JM, Fadden RP, Rice JW, Eaves J, Strachan JP, Barabasz AF, Foley BE, Barta TE, Ma W, Silinski MA, Hu M, Partridge JM, Scott A, DuBois LG, Freed T, Steed PM, Ommen AJ, Smith ED, Hughes PF, Woodward AR, Hanson GJ, McCall WS, Markworth CJ, Hinkley L, Jenks M, Geng L, Lewis M, Otto J, Pronk B, Verleysen K, Hall SE. Discovery of novel 2-aminobenzamide inhibitors of heat shock protein 90 as potent, selective and orally active antitumor agents. J Med Chem. 2009; 52:4288–4305. [PubMed: 19552433]
13. Pearl LH, Prodromou C. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem. 2006; 75:271–294. [PubMed: 16756493]
14. Hadden MK, Lubbers DJ, Blagg BSJ. Geldanamycin, radicicol, and chimeric inhibitors of the Hsp90 N-terminal ATP binding site. Curr Top Med Chem. 2006; 6:1173–1182. [PubMed: 16842154]
15. Smith DF, Whitesell L, Nair SC, Chen S, Prapapanich V, Rimerman RA. Progesterone receptor structure and function altered by geldanamycin an Hsp90-binding agent. Mol Cell Biol. 1995; 15:6804–6812. [PubMed: 8524246]

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16. Smith JR, Clarke PA, Billy Ed, Workman P. Silencing the cochaperone Cdc37 destabilizes kinase clients and sensitizes cancer cells to Hsp90 inhibitors. Oncogene. 2008; 28:157–169. [PubMed: 18931700]
17. Smith JR, Workman P. Targeting Cdc37. Cell Cycle. 2009; 8:362–372. [PubMed: 19177013]
18. Smith JR, Workman P. Targeting Cdc37: an alternative, kinase-directed strategy for disruption of oncogenic chaperoning. Cell Cycle. 2009; 8:362–372. [PubMed: 19177013]
19. Lamb J, Crawford ED, Peck D, Modell JW, Blat iC, Wrobel MJ, Lerner J, Brunet J-P, Subramanian A, Ross KN, Reich M, hieronymus h, Wei G, Armstrong SA, Haggarty SJ, Clemons PA, Wei R, Carr SA, Lander ES, Golub TR. The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease. Science. 2006; 313:1929–1935. [PubMed: 17008526]
20. Zhang T, Hamza A, Cao X, Wang B, Yu S, Zhan CG, Sun D. A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther. 2008; 7:162–170. [PubMed: 18202019]
21. Sreeramulu S, Gande SL, Gobel M, Schwalbe H. Molecular mechanism of inhibition of the human protein complex Hsp90-Cdc37, a kinome chaperone-cochaperone, by triterpene celastrol. Angew Chem Int Ed. 2009; 48:5853–5855.
22. Nemoto T, Ohara-Nemoto Y, Ota M, Takagi T, Yokoyama K. Mechanism of dimer formation of the 90-kDa heat-shock protein. Eur J Biochem. 1995; 233:1–8. [PubMed: 7588731]
23. Yamada S, Ono T, Mizuno A, Nemoto TK. A hydrophobic segment within the C-terminal domain is essential for both client-binding and dimer formation of the HSP90-family molecular chaperone. Eur J Biochem. 2003; 270:146–154. [PubMed: 12492485]
24. Scheibel T, Weikl T, Buchner J. Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. Proc Natl Acad Sci. 1998; 95:1485–1499.
25. Johnson BD, Chadli A, Felts SJ, Bouhouche I, Catelli MG, Toft DO. Hsp90 chaperone activity requires the full length protein and interaction among its multiple domains. J Biol Chem. 2000; 275:32499–32507. [PubMed: 10913439]
26. Owen BA, Sullivan WP, Felts SJ, Toft DO. Regulation of heat shock protein 90 ATPase activity by sequences in the carboxyl terminus. J Biol Chem. 2002; 277:7086–7092. [PubMed: 11751892]
27. Marcu MG, Chadli A, Bouhouche I, Catelli M, Neckers LM. The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. J Biol Chem. 2000; 275:37181–37186. [PubMed: 10945979]
28. Marcu MG, Schulte TW, Neckers L. Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. J Natl Cancer Inst. 2000; 92:242–248. [PubMed: 10655441]
29. Burlison JA, Neckers L, Smith AB, Maxwell A, Blagg BSJ. Novobiocin: redesigning a DNA gyrase inhibitor for selective inhibition of Hsp90. J Am Chem Soc. 2006; 128:15529–15536. [PubMed: 17132020]
30. Guettouche T, Boellmann F, Lane WS, Voellmy R. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. BMC Biochem. 2005; 6
31. Shamovsky I, Nudler E. New insights into the mechanism of heat shock response activation. Cell Mol Life Sci. 2008; 65:855–861. [PubMed: 18239856]
32. Burlison JA, Avila C, Vielhauer G, Lubbers DJ, Holzbeierlein J, Blagg BSJ. Development of novobiocin analogues that manifest anti-proliferative activity against several cancer cell lines. J Org Chem. 2008; 73:2130–2137. [PubMed: 18293999]
33. Donnelly AC, Mays JR, Burlison JA, Nelson JT, Vielhauer g, Holzbeierlein J, Blagg BSJ. The design, synthesis, and evaluation of coumarin ring derivatives of the novobiocin scaffold that exhibit antiproliferative activity. J Org Chem. 2008; 73:8901–8920. [PubMed: 18939877]
34. Donnelly A, Blagg BSJ. Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. Curr Med Chem. 2008; 15:2702–2717. [PubMed: 18991631]
35. Hieronymus H, lamb J, Ross KN, Peng XP, Clement C, Rodina A, nieto M, Du J, Stegmaier K, Raj SM, Maloney KN, Clardy J, Hahn WC, Chiosis G, Golub tR. Gene expression signature-based chemical genomic prediction identifies a novel class of Hsp90 pathway modulators. Cancer Cell. 2006; 10:321–330. [PubMed: 17010675]

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36. Tash JS, Chakrasali R, Jakkaraj SR, Hughes J, Smith SK, Hornbaker K, Heckert LL, Ozturk SB, Hadden MK, Kinzy TG, Blagg BSJ, Georg GI. Gamendazole, an orally active indazole carboxylic acid male contraceptive agent, targets Hsp90AB1 (Hsp90BETA) and EEF1A1 (eEf1A), and stimulates Il1a transcription in rat sertoli cells. Biol Reprod. 2008; 78:1139–1152. [PubMed: 18218611]
37. Nichols RL, Finland M. Novobiocin; a limited bacteriologic and clinical study of its use in forty-five patients. Antibiotic Med Clin Ther. 1956; 2:241–257.
38. Shen G, Yu XM, Blagg BS. Syntheses of photolabile novobiocin analogues. Bioorg Med Chem Lett. 2004; 14:5903–5906. [PubMed: 15501066]
39. Yun BG, Huang W, Leach N, Hartson SD, Matts RL. Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interations. Biochemistry. 2004; 43:8217–8229. [PubMed: 15209518]
40. Yu XM, Shen G, Neckers L, Blake H, Holzbeierlein J, Cronk B, Blagg BSJ. Hsp90 inhibitors identified from a library of novobiocin analogues. J Am Chem Soc. 2005; 127:12778–12779. [PubMed: 16159253]
41. Hartson SD, Thulasiraman V, Huang W, Whitesell L, Matts RL. Molybdate inhibits Hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. Biochemistry. 1999; 38:3837–3849. [PubMed: 10090774]
42. Hadden MK, Galam L, Gestwicki JE, Matts RL, Blagg BS. Derrubone, an inhibitor of the Hsp90 protein folding machinery. J Nat Prod. 2007; 70:2014–2018. [PubMed: 18020309]
43. Millson SH, Truman AW, Racz A, Hu B, Panaretou B, Nuttall J, Mollapour M, Soti C, Piper PW. Expressed as the sole Hsp90 of yeast, the alpha and beta isoforms of human Hsp90 differ with regard to their capacities for activation of certain client proteins, whereas only Hsp90beta generates sensitivity to the Hsp90 inhibitor radicicol. FEBS J. 2007; 274:4453–4463. [PubMed: 17681020]
44. Hjorth-Sorensen B, Hoffman ER, Lissin NM, Sewell AK, Jakobsen BK. Activation of heat shock transcription factor in yeast is not influenced by the levels of expression of heat shock proteins. Mol Microbiol. 2001; 39:914. [PubMed: 11251812]
45. Adams, A.; Gottschling, DE.; Kaiser, CA.; Stearns, T. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 1997.
46. Panaretou B, Piper PW. Isolation of yeast plasma membranes. Methods Mol Biol. 1996; 53:117–121. [PubMed: 8924973]
47. Nathan DF, Lindquist S. Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol Cell Biol. 1995; 15:3917–3925. [PubMed: 7791797]
48. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 2003; 31:3784–3788. [PubMed: 12824418]
49. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, Burrows FJ. A high-affinity conformation of Hsp90 confers selectivity on Hsp90 inhibitors. Nature. 2003; 425:407–410. [PubMed: 14508491]
50. Tash JS, Attardi B, Hild SA, Chakrasali R, Jakkaraj SR, Georg GI. A novel potent indazole carboxylic acid derivative blocks spermatogenesis and is contraceptive in rats after a single oral dose. Biol Reprod. 2008; 78:1127–1138. [PubMed: 18218612]

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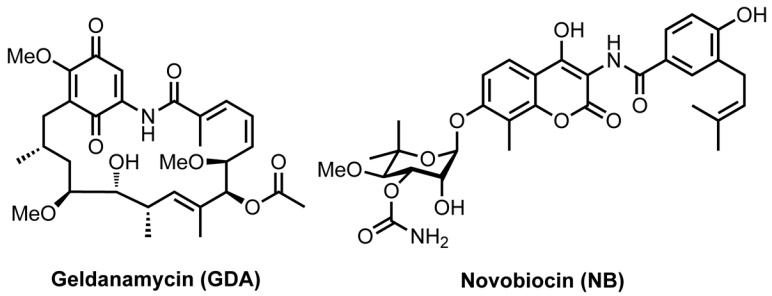

Figure 1.
Structures of the natural product Hsp90 inhibitors geldanamycin (GDA) and novobiocin (NB).

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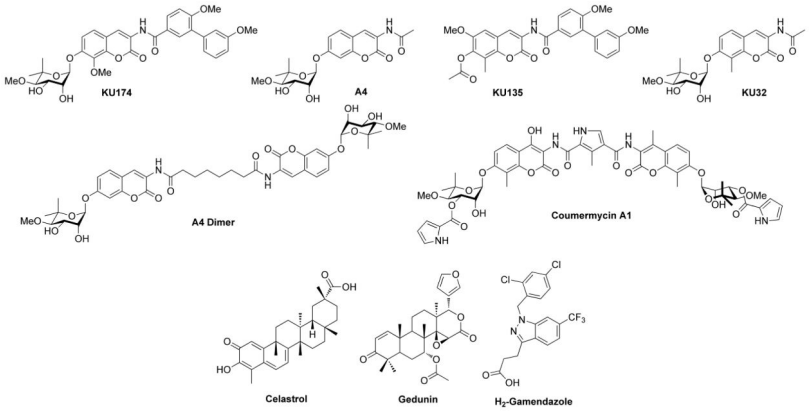

Figure 2.
Structures of Hsp90 modulators under investigation.

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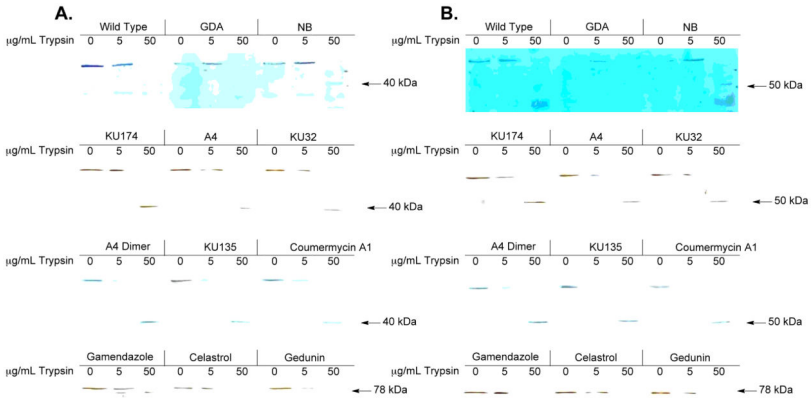

Figure 3.

Proteolytic fingerprint of Hsp90 after incubation with modulator. Rabbit reticulocyte lysate reaction mixtures containing an ATP regenerating system were incubated for 5 min at 37°C in the absence of drug (Wild type with 0, 50 and 125 µg/ml trypsin), in the presence of GDA (10 µg/ml), NB (20 mM), NB analogue, KU174, KU32, KU135, A4, A4 dimer, coumermycin A1 (10 µM) or H2-gamendazole, Celastrol and gedunin (20 µM). After drug treatment, reaction mix were chilled and incubated on ice with trypsin at the indicated concentrations. Trypsinolyzed reaction mixtures were boiled in SDS-PAGE buffer and analyze by SDS-PAGE and Western blotting with antibodies directed against the N-terminus (A) and C-terminal (B) of Hsp90.

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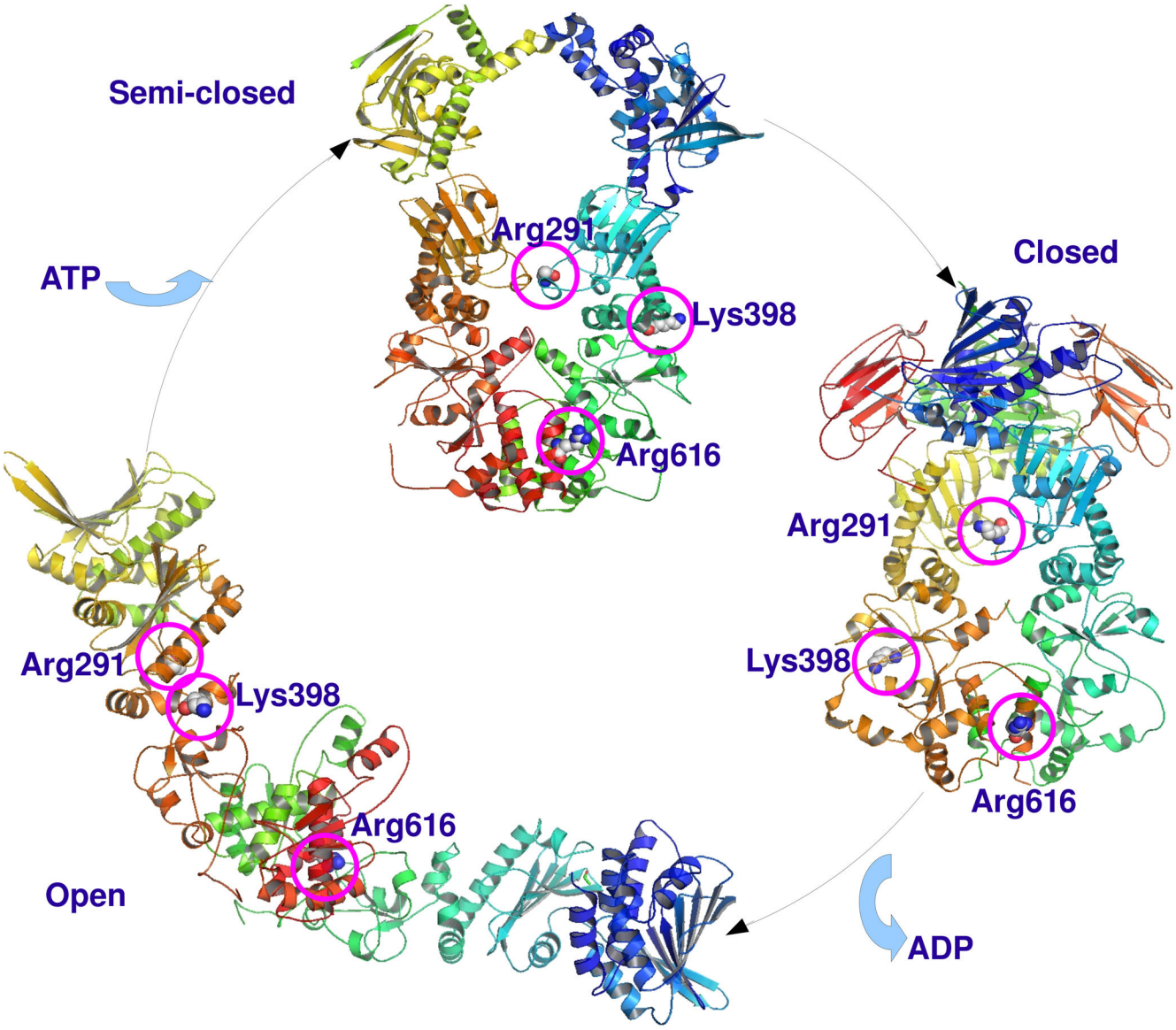

Figure 4.

The location of cleavage sites (Arg 291, Lys 398, and Arg616) in different Hsp90 conformations.

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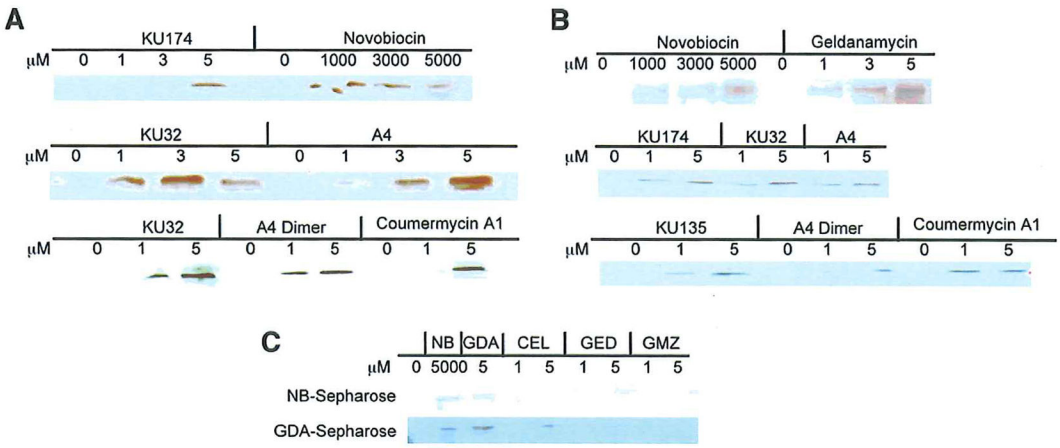

Figure 5.

Affinity Chromatography using NB bound sepharose (A), GDA bound sepharose (B), and affinity chromatography results for celastrol (CEL), gedunin (GED), and H2-gamendazole (GMZ) at the indicated concentrations. RRL system and ATP regenerating reaction mixture were incubated for 5 min at 37°C, applied for the NB-sepharose or GDA-sepharose column, after washing with 20 volume buffer, binding Hsp90 is competed by a solution of the indicated compound at the indicated concentration. Eluents were detected by anti-Hsp90 antibody. N5 (NB 5 mM), G5 (GDA 5 μM)

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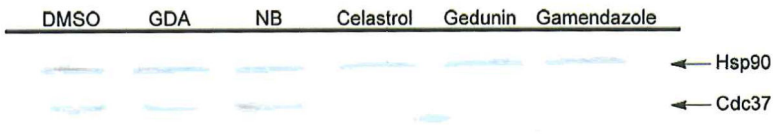

Figure 6.

Celastrol, gedunin and H2-gamendazole decreases Hsp90 interaction with Cdc37. Celastrol, gedunin and H2-gamendazole treatement of SKBR-3 cells (5 μM) decreased the amount of Cdc37 detected in co-immunoprecipitation with Hsp90, as shown by western blot of the co-immunoprecipitate and lysate. GDA (10 μM) and NB (1 mM) did not affect the interaction of Hsp90 and cdc37, which is shown as a control.

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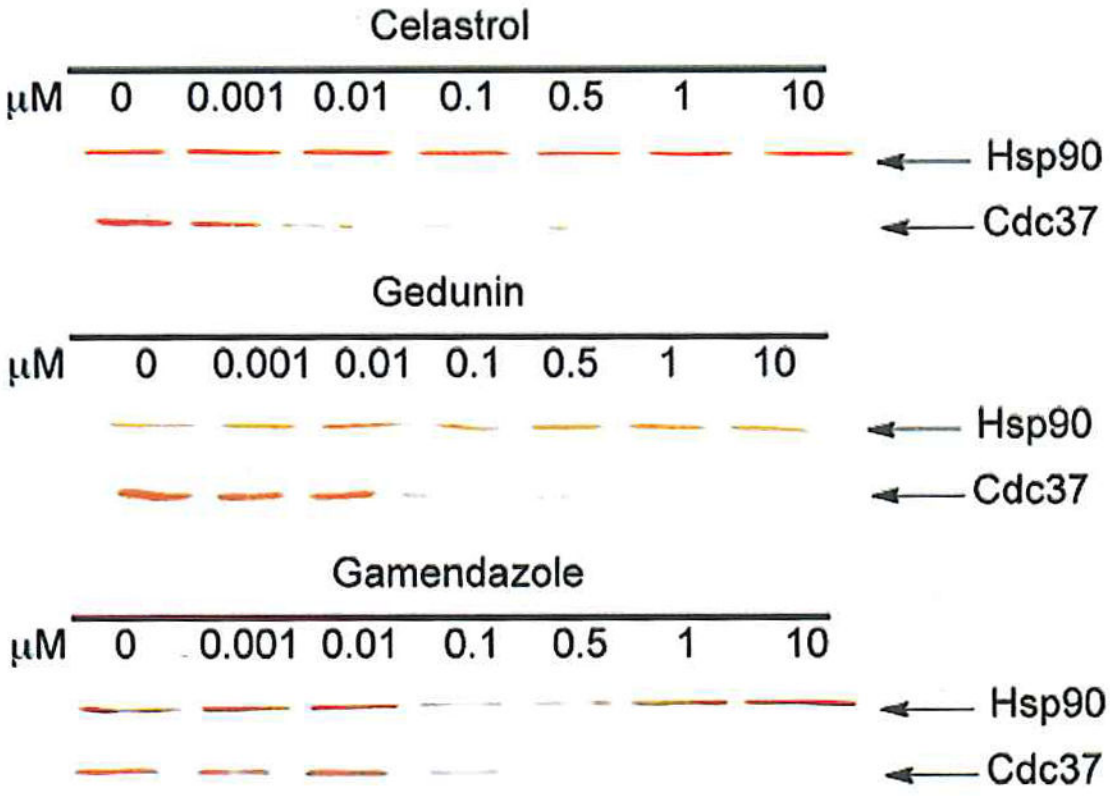

Figure 7.

Dose-dependent activity of Celastrol, gedunin and H2-gamendazole on the Cdc37/Hsp90 complex. Treatment of SKBR-3 cells (0, 0.001, 0.01, 0.1, 0.5, 1 and 10 μM) with celastrol, gedunin and H2-gamendazole decreased the cdc37 in co-immunoprecipitated with Hsp90 with the increasing concentrations of the compounds, as shown by western blot analysis of the coimmunoprecipitate (Hsp90 IP).

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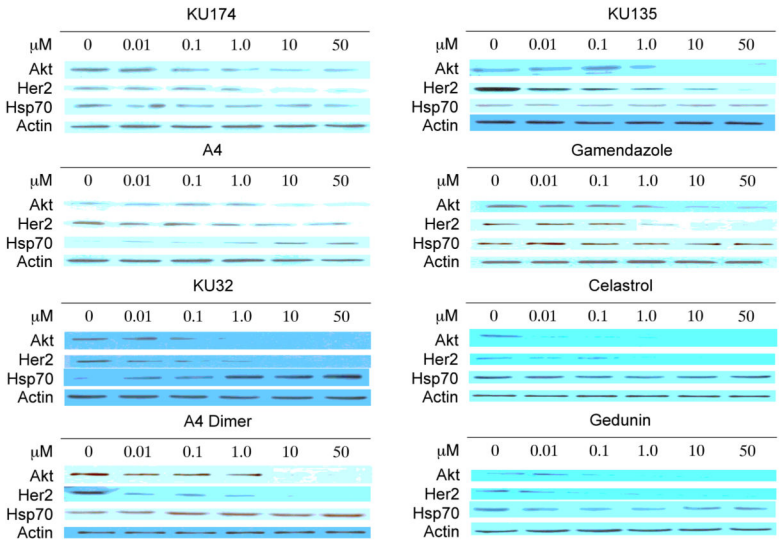

Figure 8.

Regulation of client proteins involved in the proliferation of MCF-7 cell. MCF-7 cells were grown to confluence as described above, seeded in culture dishes (1×10^6/dish) for overnight to attach (37°C, 5% CO_2). Compounds were added to the cell at varying concentrations and incubated for 48 h. Cells were harvested and analyzed for the Hsp90 client protein as described in experimental.

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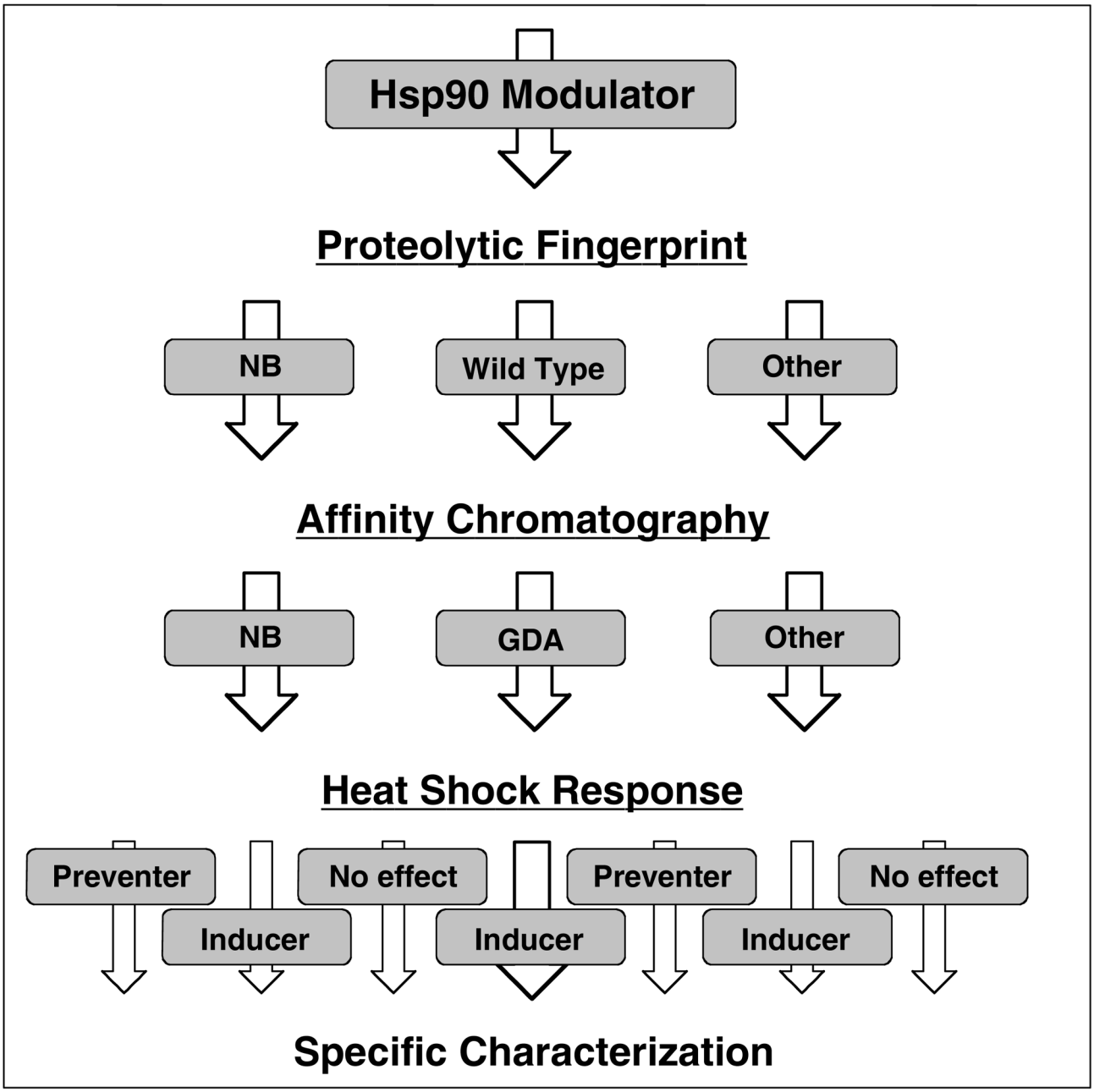

Chart 1.

Flow chart depicting systematic protocol for Hsp90 characterization. A lead compound is identified as an Hsp90 modulator and first evaluated for its effects on Hsp90 during trypsin proteolysis. Compounds will elicit proteolysis fingerprints similar to NB, wild type Hsp90, or neither (other). Next, several concentrations of the compound are used to elute Hsp90 off of GDA or NB bound sepharose beads during affinity chromatography. Again, compounds in each class should demonstrate behavior similar to NB, GDA, or neither (other). Finally, after evaluation in the β-galactosidase assay, compounds can be classified as C-terminal binders, N-terminal binders, or other, and further broken down into preventers, inducers, or

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those exhibiting no effect on the heat shock response. After these three initial evaluations, compounds in the “other” class can be characterized more specifically *via* co-immunoprecipitation, western blot analysis, NMR analysis, X-ray crystallographic techniques, etc… to determine the precise mode of action such, as allosteric modulator, co-chaperone disruptor, client protein disruptor, or other.

Evaluation of the Heat Shock Response in Yeast PP30 Cells Due to Select Modulators with and without Heat Stress.

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Table 1

OPAL prediction.

Type of site	Location	P3-P2-P1-P1′*
Class 1	291	WNRD
Class 3	398	IAKL
Class 3	616	FIRR
Class 4	617	IRRM

Class 1–4: Fastest to slowest cleavage,

*P1-P1′ is the cleavage site

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Table 2

The solvent accessible surface area of the cleavage site residues.

Position of cleavage site	HTPG	Semi-closed
291	198.44	107.38
398	59.42	55.96
616	23.80	92.85
617	25.08	56.18